

AD \_\_\_\_\_

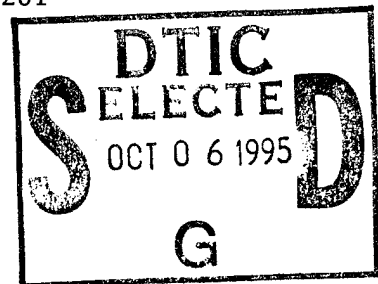
GRANT NO: DAMD17-94-J-4356

TITLE: Role of Proteases in Breast Cancer Progression

PRINCIPAL INVESTIGATOR: Dr. Rafael A. Fridman

CONTRACTING ORGANIZATION: Wayne State University  
Detroit, Michigan 48201

REPORT DATE: July 1995



TYPE OF REPORT: Annual

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

DISTRIBUTION STATEMENT: Approved for public release;  
distribution unlimited

DTIC QUALITY INSPECTED 5

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

19951003 029

# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 0704-0188

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

<b>1. AGENCY USE ONLY (Leave blank)</b>	<b>2. REPORT DATE</b> July 1995	<b>3. REPORT TYPE AND DATES COVERED</b> Annual 1 Jul 94 - 30 Jun 95
---	------------------------------------	--

<b>4. TITLE AND SUBTITLE</b> Role of Proteases in Breast Cancer Progression	<b>5. FUNDING NUMBERS</b> DAMD17-94-J-4356
--	---

<b>6. AUTHOR(S)</b> Dr. Rafael A. Fridman	
--	--

<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> Wayne State University Detroit, Michigan 48201	<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>
--	---

<b>9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	<b>10. SPONSORING/MONITORING AGENCY REPORT NUMBER</b>
--	---

**11. SUPPLEMENTARY NOTES**

<b>12a. DISTRIBUTION/AVAILABILITY STATEMENT</b> Approved for public release, distribution unlimited	<b>12b. DISTRIBUTION CODE</b>
--	-------------------------------

**13. ABSTRACT (Maximum 200 words)**  
Metastasis is the major cause of death in breast cancer patients. Metastasis formation depends on the ability of the tumor cells to degrade extracellular matrices (ECM) using speicalized proteinases including the matrix metalloproteinases (MMPs) and cathepsins. To study the role of these enzymes in breast cancer progression, we use preneoplastic MCF-10 breast epithelial cell variants and malignant MDA-MB-435 breast epithelial cells and stromal fibroblasts derived from normal and malignant breast tissue. We have studied the regulation of activation and plasma membrane localization of gelatinase A (GelA) and B (GelB), two members of the MMP family, in MCF10A cells. In this report we show that MCF10A cells treated with phorbol ester (TPA) produce high levels of GelB that is secreted into the media in a latent form and is in complex with TIMP-1. GelB was also found in association with the plasma membrane of MCF10A cells where is partially activated and free of inhibitors. Also, the plasma membrane of the MCF10A cells can activate GelA independently of TPA treatment. Experiments in progress will study the role of stromal fibroblasts on MMP cell surface localization and activation in preneoplastic and malignant breast epithelial cells.

<b>14. SUBJECT TERMS</b> matrix metalloproteinases, metastasis, stroma, cathepsin, tumor-stroma interactions	<b>15. NUMBER OF PAGES</b> 40
	<b>16. PRICE CODE</b>

<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified	<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified	<b>20. LIMITATION OF ABSTRACT</b> Unlimited
--	---	--	--

*Enclosure*

## GENERAL INSTRUCTIONS FOR COMPLETING SF 298

The Report Documentation Page (RDP) is used in announcing and cataloging reports. It is important that this information be consistent with the rest of the report, particularly the cover and title page. Instructions for filling in each block of the form follow. It is important to *stay within the lines* to meet *optical scanning requirements*.

**Block 1. Agency Use Only (Leave blank).**

**Block 2. Report Date.** Full publication date including day, month, and year, if available (e.g. 1 Jan 88). Must cite at least the year.

**Block 3. Type of Report and Dates Covered.** State whether report is interim, final, etc. If applicable, enter inclusive report dates (e.g. 10 Jun 87 - 30 Jun 88).

**Block 4. Title and Subtitle.** A title is taken from the part of the report that provides the most meaningful and complete information. When a report is prepared in more than one volume, repeat the primary title, add volume number, and include subtitle for the specific volume. On classified documents enter the title classification in parentheses.

**Block 5. Funding Numbers.** To include contract and grant numbers; may include program element number(s), project number(s), task number(s), and work unit number(s). Use the following labels:

<b>C</b> - Contract	<b>PR</b> - Project
<b>G</b> - Grant	<b>TA</b> - Task
<b>PE</b> - Program Element	<b>WU</b> - Work Unit Accession No.

**Block 6. Author(s).** Name(s) of person(s) responsible for writing the report, performing the research, or credited with the content of the report. If editor or compiler, this should follow the name(s).

**Block 7. Performing Organization Name(s) and Address(es).** Self-explanatory.

**Block 8. Performing Organization Report Number.** Enter the unique alphanumeric report number(s) assigned by the organization performing the report.

**Block 9. Sponsoring/Monitoring Agency Name(s) and Address(es).** Self-explanatory.

**Block 10. Sponsoring/Monitoring Agency Report Number.** (If known)

**Block 11. Supplementary Notes.** Enter information not included elsewhere such as: Prepared in cooperation with...; Trans. of...; To be published in.... When a report is revised, include a statement whether the new report supersedes or supplements the older report.

**Block 12a. Distribution/Availability Statement.**

Denotes public availability or limitations. Cite any availability to the public. Enter additional limitations or special markings in all capitals (e.g. NOFORN, REL, ITAR).

**DOD** - See DoDD 5230.24, "Distribution Statements on Technical Documents."

**DOE** - See authorities.

**NASA** - See Handbook NHB 2200.2.

**NTIS** - Leave blank.

**Block 12b. Distribution Code.**

**DOD** - Leave blank.

**DOE** - Enter DOE distribution categories from the Standard Distribution for Unclassified Scientific and Technical Reports.

**NASA** - Leave blank.

**NTIS** - Leave blank.

**Block 13. Abstract.** Include a brief (*Maximum 200 words*) factual summary of the most significant information contained in the report.

**Block 14. Subject Terms.** Keywords or phrases identifying major subjects in the report.

**Block 15. Number of Pages.** Enter the total number of pages.

**Block 16. Price Code.** Enter appropriate price code (*NTIS only*).

**Blocks 17. - 19. Security Classifications.** Self-explanatory. Enter U.S. Security Classification in accordance with U.S. Security Regulations (i.e., UNCLASSIFIED). If form contains classified information, stamp classification on the top and bottom of the page.

**Block 20. Limitation of Abstract.** This block must be completed to assign a limitation to the abstract. Enter either UL (unlimited) or SAR (same as report). An entry in this block is necessary if the abstract is to be limited. If blank, the abstract is assumed to be unlimited.

FOREWORD

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

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

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

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

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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

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

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

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

  
\_\_\_\_\_  
PI - Signature

Rafael Fridman, Ph.D.

Date

July 26, 1995

#### 4. TABLE OF CONTENTS

	<u>Page Number</u>
<b>Introduction</b>	<b>5-7</b>
<b>Body of Report</b>	<b>7-18</b>
<b>Conclusions</b>	<b>18-19</b>
<b>References</b>	<b>19-21</b>
<b>Appendix No.</b>	

1. "Enhanced expression of tissue inhibitor of metalloproteinase-2 (TIMP-2) in the stroma of breast carcinomas correlates with tumor recurrence." Int. J. Cancer, 59, 339-344. Page 22
2. "Progelatinase B activation by gelatinase A. Cancer Res., 55, 2548-2555." (supported in part by a Grant from the Department of Defense DAMD17-94-J-4356) Page 28
3. "The effect of platelets on invasiveness and protease production of human mammary tumor cells." Int. J. Cancer, 60, 413-417. Page 36

Accession For	
NTIS CRA&I	<input checked="" type="checkbox"/>
DTIC TAB	<input type="checkbox"/>
Unannounced	<input type="checkbox"/>
Justification .....	
By .....	
Distribution /	
Availability Codes	
Dist	Avail and/or Special
A-1	

## 5. INTRODUCTION

Tumor metastasis is the major cause of treatment failure in breast cancer patients. Numerous studies have shown that metastasis formation depends on the ability of the tumor cells to invade basement membranes and connective tissue matrices in a process involving a specialized group of enzymes capable of degrading extracellular matrix (ECM) components (1-3). Studies with various enzyme systems including the serine proteases (4), the matrix metalloproteinases (MMPs) (5-11) and the cathepsins (12) have shown that the degradation of ECM during tumor cell invasion is the result of a collaborative action between enzymes, enzyme receptors and enzyme inhibitors produced by both the tumor and the stroma. For example, tumor cells may utilize stromal enzymes during their invasion of ECM by binding enzymes to specific cell surface receptors (13) and/or by producing enzymes capable of activating stromal proteases (14). In turn, stromal cells can induce the expression of ECM-degradative enzymes in tumor cells (15) or they can produce specific enzyme inhibitors in response to the degradative activity produced by tumor cells (16).

**5.1 Matrix Metalloproteinases.** The MMPs are a family of highly conserved zinc-dependent proteinases capable of degrading many extracellular matrix (ECM) components (17). Although all the MMPs can degrade ECM, several of them, in particular, were shown to be associated with breast cancer. These include gelatinase A (72 kDa type IV collagenase or MMP-2), gelatinase B (92 kDa type IV collagenase or MMP-9), stromelysin-3, matrylisin, collagenase-3 and membrane-type MMP (MT-MMP).

The MMPs, except stromelysin-3 and MT-MMP, are secreted in a latent form that requires activation to become proteolytically active (17). In the case of stromelysin-3, activation occurs intracellularly and it is mediated by a furin enzyme (18). The mechanism of MT-MMP activation is still unknown. Proenzyme activation is a critical event in regulation of MMP activity and may be essential for ECM degradation during tumor cell invasion. The physiological mechanisms responsible for MMP activation in breast cancer are not completely understood, but may involve the action of other proteases, including other MMPs. Previous studies have shown that MT-MMP, an MMP present in the plasma membrane, may be the physiological activator of progelatinase A (19-22). The plasma membrane-dependent activation of progelatinase A is induced in cultured cells by treatment with phorbol ester (TPA) (20,21), concanavalin A (19,20,23,24), transforming growth factor- $\beta$  (20), or a collagen substrate (25).

The MMPs are all inhibited by the tissue inhibitor of metalloproteinases (TIMPs), a conserved family of low molecular weight proteins that presently includes TIMP-1 (30 kDa) (26), TIMP-2 (21 kDa) (27,28) and TIMP-3 (22 kDa) (29). TIMP-1 and TIMP-2 have been shown to inhibit MMPs by forming a stoichiometric complex with the active species (1,17). The association of TIMP-1 and TIMP-2 with the gelatinases, however, is unique since the inhibitors can also form a stable complex with the latent enzymes. For instance, TIMP-1 is capable of binding to the latent form of progelatinase B (30) whereas TIMP-2 can form a complex with progelatinase A (27). This unique interaction of the TIMPs with the proenzymes may provide an additional level of regulation by preventing generation of full enzymatic activity (31,32). However, recent studies have suggested a possible role of TIMP-2 on the activation of progelatinase A by MT-MMP on the cell surface (33).

**5.3 Epithelial-Stromal Regulation of ECM-degrading Proteases in Breast Cancer.** In breast cancer, expression of MMPs, in particular of the gelatinases (8,34-36) and stromelysin-3 (5), have

been suggested to play a role in tumor progression. However, the molecular, biochemical and tissue regulation of these enzymes at early and late stages of breast cancer development are still unknown. Immunohistochemical studies showed elevated expression of the 72 and 92 kDa gelatinases in the tumor cells (34,35, **Appendix 1**). *In situ* hybridization studies, however, showed mRNA expression for enzymes and TIMP-2 in the fibroblasts around invasive tumor cell clusters (8). Zymograms of tissue extracts of invasive breast carcinoma samples contained mostly the activated form (62 kDa) of the 72 kDa gelatinase (15/20) whereas only 2/20 of the samples showed active 92 kDa gelatinase (36). The studies of Basset et al. (5) demonstrated that the stroma of breast carcinomas expresses a new member of the MMP family, stromelysin-3. This study also showed that mRNAs for stromelysin-3, 92 kDa gelatinase and interstitial collagenase (MMP-1) were the only MMP mRNAs overexpressed in breast carcinoma whereas mRNAs for stromelysin-1, 72 kDa gelatinase, and pump-1 were also expressed in adenomas (5). In other study, both the 72 and 92 kDa gelatinase mRNAs were expressed at high levels in breast carcinomas whereas low levels of stromelysin-1 mRNA were detected (37). Recent *in situ* hybridization studies in seventeen cases of breast cancer showed high levels of gelatinase mRNAs in 60-80% of the cases, whereas moderate levels of stromelysin-1 mRNA were detected in only 30% of the cases (Dr. Lynn Matrisian, Vanderbilt University, personal communication).

In all these studies, mRNA expression of these MMPs was restricted to the breast stroma suggesting an important role for the stromal cells in MMP expression in breast cancer. In the case of the 72 kDa gelatinase, it was speculated that the activator of this enzyme, the recently identified MT-MMP (22), was probably localized in the breast cancer cells. This would allow activation of the stromal 72 kDa enzyme on the surface of the tumor cells. However, in a recent study the mRNA for MT-MMP was also localized in the tumor stroma of breast cancer tissues (38). This raises the question of (i) how the degradation of ECM is regulated in breast cancer, (ii) what is the role of the tumor and stromal cells in MMP expression and activity and (iii) how the tumor cells utilize the stromal MMPs for ECM degradation? Here we report preliminary studies suggesting that malignant breast cancer cells can induce the secretion of MMP-2 by fibroblast cells.

Another important aspect of MMPs in breast cancer that may be regulated by tumor-stroma interactions is the conversion of the zymogen to the active enzyme. As mentioned before, activation of the 72 kDa gelatinase may be achieved by MT-MMP (22). Interestingly, this activation has been shown to require binding of TIMP-2 to MT-MMP on the cell surface (33). This TIMP-2-MT-MMP bimolecular complex can in turn bind the proenzyme form of the 72 kDa gelatinase through the C-terminal end of the enzyme, causing zymogen activation (33). Thus, this paradoxical model of gelatinase A activation requires the participation of TIMP-2. It is tempting to speculate that the overexpression of TIMP-2 in the stroma of invasive breast cancer (please, see Results section) may also play a role in enzyme activation and contribute to the degradative activity in the tumor. Current studies in our laboratory are addressed to define the role of TIMP-2 in the regulation of MMP activity in breast cancer progression.

The 92 kDa gelatinase is also expressed at high levels in breast tumors (5,37, **Appendix 1**). *In vitro*, expression of this enzyme can be induced in immortalized breast epithelial cells, MCF10A, in response to phorbol ester and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) (please, see Results section). In contrast, cultured breast fibroblasts produce only constitutive levels of the 72 kDa gelatinase. The 92 kDa enzyme, in contrast to the 72-kDa enzyme, is not activated by MT-MMP (20,21) and is usually detected in the culture media of normal and tumor cells in a latent form. Studies with purified enzymes, however, have shown the ability of several proteases to activate the 92 kDa

gelatinase including stromelysin-1 (MMP-3) (39,40), plasmin (41) and tissue kallikrein (42). Activation of the 92 kDa gelatinase with stromelysin-1, which appears most efficient, generates an 82-kDa active species with enzymatic activity (40,41). The coordinated regulation of the 92 kDa enzyme and stromelysin-1 expression by cytokines in certain cells has been suggested to facilitate progelatinase B activation (39). However, in breast tumors, these enzymes are not always co-expressed as opposed to gelatinase A and B. We have hypothesized that the high levels and colocalization of the two gelatinases in breast tumors may facilitate the interaction of these two enzymes. We have recently found that MT-MMP-activated 72 kDa gelatinase can activate the latent form of the 92 kDa enzyme (please, see Results section). In addition, here we report the presence of activated MMP-9 in the plasma membranes of breast epithelial cells treated with phorbol ester.

## 6. BODY OF REPORT

### 6.1 EXPERIMENTAL METHODS

**6.1.a. Cell Culture. *The MCF10A breast epithelial system.*** As a model to study the expression of proteases in various stages of breast cancer progression *in vitro*, we have been using the MCF10 system of immortalized breast epithelial cells (MCF-10) (43-45). These cells were obtained from a patient with fibrocystic breast disease and have undergone spontaneous immortalization in culture (43). The MCF-10A line is near-diploid and monosomic for chromosomes 3 and 9. This line has been transfected with protooncogenic (MCF-10AneoN) and oncogenic (MCF-10AneoT) forms of c-H-ras plus the neomycin resistance gene (46). The MCF-10AneoT cells appear to have some characteristics of atypical breast epithelial stem cells as they are capable of indefinite proliferation and have a wide range of differentiation from normal to atypical. With prolonged xenograft growth, they occasionally evolve (30%) to a fully malignant histological appearance (44). Dr. Miller and collaborators have developed a series of transplant generations (TG1, TG2, and TG3) from lesions of MCF-10AneoT cells implanted in subcutis of nude/beige mice (44). These transplant generations were established in culture.

MCF10A, MCF10AneoT, and the TG (1 to 3) cells were grown in DMEM/F-12 (1:1) medium supplemented with 5% horse serum, insulin (10 µg/ml), cholera toxin (0.1 µg/ml), penicillin (100 i.u./ml), streptomycin (100 µg/ml), hydrocortisone (0.5 µg/ml), and epidermal growth factor (20 ng/ml). For some experiments, the cells will be maintained without the growth factors and hormones. Malignant breast carcinoma MDA-MB-435 cells were obtained from Dr. Janet Price (MD Anderson, TX). The metastatic properties of these cells have been very well characterized (47). The cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics.

***Stromal Cells.*** Fibroblasts derived from normal (reduction mammoplasty) or breast bearing tumor were kindly provided by Drs. Helene Smith and Shanaz Derkie (UCSF, CA). These cells were grown in DMEM-H16/F-12 (1:1) medium supplemented with 15% FBS, non-essential amino acids and antibiotics. Cells were split (1:2 to 1:5) using a trypsin-EDTA solution.

#### 6.1.b. Preparation of Conditioned Media.

To prepare serum-free conditioned (SF-CM) media of the breast epithelial cells or fibroblasts, 80 % confluent cultures were washed four times with warm serum-free media without growth factors

and hormones after aspirating the growth media and then incubated with a reduced volume of the serum-free media for 24-48 hrs at 37°C. The media was then collected, centrifuged to remove cell debris (2000 rpm, 10 min) and filtered through a 0.2 µm filter. The samples were analyzed immediately for expression of enzymes or enzyme inhibitors by zymography and immunoblotting. The SF-CM was also used to test their effect on other cell types. For these studies, the SF-CM was concentrated 10-folds using Amicon concentrators. To determine the protein concentration of the SF-CM, 1 ml of the media was dialyzed against water followed by concentration with a speed-vac. The lyophilized media was then resuspended in 50 µl water and the protein content was determined with the BCA kit (Pierce).

### **6.1.c. Analysis of Enzyme and Inhibitor Expression.**

**Zymography**. This was performed in 10% SDS-polyacrylamide gels containing 0.1% gelatin as described earlier (32). Samples of the SF-CM or purified enzymes were resuspended in Laemmli sample buffer (4X) without reducing agents and were not subsequently heated. At the end of the electrophoresis, the gels were incubated (20 min., 22°C) in a solution of 2.5% Triton-X100 in water to remove the SDS, and then washed in 50 mM Tris/HCl buffer pH 8 for another 20 min. The gels were then incubated overnight in 50 mM Tris/HCl, 5 mM CaCl<sub>2</sub> pH 8, at 37°C and stained with 0.25% Coomassie Blue in 10% methanol and 5% acetic acid. Bands of gelatinolytic activity were easily visualized after destaining the gels with 10% methanol-5% acetic acid as cleared bands against the blue-stained gelatin background.

**Immunoblot Analysis**.. Samples were subjected to SDS-PAGE under reducing conditions. The separated proteins were then transferred to nitrocellulose paper. After blocking with 3% bovine serum albumin and 3% non-fat dry milk in 50 mM Tris/HCl pH 7.5, the blots were incubated with the corresponding primary antibody diluted in 50 mM Tris/HCl pH 7.5, 150 mM NaCl and 0.1% Tween-20. The immunodetection of the antigen was performed using the immunoperoxidase ABC kit (Vector Laboratories). Immunoblot analysis were also performed using the ECL kit system (Amersham) as described by the manufacturer.

### **6.1.d. Expression and Purification of Recombinant Enzymes and Inhibitors**

Human recombinant progelatinase A, progelatinase B, TIMP-2 and TIMP-1 were all expressed in mammalian cells using a recombinant vaccinia virus expression system (Vac/T7), as previously described (32). Recombinant vaccinia viruses containing either the progelatinases or TIMP cDNAs were obtained by homologous recombination as previously described (32). Progelatinase A and B were purified from the media (Opti-MEM I, Gibco, Grand Island, NY) of HeLa cells infected with the appropriate recombinant viruses, as described previously (32). TIMP-2 was purified by affinity chromatography using a monoclonal antibody (mAb) (CA-101) against human TIMP-2, as described previously (32). TIMP-1 was purified from the media of infected HeLa cells using a lentil lectin-Sepharose 4B (Sigma); column equilibrated with 20 mM Hepes pH 7.5, 500 mM NaCl, 1 mM CaCl<sub>2</sub>, 10% glycerol, 0.05% Brij-35 and 0.02% NaN<sub>3</sub>. TIMP-1 was eluted with 500 mM methyl α-D-mannopyranoside (Sigma) diluted in the same buffer, dialyzed against collagenase buffer, concentrated in a Centricon-10 concentrator (Amicon, Beverly, MA) and purified by reverse phase-HPLC. The concentrations of the purified enzymes and TIMPs were determined by amino-acid analysis.

### 6.1.e. Triton-X114 Extraction of Cell Surface-associated Gelatinases.

Cultures of MCF10A cells (in 6-well plates) treated or not with TPA were washed three times with cold tris-buffered saline (TBS) and then incubated (15 min, 4°C) with 0.2 ml/well of 1.5% triton-X114 in TBS. The cells were then scrapped and the extracts centrifuged at 10,000 g at 4°C for 2 min. The supernatant was collected and incubated at 37°C for 2 min and then centrifuged at 14,000 rpm for 2-3 min. This procedure results in the formation of two phases, a detergent lower phase and an aqueous upper phase.

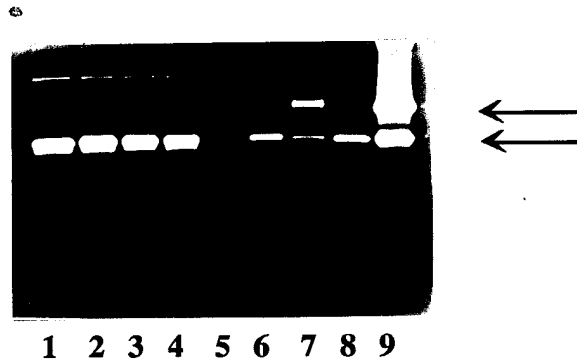
### 6.1 f. Isolation of Plasma Membranes

Plasma membranes were isolated from MCF10A and HT1080 cells cultured in roller bottles. To this end, 80% confluent cultures were treated or not with phorbol ester (100 nM) overnight in serum-free media. The next day the media was removed and the cells were washed twice with cold PBS. The cells were then scrapped in PBS followed by a low speed (1200 rpm) spin for 5 min. The cell pellet was then resuspended in 25 mM Tris pH 7.4, 8.5% sucrose and 50 mM NaCl containing protease inhibitors (NEM, PMSF, aprotinin, leupeptin, pepstatin) and homogenized with a Dounce homogenizer. The homogenate was centrifuged at 3000 rpm for 10 min at 4°C and the supernatant was collected. The supernatant containing the plasma membranes was centrifuged at 100,000 g for 2 hrs. and the pellet was resuspended in 25 mM Tris pH 7.4, 50 mM NaCl containing protease inhibitors. The plasma membranes were further fractionated on a discontinuous sucrose gradient at 100,000 g for 2 hrs at 4°C in a SW40 rotor. The 50%/30% interface band representing the enriched plasma membrane fraction was collected in 25 mM Tris pH 7.4, 50 mM NaCl containing protease inhibitors and centrifuged again at 100,000 g for 2 hrs. Finally, the plasma membrane pellet was resuspended in 25 mM Tris pH 7.4, 50 mM NaCl containing protease inhibitors and stored at -80°C. The protein concentration of the plasma membrane preparation was determined using the BCA kit (Pierce).

## 6.2 RESULTS.

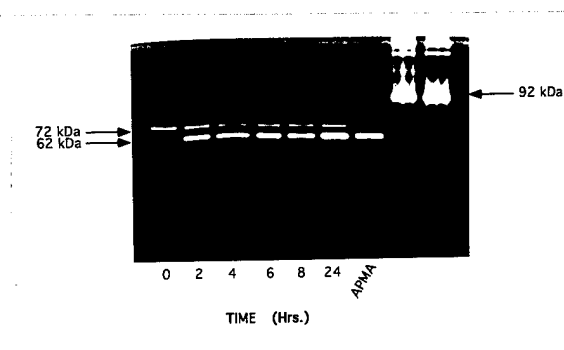
### 6.2.a. Expression of MMPs, and TIMPs.

We tested the expression of MMPs and TIMPs in immortalized breast epithelial MCF10A cells, invasive TG3b and malignant breast cancer cells, and various human fibroblasts. **Figure 1** is a zymogram showing the expression of MMPs in the conditioned media of human lung CCL153 fibroblasts (**lane 8**), MCF10A (**lane 7**), TG2b (**lane 6**), MDA-435 (**lane 5**) and four different fibroblasts isolated from normal (**lanes 1 and 2**) or tumor-bearing breast tissue (**lanes 3 and 4**). Except for MDA-435 all these cell lines secreted proMMP-2 at various degrees or proMMP-9. Only MCF10A cells produced higher levels of proMMP-9 (**lane 7**). The media of the breast fibroblasts showed a higher molecular weight gelatinase that its identity is unknown. As control, we utilized media of HT1080 cells (**lane 9**) known to produce both proMMP-2 and proMMP-9. Same results were obtained by immunoblot analysis (not shown).



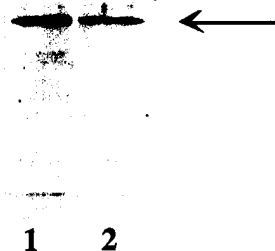
**Figure 1.** Gelatin-zymogram of gelatinases secreted by breast epithelial cells and fibroblasts. Lane 1, M106 (normal breast fibroblast); lane 2, M7 (normal breast fibroblast); lane 3, B74 (tumor breast fibroblast); lane 4, B394 (tumor breast fibroblast); lane 5, MDA-435; lane 6, TG3b; lane 7, MCF10A; lane 8, lung fibroblasts (CCL153); and lane 9, human fibrosarcoma HT1080 cells. Upper arrow, proMMP-9; lower arrow, proMMP-2.

We will test the breast epithelial and fibroblasts cells for other MMPs including MMP-1 (interstitial collagenase) MMP-3 (stromelysin-1), collagenase 3 and MT-MMP. The latter is important since this enzyme has been suggested to be the activator of proMMP-2 (22). As a source of this novel MMP we are currently using plasma membranes isolated from HT1080 human fibrosarcoma cells treated with phorbol ester. It has been shown that these plasma membranes can activate proMMP-2 into its active forms due to the presence of MT-MMP (20,21,22). As shown in **Figure 2**, plasma membranes (100  $\mu\text{g/ml}$ ) of the HT1080 cells activated recombinant proMMP-2 (1.25  $\mu\text{g/ml}$ ) into a major 62-kDa and a minor 45-kDa forms (not detectable in this Figure) after 2 hrs incubation. Latent MMP-2 was not activated in the absence of plasma membrane even after a 24 hrs incubation period at 37°C (not shown).



**Figure 2.** Activation of proMMP-2 by plasma membranes of HT1080 cells. Purified proMMP-2 was incubated for various periods of time with isolated plasma membranes and then the reaction mixture was analyzed by gelatin-zymography. APMA-activated MMP-2 (APMA) and proMMP-9 (92 kDa) were used as controls.

Using a polyclonal antibody against the catalytic domain of human MT-MMP (kindly provided by Dr. S. Weiss (University of Michigan), we detected a 60-65-kDa protein representing MT-MMP in two independent isolations of HT1080 plasma membranes (**Figure 3, lanes 1 and 2, next page**).

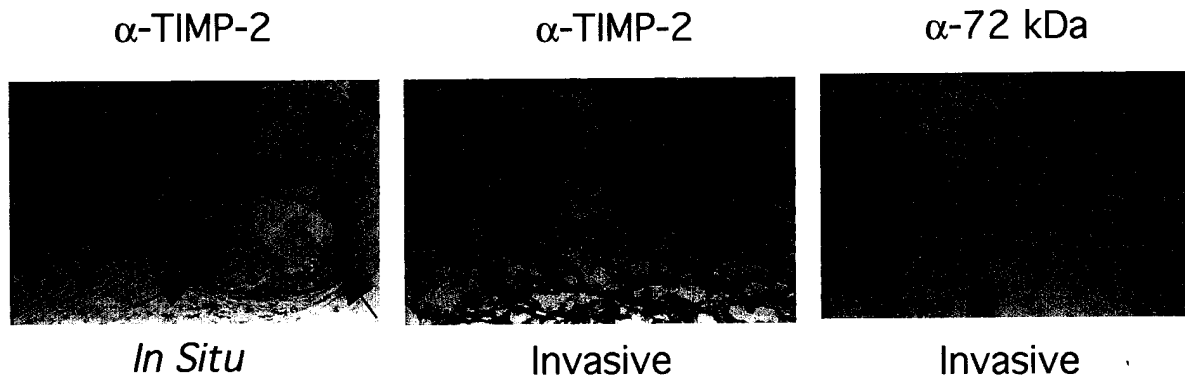


**Figure 3.** Immunoblot analysis of MT-MMP (arrow) in the plasma membranes of TPA-treated HT1080 cells.

Since we do not have in our possession sufficient anti-MT-MMP antibody for our studies, it was necessary to produce specific antibodies against this protein. The production of the polyclonal antibody was ordered from Research Genetics Inc. and the final antiserum was recently received. A synthetic peptide comprising residues 450-466 (KNIKVWEGIPESPRGSF) starting from the first Met of the human MT-MMP sequence was used to immunized rabbits. The antiserum is now being tested for recognition of MT-MMP. Eventually, this antibody will be used to test the expression of MT-MMP in the breast epithelial and fibroblast cells and in immunohistochemical studies in breast carcinomas.

**6.2.b. Stromal-Epithelial Regulation of MMP And TIMP Expression.**

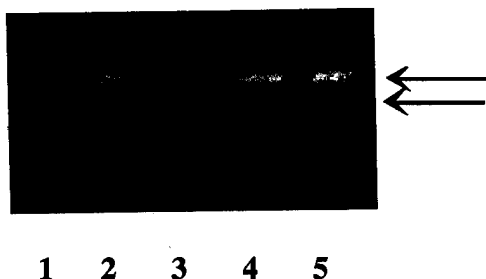
In our immunohistochemical studies in sections of invasive breast carcinoma tissues we reported an upregulation of MMP-2, MMP-9 and TIMP-2 (Appendix 1 and Figure 4). TIMP-2 was localized in the tumor stroma and was absent from benign areas of the same section suggesting a stromal specific expression of TIMP-2. Moreover, *in situ* carcinoma showed presence of TIMP-2 closely associated with the basement membrane/stroma interface (Fig. 4). Whereas the stroma in areas of carcinoma *in situ* was weakly stained or negative for TIMP-2 staining, the stroma surrounding the invasive nests of cancer cells was strongly positive for TIMP-2. MMP-2 (72 kDa) was localized in the tumor cells.



**Figure 4.** Immunohistochemical distribution of MMP-2 (72 kDa) and TIMP-2 in *in situ* and invasive breast carcinomas.

These studies suggested a role for the stroma in breast cancer progression. We have hypothesized that the tumor cells may be stimulating the stroma to produce TIMP-2 and MMPs. In addition, the stroma may also play a role in the localization of MMP-2 and MMP-9 on the tumor cells since previous data indicated that these enzymes are synthesized mainly in the stromal cells.

**Induction of MMP-2 in fibroblast cells by malignant breast cancer cells.** Based in our immunohistochemical studies and previous *in situ* hybridization studies we decided to examine the role of tumor-stroma interactions on the expression of MMPs and TIMPs. Initially, we tested the effect of breast epithelial and breast cancer cell conditioned media on the MMP and TIMP-2 expression in human fibroblasts. To this end, SF-CM of MDA-435, at various dilutions (100 and 50%) were applied to human lung fibroblasts (CCL153) for 24 hrs. Then, the media was collected and analyzed by zymography. As shown in **Figure 5**, the MDA-435 media does not contain detectable levels of gelatinases (**lane 1**) and the CCL153 cells produced only proMMP-2 (**lane 3**). Incubation of the CCL153 cells with either 50 or 100% SF-CM from MDA-435 cells resulted in an enhanced secretion of MMP-2 as suggested by the wider gelatinolytic band of 72 kDa (**lanes 4 and 5**) as compared to CCL153 cells incubated with plain SF-media (**lane 3**). Interestingly, the 72 kDa enzyme in the CCL153 cells exposed to the SF-CM of MDA cells (**lanes 4 and 5**) showed presence of the active form which was not detected in the untreated CCL153 cells (**lane 3**). **Lane 2** shows purified recombinant MMP-2 as standard.



**Figure 5.** Zymogram of CCL153 cells incubated with SF-CM of MDA-435 cells. Lane 1, SF-CM of MDA-435 cells; lane 2, recombinant MMP-2; lane 3, CCL153 cells alone; lane 4, CCL153 cells with 50% MDA SF-CM and lane 5, CCL153 cells with 100% MDA SF-CM. Upper arrow, latent proMMP-2; lower arrow, activated MMP-2.

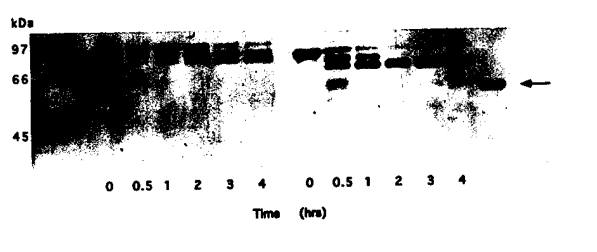
This preliminary experiment suggests that conditioned media from MDA-435 cells stimulates the secretion of latent and possible the activation of MMP-2 in human lung fibroblasts. We do not know yet the effect on TIMPs secretion.

**Note:** The previous experiment was performed with human lung fibroblasts instead of the breast fibroblasts due to the difficulty we encountered maintaining the primary breast fibroblasts. The primary human breast fibroblasts from normal and tumor-bearing breasts were obtained from Dr. Helene Smith. The following cells were obtained: Tumor derived: B-357, B-21, B-394, B-383 and B-74; Normal: M-106, and M-7. These cells were grown as described in **section 6.1.a**. However, difficulties were encountered when large number of cells were required for the various experiments due to their slow growth. The doubling time was estimated to be around 48 hrs at a splitting ratio of 1:2. Although we obtained SF-CM from these cells, their viability was compromised after several passages. We are aware of the difficulties of working with primary cultures. The most

important include long doubling times, loss of phenotypic markers and lack of reproducibility. In terms of expression of MMP-2 and TIMPs, these do not appear to change. However, whether their response to the epithelial cells will be affected cannot be predicted at this time. We have recently contacted Dr. Derkie which assumed the role of Director of the Tissue Culture Core Facility of the Geraldine Brush Cancer Research Institute. Dr. Derkie informed us that the primary fibroblasts that we received earlier were late passage cells and this may explain their slow proliferation. Dr. Derkie has selected a new set of fibroblasts that will be sent to us in the near future. Furthermore, she will also collect SF-CM from these cells that will be forwarded to us to be tested in our system. This will prevent possible variations due to different tissue culture conditions. As an alternative, we will use established fibroblasts cell lines such as the CCL153 and WI-38 cell lines, both human lung fibroblasts.

### 6.2.c. Regulation of MMP Activation and Plasma Membrane Localization in Breast Epithelial MCF10A Cells.

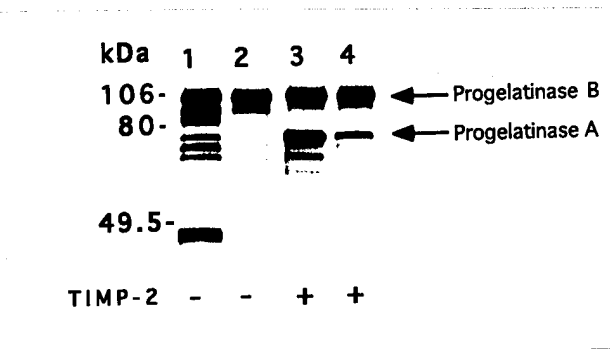
***In Vitro Activation of ProMMP-9.*** Our previous immunohistochemical studies indicated a co-expression of MMP-2 and MMP-9 in invasive breast carcinomas (**Appendix 1**). This raised the question of the mechanism of activation of these enzymes in breast tissues. Previous studies demonstrated that MMP-9 can be activated efficiently by stromelysin-1 (MMP-3) (39). However, the expression of stromelysin-1 in breast carcinoma is unclear. Due to the frequent co-expression of the 72- and 92-kDa gelatinases in breast tumors, we wished to investigate the ability of these enzymes to activate each other (**Appendix 2**). Incubation of proMMP-9 with APMA-activated MMP-2 resulted in a time- and dose-dependent conversion of the 92-kDa zymogen to the 82-kDa species (**Figure 6**). An intermediary form of 86 kDa could be observed as early as 30 min after addition of MMP-2. Full conversion to the 82-kDa species was observed after a 2-hr incubation period (**Figure 6, right panel**; 1:50 MMP-2 : proMMP-9 molar ratio). The N-terminal sequences of the 86- and the 82-kDa MMP-9 species formed after activation by gelatinase A were determined and found to be Met<sup>41</sup> for the 86-kDa form and Phe<sup>88</sup> for the 82-kDa form. These sequences are the same as those reported previously for the activation of proMMP-9 by stromelysin-1 (39). These studies have been published in Cancer Research (Appendix 2).



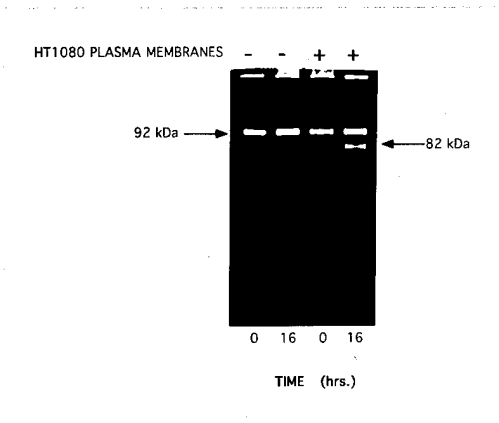
**Figure 6.** SDS-PAGE analysis of the activation of progelatinase B by gelatinase A. APMA-activated gelatinase A was incubated (37°C) with human recombinant progelatinase B for various time periods at a 1:100 (left panel) or 1:50 (right panel) gelatinase A : progelatinase B molar ratios. At the end of the indicated incubation times, the samples (1 µg/lane of progelatinase B) were analyzed in 10% SDS-polyacrylamide gel under reducing conditions, followed by staining of the gel with 0.25% Coomassie Brilliant Blue. The left lane shows the molecular-weight standards (Bio-Rad). The arrow in the right shows the activated gelatinase A used in the experiment.

Since APMA activated-gelatinase A caused activation of proMMP-9, we examined the effect of plasma membrane-activated gelatinase A (MT-MMP-activated MMP-2) on proMMP-9 activation.

Plasma membranes from phorbol ester treated-HT1080 cells were incubated (3 hr, 37°C) with proMMP-2 and then proMMP-9 was added to the reaction mixture for an additional 3-hr incubation period. The reaction mixture was then analyzed by immunoblot using a mAb (CA-209) against proMMP-9 and a mAb (CA-801) against proMMP-2. These studies (**Figure 7**) showed that plasma membrane activated-MMP-2 caused the conversion of proMMP-9 to the 86- and 82-kDa species (**Figure 7, lanes 1 and 2**). The immunoblot also showed the bands corresponding to the plasma membrane-activated gelatinase A including a 64-, 62- and a 45-kDa form. When proMMP-2 was incubated (3 hr, 37°C) with plasma membranes in the presence of recombinant TIMP-2, there was a significant reduction in the formation of the 62- and 45-kDa species and most of the 72-kDa enzyme remained in the latent form (**Figure 3, lanes 3 and 4**). Consistently, addition of proMMP-9 to the mixture of plasma membranes, proMMP-2 and TIMP-2 followed by another 3-hr incubation period, had no effect on activation of proMMP-9 (**Figure 7, lanes 3 and 4**). Also, proMMP-9 incubated with HT1080 plasma membranes in the absence of proMMP-2 was not activated (not shown).



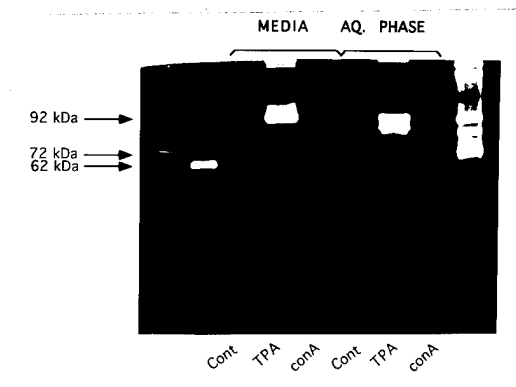
**Figure 7.** Immunoblot of proMMP-9 activation by plasma membrane-activated MMP-2 and inhibition by TIMP-2. Plasma membranes (10 µg/reaction) were incubated (3 hrs, 37°C) with proMMP-2 (200 ng/reaction) in 25 mM Hepes/KOH pH 7.5, 0.1 mM CaCl<sub>2</sub> in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of stoichiometric amounts (60 ng/reaction) of TIMP-2 to proMMP-2. An aliquot of the reaction mixture was then incubated with proMMP-9 (200 ng/lane) at 1:10 (lane 1 and 3) and 1:50 (lane 2 and 4) molar ratios (MMP-2 : proMMP-9) for another 3 hrs at 37°C. Molecular weights on the left represent the prestained molecular-weight standards (low range, Bio-Rad).



**Figure 8.** Zymogram of proMMP-9 activation by plasma membrane-activated MMP-2. ProMMP-9 (5 ng/lane) activated by a MMP-2 activated by HT1080 plasma membranes after 16 hrs incubation. The activated MMP-2 cannot be seen in this zymogram due to the low amounts of the enzyme loaded in the gel.

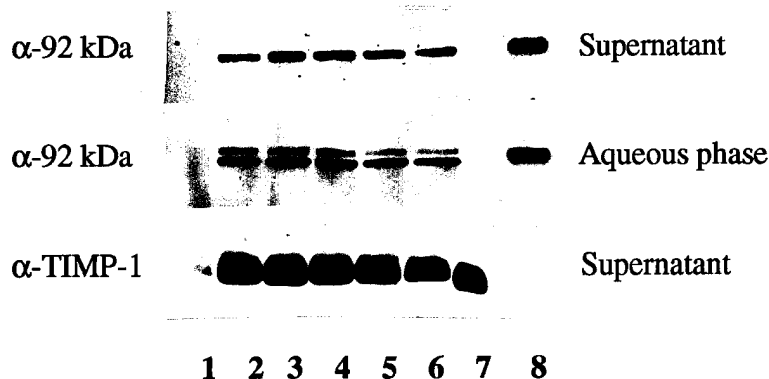
The zymogram of **Figure 8** (previous page) shows that plasma membrane-activated MMP-2 can activate proMMP-9 into the 82-kDa form. This process requires MMP-2 activation since incubation of latent MMP-2 and MMP-9 without plasma membrane did not cause proMMP-9 activation. Taken together these studies demonstrate that both APMA- or plasma membrane-activated gelatinase A can activate proMMP-9. We speculate that this process of activation may play a role in the regulation of MMP activity in breast cancer.

**Cell Surface Localization of MMP-9 in MCF10A Cells.** The ability of MT-MMP-activated MMP-2 to activate proMMP-9 suggested a role for a cell surface activation of proMMP-9. When we examined MCF10A cells for MMP expression, we found that they produce mainly proMMP-9 and very low (sometimes undetectable) levels of proMMP-2. Treatment (16 hrs, 37°C) of MCF10A cells with 100 nM of phorbol ester resulted in enhanced secretion of proMMP-9 into the media (**Figure 9**). Concanavalin A (conA) had no effect on proMMP-9 secretion. Also, phorbol ester or conA treatment did not cause an increase in proMMP-2 secretion or activation. Using the biphasic triton-X114 detergent that extracts intrinsic and peripheral plasma membrane proteins into a detergent and aqueous phase, respectively, we have found that MMP-9 was associated with the cell surface in the cells treated with TPA. The enzyme was found in the aqueous phase suggesting that is a peripheral plasma membrane component (**Figure 9**). Interestingly, the MMP-9 enzyme detected in the aqueous phase was partially activated as determined by the presence of a lower molecular weight form.



**Figure 9.** Distribution of gelatinases in human breast epithelial MCF10A cells.

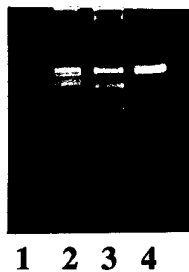
**Figure 10 (next page)** shows an immunoblot of a time course experiment showing proMMP-9 secretion and aqueous phase localization in MCF10A cells treated with TPA. In the supernatant (**top panel**), the enzyme was in the latent form and could be detected after a 30 min of TPA treatment. In the aqueous phase (**middle panel**), the enzyme was partly activated. TIMP-1 was also induced by TPA treatment but was only localized in the supernatant (**bottom panel**).



**Figure 10.** Immunoblot of a time course and cellular distribution of gelatinase B and TIMP-1 in MCF10A cells treated with TPA. Lane 1, untreated; lane 6, 30 min; lane 5, 1 hr.; lane 4, 2 hrs.; lane 3, 4 hrs.; lane 2, 6 hrs.; lane 7, purified recombinant TIMP-1; and lane 8, purified recombinant proMMP-9.

We have also found that the latent enzyme secreted into the supernatant in response to TPA was in complex with TIMP-1 (not shown). Presence of MMP-9 and its active form in the cell surface of MCF10A cells was not affected by triton-X114 extraction in the presence of protease inhibitors (PMSF, aprotinin, leupeptin, EDTA) suggesting that the origin of the lower molecular weight form of MMP-9 was not due to a non-specific proteolytic activity released during the triton-X114 treatment. Also, the same treatment did not cause the spontaneous activation of a purified preparation of recombinant proMMP-9 suggesting that cell surface activation of MMP-9 was not due to an effect of the detergent.

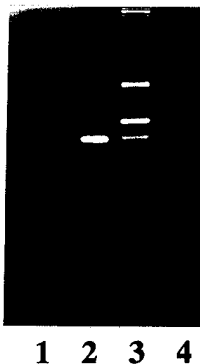
**Presence of Activated MMP-9 in Plasma Membranes of MCF10A Cells.** We next prepared plasma membranes of MCF10A cells treated or not with TPA. As shown in **Figure 11**, only the plasma membranes of TPA-treated MCF10A cells showed presence of latent and activated MMP-9 as determined by zymography (**lane 2**). When the plasma membranes were treated with triton-X114, MMP-9 was found only in the aqueous phase suggesting that the association of MMP-9 to the membranes does not involved binding to a lipid component.



**Figure 11.** Zymogram of plasma membranes isolated from MCF10A cells. Lane 1, untreated MCF10A plasma membranes; lane 2, TPA-treated MCF10A plasma membranes; lane 3, recombinant APMA-activated MMP-9 and lane 4, latent recombinant proMMP-9. Note that the recombinant APMA-activated MMP-9 (lane 3) shows an 82 kDa activated form and a 67-70 kDa activated form.

We tested whether TPA-treated MCF10A cells or their isolated plasma membranes could activate exogenous proMMP-9 by providing the cells or the membranes with purified  $^{35}\text{S}$ -proMMP-9 and found no activation. Thus, in contrast to TPA-treated HT1080 cells or their plasma membranes which can activate exogenous proMMP-2 through MT-MMP, this mechanism appears not to be involved in the cell surface activation of proMMP-9 by MCF10A cells. Taken together, these studies suggest that *in vitro* induction of MMP-9 production and secretion in normal immortalized breast epithelial cells results in the secretion of a TIMP-1/latent enzyme complex into the media whereas a fraction of activated TIMP-1 free-MMP-9 binds to the cell membrane. The mechanism of activation and plasma membrane association of MMP-9 in MCF10A cells remain to be determined.

**Plasma membranes of breast epithelial MCF10A cells activate proMMP-2.** We examine the ability of plasma membranes of untreated or TPA-treated MCF10A cells to activate proMMP-2. To this end, the isolated plasma membranes were incubated (16 hrs., 37°C) with recombinant proMMP-2 and the reaction mixture analyzed by zymography. As shown in **Figure 12**, the TPA-treated plasma membranes activated proMMP-2 into the 62-kDa form (**Fig. 12, lane 3**) as reported with the HT1080 plasma membranes (see also **Figure 2**). Incubation (16 hrs., 37°C) of proMMP-2 alone did not result in proenzyme autoactivation (**lane 4**) demonstrating the specific effect of the plasma membranes on activation of proMMP-2. The zymogram also shows that the plasma membranes of TPA-treated MCF10A cells contain both the latent and activated form of MMP-9 (**lane 3**). As control, proMMP-2 was activated with APMA generating the 62-kDa form (**lane 2**).



**Figure 12.** Activation of proMMP-2 by plasma membranes of TPA-treated MCF10A cells. Lane 1, molecular weight markers; lane 2, APMA-activated MMP-2; lane 3, plasma membrane-activated MMP-2; lane 4, latent proMMP-2.

We have recently found that plasma membranes from control untreated MCF10A cells can also activate proMMP-2 (not shown). Thus, the plasma membrane-dependent activation of proMMP-2 by MCF10A is not inducible by TPA as opposed to HT1080 cells. At the present time, we do not know whether MCF10A express the same MT-MMP expressed by HT1080 cells. We are currently testing the new polyclonal antibody with the MCF10A plasma membrane preparations to determine if these cells possess MT-MMP. Since the identification of at least three novel MT-MMPs was recently reported (Gordon Conference on MMPs, unpublished), will be of interest to determine the type of MT-MMP expressed by the MCF10A cells and if different from the MT-MMP found in HT1080 cells, whether this novel MT-MMP is the responsible for the activation of proMMP-9 in the plasma membranes of MCF10A cells.

**Plasma Membrane localization of MMPs. Relevance in Tumor-Stroma Interactions in Breast Cancer Progression.** Recent findings from our laboratory and others have suggested the importance of the tumor stroma in the expression of MMPs and TIMPs in breast cancer. Whereas *in situ* hybridization studies showed mRNA expression in the stroma, immunohistochemical studies demonstrated a pericellular distribution of MMP-2 and MMP-9 in the tumor cells. Thus, it is possible that the stromal cells secrete the enzymes which then bind to the surface of the tumor cells. Our studies with MCF10A cells demonstrate the importance of the plasma membrane localization of MMPs in breast epithelial cells and suggest that this localization may play a role in enzyme activation.

### **6.2.c. Future Studies.**

We will continue, during the second year, to test the effect of stroma-tumor interactions on MMP and TIMP expression using the premalignant and malignant breast epithelial cells. In particular, we will try to determine whether there is a signal from the breast epithelial cells that may stimulate the secretion and activation of MMPs by the stromal cells. Regarding fibroblasts, we will continue our efforts with the primary fibroblasts provided by Dr. Shanaz Derkie. We will also study tumor-stroma interactions in a mouse model of mammary carcinoma (kindly provided by Dr. Fred Miller, Michigan Cancer foundation) that consists of low and highly metastatic mammary carcinoma cells and mammary-derived fibroblasts. In these experiments, we will pay special attention to plasma membrane localization. For example, cancer cells may induce secretion of latent MMPs into the media by the fibroblasts cells, as shown in **Figure 5**. However, active enzymes may localize in the cell surface. RT-PCR will be used in the co-culture experiments to determine the source of enzyme production. The system of breast tumor and stroma cells will be also examined for cathepsin expression and regulation (in collaboration with Dr. Bonnie Sloane)

During the second year, we will establish the *in situ* hybridization technique and species specific RT-PCR method (in collaboration with Dr. Markku Kurkinen) with the goal to study the expression of MMPs, TIMPs and cathepsins in the xenograft model of breast tumors using the MCF10 variants and malignant MDA-MB-435 cells.

## **7. CONCLUSIONS**

- 1.** Immortalized human breast epithelial cells secrete mainly proMMP-9 and very low levels of proMMP-2, and TIMPs. Phorbol ester and TNF- $\alpha$  induce proMMP-9 and TIMP-1 secretion into the media.
- 2.** Malignant MDA-435 breast cancer cells do not secrete MMPs in culture and are not inducible by phorbol ester.
- 3.** Primary fibroblasts from normal and malignant breast tissue secrete only proMMP-2, and TIMPs.
- 4.** Conditioned media from MDA-435 cells induce secretion and possibly activation of proMMP-2 in human lung fibroblasts.

5. Latent and activated MMP-9 free of TIMP-1 are associated with the plasma membrane of TPA-treated MCF10A cells. In the media, only latent enzyme in complex with TIMP-1 can be found.

6. Plasma membranes of untreated or TPA-treated MCF10A cells can activate proMMP-2.

## 8. REFERENCES

1. Liotta, L.A., Steeg, P.A., and Stetler-Stevenson, W.G. (1991) Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* 64, 327-336.
2. Testa, J.E., and Quigley, J.P. (1990) The role of urokinase-type plasminogen activator in aggressive tumor cell behavior. *Cancer Metastasis Rev.*, 9,353-367.
3. Sloane, B.F., Moin, K., and Lah, T. T. (1993) regulation of Lysosomal Endopeptidases in Malignant Neoplasia. In: *Aspects of the Biochemistry and Molecular Biology of Tumors*. T.G. Pretlow and T.P. Pretlow, eds. (Academic Press, NY), in press.
4. Pyke, C., Kristensen, P., Ralfkiaer, E., Grondhal-hansen, J., Eriksen, J., Blasi, F., and Dano, K. (1991) Urokinase-type plasminogen activator is expressed in stromal cells and its receptor in cancer cells at invasive foci in human colon adenocarcinomas. *Am. J. Pathol.*, 138, 1059-1067.
5. Basset, P., Bellocq, J.P., Wolf, C., Stoll, I., Hutin, P., Limacher, J.M., Podhajcer, O.L., Chenard, M.P., Rio, M.C. and Chambon, P. (1990) A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. *Nature* 348, 699-704.
6. Dabbous, M.K., North, S.M., Haney, L., and Nicolson, G.L. (1988) Macrophage and lymphocyte potentiation of syngeneic tumor cells and host fibroblast collagenolytic activity in rats. *Cancer Res.*, 48, 6832-6836.
7. Kataoka, H., DeCastro, R., Zucker, S., and Biswas, C. (1993) Tumor-cell derived collagenase-stimulatory factor increases expression of interstitial collagenase, stromelysin and 72 kDa gelatinase. *Cancer Res.* 53, 3154-3158.
8. Poulson, R., Hanby, A.M., Pignatelli, M., Jeffrey, R.E., Longcroft, J.M., Rogers, L., and Stamp, G.W. (1993) Expression of gelatinase A and TIMP-2 mRNAs in desmoplastic fibroblasts in both mammary carcinomas and basal cell carcinomas. *J. Clin. Pathol.* 46, 429-436.
9. Pyke, C., Ralfkiaer, E., Tryggvason, K., and Dano, K. (1993) Messenger RNA for two type IV collagenases is located in stromal cells in human colon cancer, *Am. J. Pathol.* 142, 359-365.
10. Poulson, R., Pignatelli, M., Stetler-Stevenson, W.G., Liotta, L.A., Wright, P.A., Jeffrey, R.E., Longcroft, J.A., Rogers, L., and Stamp, G.W.H. (1992) Stromal expression of 72 kDa type IV collagenase (MMP-2) and TIMP-2 mRNAs in colorectal neoplasia. *Am. J. Pathol.* 141, 389-396.
11. Autio-Harmanen, H., Karttunen, T., Hurskainen, Hoyhtya, M., Kauppila, A., and Tryggvason, K. (1993) Expression of 72 kDa type IV collagenase (gelatinase A) in benign and malignant ovarian tumors. *Lab. Invest.* 69, 312-321.
12. Baisi, A., Knopfel, M., and Keist, R. (1988) Tumor-host interactions in rabbit V2 carcinoma: stimulation of cathepsin B in host fibroblasts by a tumor-derived cytokine. *Invasion Metastasis*, 8, 143-158.
13. Ossowski, L., Clunie, G., Massucci, M-T., and Blasi, F. (1991) In vivo paracrine interaction between urokinase and its receptor: effect on tumor cell invasion. *J. Cell Biol.*, 115, 1107-1112.
14. He, C., Wilhelm, S.M., Pentland, A.P., Marmer, B.L., Grant, G.a., Eisen, A.Z., and Goldberg, G.I. (1989) Tissue cooperation in a proteolytic cascade activating human interstitial collagenase. *Proc. Natl. Acad. Sci. USA*, 86, 2632-2636.

15. Henry, N., van Lamsweerde A.L., and Vaes G. (1983) Collagen degradation by metastatic variants of Lewis lung carcinoma: cooperation between tumor cells and macrophage. *Cancer Res.* 43, 5321-5327.
16. Pyke, C., Kristensen, P., Ralfkiaer, E., Eriksen, J., and Dano, K. (1991) The plasminogen activation system in human colon cancer: messenger RNA for the inhibitor PAI-1 is located in endothelial cells in the tumor stroma. *Cancer Res.*, 51, 4067-4071.
17. Woessner, J.F. (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodelling. *FASEB J.* 5, 2145-2154.
18. Pei, D., and Weiss, S.J. (1995) Furin-dependent intracellular activation of the human stromelysin-3 zymogen. *Nature*, 375, 244-247.
19. Ward, R.V., Atkinson, S.J., Slocombe, P.M., Docherty, A.J.P., Reynolds, J.J., and Murphy, G. Tissue inhibitor of metalloproteinases-2 inhibits the activation of 72 kDa progelatinase by fibroblasts membranes. *Biochim. Biophys. Acta* 1079, 242-246, 1991.
20. Brown, P.D., Kleiner, D.E., Unsworth, E.J., and Stetler-Stevenson, W.G. Cellular activation of the 72 kDa type IV procollagenase/TIMP-2 complex. *Kidney Int.* 43, 163-170, 1993.
21. Strongin, A.Y., Marmer, B.L., Grant, G.A., and Goldberg, G.I. Plasma membrane-dependent activation of the 72 kDa type IV collagenase is prevented by complex formation with TIMP-2. *J. Biol.* 268, 14033-14039, 1993.
22. Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. A matrix metalloproteinase expressed in the surface of tumor cells. *Nature (Lond.)* 370, 61-65, 1994.
23. Overall, C.M. and Dodek, J. Concanavalin-A produces a matrix-degradative phenotype in human fibroblasts. Induction/endogenous activation of collagenase, 72 kDa gelatinase and pump-1 is accompanied by suppression of tissue inhibitor of metalloproteinases. *J. Biol. Chem.* 265, 21141-21151, 1990.
24. Overall, C.M. Regulation of tissue inhibitor of matrix metalloproteinase expression. *Ann. New York Acad. Sci.* 732, 51-64, 1994.
25. Azzam, H.S. and Thompson, E.W. Collagen-induced activation of 72,000 type IV collagenase in normal and malignant fibroblastoid cells. *Cancer Res.* 52, 4540-4544, 1992.
26. Carmichael, D.F., Sommer, A., Thompson, R.C., Anderson, D.C., Smith, C.G., Welgus, H.G., and Stricklin, G.P. (1986) Primary structure and cDNA cloning of human fibroblast collagenase inhibitor. *Proc. Natl. Acad. Sci. USA* 83, 2407-2411.
27. Goldberg, G.I., Marmer, B.L., Grant, G.A., Eisen, A.Z., Wilhelm, S.M., and He, C. (1989) Human 72-kilodalton type IV collagenase forms a complex with tissue inhibitor of metalloproteinase designated TIMP-2. *Proc. Natl. Acad. Sci. USA* 86, 8207-8211.
28. Stetler-Stevenson, W.G., Krutzsch, H.C., and Liotta, L.A. (1989) Tissue inhibitor of metalloproteinase (TIMP-2) *J. Biol. Chem.* 264, 17374-17378.
29. Pavloff, N., Staskus, P.W., Kishnani, N.S., and Hawkes, S.P. (1992) A new inhibitor of metalloproteinases from chicken: ChIMP-3. A third member of the TIMP family. *J. Biol. Chem.* 267, 17321-17326.
30. Wilhelm, S.M., Collier, I.E., Marmer, B.L., Eisen, A.Z., Grant, G.A., and Goldberg, G.I. (1989) SV40-transformed human lung fibroblasts secrete a 92 kDa type IV collagenase which is identical to that secreted by normal human macrophages. *J. Biol. Chem.* 264, 17213-17221.
31. Goldberg, G.I., Strongin, A., Collier, I.E., Genrich, L.T., and Marmer, B.L. (1992) Interaction of 92-kDa type IV collagenase with the tissue inhibitor of metalloproteinases prevents dimerization, complex formation with interstitial collagenase, and activation of the proenzyme with stromelysin. *J. Biol. Chem.* 267, 4583-4591.
32. Fridman, R., Bird, R.E., Hoyhtya, M., Oelkuct, M., Komarek, D., Liang, C-M., Berman, M.L., Liotta, L.A., Stetler-Stevenson, W.G., and Fuerst, T.R. (1993) Expression of human recombinant 72

kDa gelatinase and tissue inhibitor of metalloproteinase-2 (TIMP-2): characterization of complex and free enzyme. *Biochem. J.* 289, 411-416.

33. Strongin, A.Y., Collier, I., Bannikov, G., Marmer, B.L., Grant, G.A., and Goldberg, G.I. Mechanism of cell surface activation of 72 kDa type collagenase: Isolation of the activated form of the membrane metalloprotease. *J. Biol. Chem.* 270, 5331-5338, 1995.

34. Monteagudo, C., Merino, M.J., San-Juan, J., Liotta, L.A., and Stetler-Stevenson, W.G. (1990) Immunohistochemical distribution of type IV collagenase in normal, benign and malignant breast tissue. *Amer. J. of Path.* 136, 585-592.

35. D'Errico, A., Garbisa, S., Liotta, L.A., Castranovo, V., Stetler-Stevenson, W.G., and Grigioni, W.F. (1991) Augmentation of type IV collagenase, laminin receptor and Ki62 proliferation antigen associated with human colon, gastric and breast carcinoma progression. *Modern Path.* 4, 239-246.

36. Brown, P.D., Bloxidge, R.E., Anderson, E., and Howell, A. (1993) Expression of activated gelatinase in human invasive breast carcinoma. *Clin. Exp. Metastasis* 11, 183-189.

37. Polette, M., Clavel, C., Cockett, M., Bentzmann, S.G., Murphy, G., and Birembaut, P. Detection and localization of mRNAs encoding matrix metalloproteinases and their inhibitors in human breast pathology. *Invasion & Metastasis*, 13, 31-37, 1993.

38. Okada, A., Bellico, J-P, Rouyer, N., Chenard, M-P, Rio, M-C, Chambon, P., and Basset, P. (1995) Membrane-type matrix metalloproteinase (MT-MMP) gene is expressed in stromal cells of human colon, breast, and head and neck carcinomas. *Proc. Natl. Acad. Sci. USA*, 92, 2730-2734.

39. Ogata, Y., Enghild, J.J. and Nagase, H. Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. *J. Biol. Chem.* 267, 3581-3584, 1992.

40. O'Connell, J.P., Willenbrock, F., Docherty, A.J.P., Eaton, D., and Murphy, G. Analysis of the role of the COOH-terminal domain in the activation, proteolytic activity and tissue inhibitor of metalloproteinase interactions of gelatinase B. *J. Biol. Chem.* 269, 14967-14973, 1994.

41. Okada, Y., Gonoji, Y., Naka, K., Tomita, K., Nakanishi, I., Iwata, K., Yamashita, K., and Hayakawa, T. Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) from HT1080 human fibrosarcoma cells. *J. Biol. Chem.* 267, 21712-21719, 1992.

42. Menashi, S., Fridman, R., Desrevieres, S., Lu, H., Legrand, Y., and Soria, C. Regulation of 92-kDa gelatinase B activity in the extracellular matrix by tissue kallikrein. *Ann. New York Acad. Sci.* 732, 466-468, 1994.

43. Soule, H.D., Maloney, T.M., Wolman, S.R., Peterson, W.D., Brenz, R., McGrath, C.M., Russo, J., Pauley, R.J., Jones, R.F., and Brooks, S.C. (1990) Isolation and characterization of a spontaneously immortalized human breast epithelial cell line, MCF-10. *Cancer Res.* 50, 6075-6086.

44. Miller, F.R., Soule, H.D., Tait, L., Pauley, R.J., Wolman, S.R., Dawson, P.J., and Heppner, G.H. (1993) Xenograft model of progressive human proliferative Breast Disease. *J. Natl. Cancer Inst.* 85, 1725-1732.

45. Bartow, S.A. (1993) Toward a model for early stages of human breast carcinogenesis. *J. Natl. Cancer Inst.* 85, 1710.

46. Basolo, F., Elliot, J., Tait, L., Chen, X.Q., Maloney, T., Russo, I.H., Pauley, R., Momoki, S., Caamano, J., Klein-Szanto, A.J.P., Koszalka, M., and Russo, J. (1991) Transformation of human breast epithelial cells by c-Ha-ras oncogene. *Mol. Carcinog.* 4, 25-35.

47. Price, J.E., Polyzos, A., Zhang, R.D., and Daniels, L.M. (1990) Tumorigenicity and metastasis of human breast carcinoma cell lines in nude mice. *Cancer Res.* 50, 717-721.



## ENHANCED EXPRESSION OF TISSUE INHIBITOR OF METALLOPROTEINASE-2 (TIMP-2) IN THE STROMA OF BREAST CARCINOMAS CORRELATES WITH TUMOR RECURRENCE

Daniel W. VISSCHER<sup>1</sup>, Matti HÖYHTYÄ<sup>2</sup>, Susan K. OTTOSEN<sup>1</sup>, Chi-Ming LIANG<sup>3</sup>, Fazlul H. SARKAR<sup>1</sup>, John D. CRISSMAN<sup>1</sup> and Rafael FRIDMAN<sup>1,4</sup>

<sup>1</sup>Department of Pathology, Wayne State University School of Medicine, Detroit, MI 48201, USA; <sup>2</sup>Biocenter and Department of Biochemistry, University of Oulu, FIN-90570, Oulu, Finland; and <sup>3</sup>Oncologix, Inc., Gaithersburg, MD 20878, USA.

**The 72-kDa (MMP-2, gelatinase A) and the 92-kDa (MMP-9, gelatinase B) matrix metalloproteinases have been associated with tumor cell invasion and metastasis. Immunohistological staining of MMP-2 and MMP-9, basal lamina collagen IV and TIMP-2 were performed on frozen sections of 83 invasive breast carcinomas. MMP-2 and MMP-9 were associated with neoplastic cell plasma membrane in 72% of cases and exhibited inter-tumoral variability of staining intensity. MMP-2 and MMP-9 staining was not correlated with presence of metastases at time of diagnosis or with disease outcome. TIMP-2 was detected in the peri-tumoral stroma and was present in 87% of cases. Residual benign breast tissue was negative for TIMP-2 staining. Neoplasms with diffuse TIMP-2 staining (24%) recurred significantly more frequently (75% recurred) than cases with focal (42% recurred) or absent (27% recurred) TIMP-2. Presence of collagen IV was negatively correlated with gelatinase staining. We conclude that up-regulation of MMP-2 and MMP-9 expression in breast tumor cells is reciprocally correlated to collagen IV staining. Clinical outcome, however, is more closely related to the presence of TIMP-2 than the corresponding MMPs. Enhanced TIMP-2 expression, therefore, may denote a stromal response to tumor invasion, indicative of aggressive behavior in a subset of breast carcinomas.**

© 1994 Wiley-Liss, Inc.

The early proliferative stages of breast cancer are characterized by a continuous basement membrane (BM) separating the hyperplastic epithelial cells from the surrounding stroma. Pathologically, the transition from *in situ* to invasive carcinoma is usually accompanied by disorganization and discontinuity of the BM. It is generally believed that an enhanced process of proteolysis of both the BM and the stromal extracellular matrix (ECM) contributes to the escape of breast cancer cells into the neighboring tissues, eventually leading to the formation of distant metastases. A group of enzymes thought to play a role in tumor cell invasion are the matrix metalloproteinases (MMPs), a family of related zinc-dependent proteinases capable of degrading ECM components (Matrisian, 1990). Much attention has been focused on the 72-kDa (MMP-2, gelatinase A) and the 92-kDa (MMP-9, gelatinase B) gelatinases/type IV collagenases (Liotta *et al.*, 1991), 2 members of the MMP family active against collagen IV, the major structural collagen of BM. Immunohistochemical studies showed elevated expression of MMP-2 in the tumor cells of malignant breast tissue (Monteagudo *et al.*, 1990; D'Errico *et al.*, 1991). In another study, mRNAs for MMP-2 and TIMP-2 were localized in desmoplastic fibroblasts of the stroma of breast carcinomas (Poulsom *et al.*, 1993). Basset *et al.* (1990) reported elevated mRNA levels for MMP-9 in breast carcinoma as compared to adenomas. Gelatin zymography of breast tumor extracts showed activation of MMP-2 in 15/20 tumors, whereas no activation or trace amounts of the active form of MMP-9 were observed (Brown *et al.*, 1993). Therefore, elevated expression of MMP-2 and MMP-9 is associated with breast carcinomas. However, little is known about the correlation between enzyme expression and disease outcome in breast tumors.

The enzymatic activity of MMP-2 and MMP-9 is inhibited by the tissue inhibitors of metalloproteinases (TIMPs) (Matrisian, 1990), a family of specific naturally occurring inhibitors of

MMPs. TIMP-2, one member of the TIMP family, forms a complex with latent pro-MMP-2 and inhibits the active forms of both the 72- and the 92-kDa enzymes (Goldberg *et al.*, 1992; Fridman *et al.*, 1993). TIMP-2 inhibits *in vitro* tumor cell invasion, and it has been suggested that a balance between TIMPs and activated gelatinases may determine the extent of ECM degradation and remodeling occurring during tumor cell invasion (Liotta *et al.*, 1991). We have previously shown that breast carcinomas contain elevated levels of TIMP-2 localized, usually, in the tumor stroma (Höyhty *et al.*, 1994). However, the findings were not correlated with markers of breast cancer progression. In this study, we evaluated the immunohistochemical distribution of TIMP-2 along with that of MMP-2 and MMP-9 and collagen IV in 83 cases of invasive breast carcinoma. The staining of these proteins was correlated with tumor stage, lymph node status, metastases and disease outcome. The data show a positive correlation of TIMP-2 stromal immunostaining with tumor recurrence and suggest a role for TIMP-2 in breast cancer progression.

### MATERIAL AND METHODS

#### Monoclonal antibodies (MAbs)

Female BALB/c mice were immunized with emulsion of Freund's complete adjuvant and natural pro-MMP-2/TIMP-2 complex (10 µg antigen in 100 µl/mouse) isolated from conditioned media of human melanoma A2058 cells. The final boost and the pre-fusion boost were performed with either purified human recombinant TIMP-2 or recombinant MMP-2 produced in a recombinant vaccinia virus mammalian cell expression (Vac/T7), system as previously described (Fridman *et al.*, 1993). Another group of mice was immunized with purified human recombinant pro-MMP-9 obtained in the Vac/T7 system. Mouse myeloma cells (P3-X63-Ag8.653) and spleen cells from the immunized mice were fused using standard protocols. Hybrid cells were selected on 96-well plates in HAT media (Dulbecco's modified Eagle media supplemented with 20% FPS, 2% Origen, 0.1 mM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine). Wells containing colonies after 10-14 days were screened for antibody production by ELISA, immunoblots and immunoprecipitation as previously described (Höyhty *et al.*, 1994). Characterization of the mouse MAbs CA-406 (anti-MMP-2) and T2-101 (anti-TIMP-2) has been previously reported (Höyhty *et al.*, 1994). The MAb CA-209 against human MMP-9 recognizes both the native and the denatured enzyme as well as the precursor (92-kDa) and active (85-kDa) forms and a pro-MMP-9/TIMP-1 complex, suggesting that the epitope of the antibody does not involve the TIMP-1 binding region. In addition, CA-209 recognizes pro-MMP-9 produced by human fibrosar-

<sup>4</sup>To whom correspondence and reprint requests should be addressed, at Department of Pathology, Wayne State University School of Medicine, 540 E. Canfield, Detroit, MI 48201, USA. Fax: (313) 577-8180.

## DISCUSSION

MABs were used to evaluate the distribution of MMP-2, MMP-9 and TIMP-2 in invasive breast carcinomas. These findings were correlated with collagen IV staining, lymph node status, presence of distant metastases and tumor recurrence. We have found that TIMP-2 is localized in the stroma of invasive tumors and in the tumor-stroma interface region in areas with *in situ* carcinoma. A similar localization of TIMP-2 in breast tumors was also reported in previous studies using immunohistochemistry (Höyhty *et al.*, 1994) and *in situ* mRNA hybridization (Poulsom *et al.*, 1993). The major finding of this study, however, was the apparent association between elevated TIMP-2 immunoreactivity and disease aggressiveness in breast carcinoma. However, we would caution that the clinical outcome data are limited by relatively short follow-up intervals, the stage heterogeneity of our series as well as the statistical methods employed. Multivariate analysis would be required to demonstrate that TIMP-2 immunostaining was independent of other parameters such as tumor differentiation. The correlation of TIMP-2 over-expression with impaired survival may seem paradoxical given the known ability of TIMP-2 to inhibit MMP activity and tumor cell invasion (Matrisian, 1990; Liotta *et al.*, 1991). However, elevated levels of TIMP-1 were also found in human colon tumors compared to normal mucosa (Lu *et al.*, 1991). We have recently observed a positive correlation between intense stromal TIMP-2 immu-

nostaining and adverse outcome in transitional carcinoma of the bladder (Grignon *et al.*, 1994). Thus strong immunoreactivity with our anti-TIMP-2 MAB (T2-101) characterizes an aggressive subset of epithelial malignancies. The high levels of TIMPs may represent a mechanism by which the tumor stroma controls the proteolysis and remodeling of ECM that occurs during invasion of tumor cells with abundant and activated MMPs. It was interesting to find TIMP-2 in the peri-ductal stroma in areas of carcinoma *in situ*, suggesting that TIMP-2 expression may constitute an early stromal response to tumor invasion. Stromal TIMP-2 may also exert other effects on cells possibly related to its growth promoting activity (Nemeth and Goolsby, 1993), which may play a role in breast cancer progression.

Our data with TIMP-2 are analogous with other protease systems in breast carcinoma. Jänicke *et al.* (1993) reported that levels of plasminogen activator inhibitor-1 were more closely correlated with impaired survival in breast carcinoma patients than levels of urokinase plasminogen activator. Thus enhanced protease inhibitor expression in some tumor systems may be a more accurate reflection of ECM remodeling, and thereby invasive phenotype, than levels of corresponding protease. In this study, for example, staining of both the 72- and the 92-kDa enzymes failed to correlate significantly with distant metastases or poor outcome. A lack of correlation between the level of MMP-2 mRNA and disease severity was reported in 32

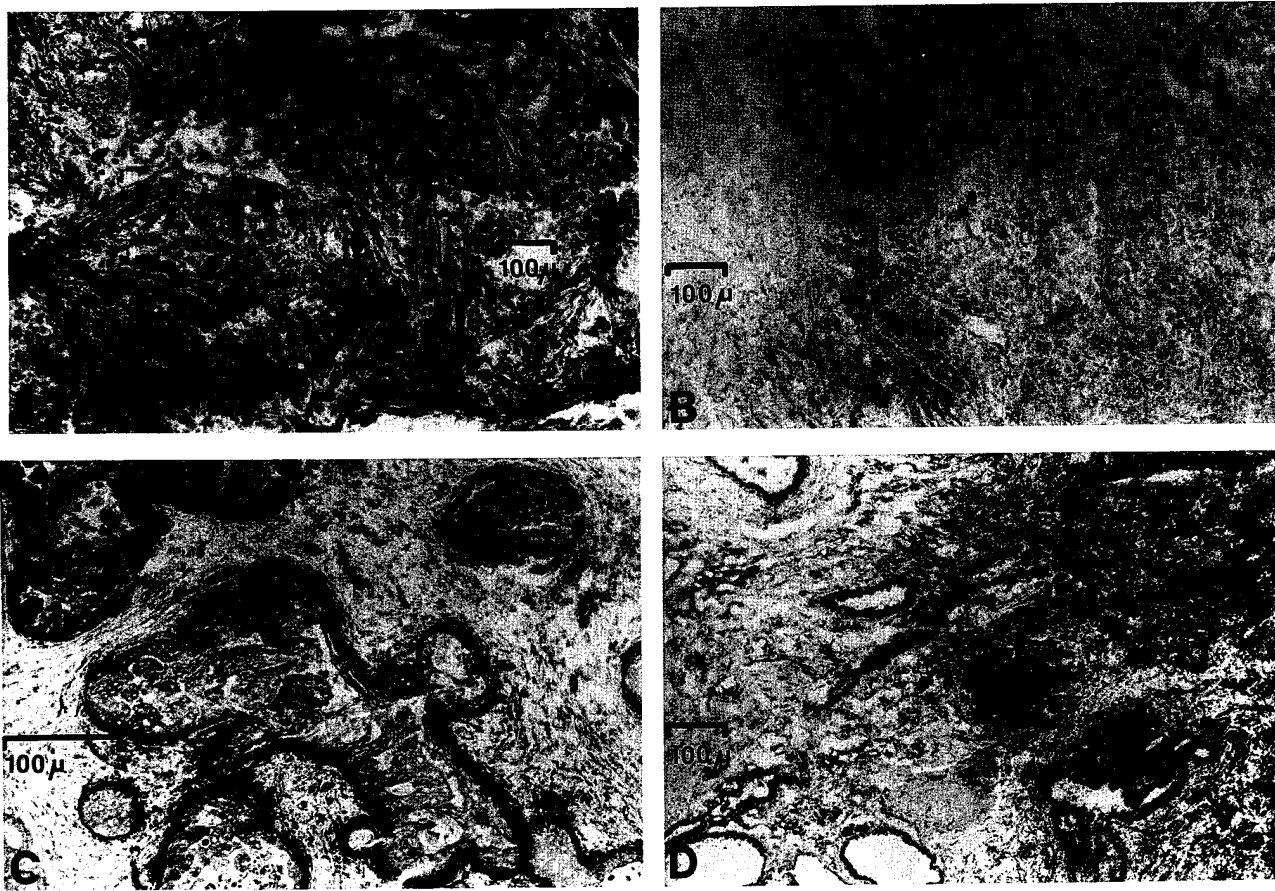


FIGURE 2 – TIMP-2 immunostaining in breast carcinoma tissue. Frozen sections of invasive breast carcinoma specimens were stained with a MAB to human TIMP-2 as described in “Material and Methods”. (a) Diffuse strong TIMP-2 staining in peri-tumoral stroma. (b) Stromal TIMP-2 immunostaining with accentuation at the “advancing front” of the neoplasm (tumor on right, benign on left). (c) Peri-ductal TIMP-2 immunostaining in area of carcinoma *in situ*. (d) Presence of TIMP-2 immunostaining in area of neoplasm (top right) but not in residual benign breast tissue (left). Magnification (a, b, d)  $\times 100$ , (c)  $\times 200$ .

biopsies of malignant breast tumors (Davies *et al.*, 1993a). Likewise, MMP-2 levels did not correlate with tumor grade or stage in prostatic adenocarcinoma. However, MMP-2 expression was higher in the prostate cancer cells than in the benign hyperplastic epithelial cells (Boag and Young, 1994). In bladder carcinomas, however, the level of MMP-2 and MMP-9 expression correlated with tumor grade (Davies *et al.*, 1993b). Differences between tumor types, sample size, fixation and method of detection may account for these differences. The MABs used here, however, cannot distinguish between latent and active enzymes. Thus a correlation between metastasis/recurrence and presence of active enzymes could not be done. In the study of Davies *et al.* (1993a), however, the amount of active MMP-2, as determined by zymography, was higher in breast malignant tissue. In any case, the molecular complexity of the ECM is such that invasion of neoplastic cells may require the cooperative activity of multiple protease species. We have previously shown that in breast carcinomas clinical outcome is more closely correlated with immunostaining for multiple protease species (Visscher *et al.*, 1993).

An interesting finding of our study was the correlation between absence of collagen IV in the basal lamina and intense staining of the 2 gelatinases. Also, most of the tumors with abundant basal lamina showed low levels of TIMP-2 staining. These results suggest that degradation of collagen IV in breast tumors may be a consequence of a balance between gelatinases and TIMPs, though the presence of TIMP-1 was not examined in these specimens. However, abnormal collagen IV gene expression and secretion in the tumors may also account for the absence of basal lamina.

The location of the gelatinases or TIMP-2 as epithelial or stromal described here does not necessarily mean origin, or synthesis, within these particular cellular components of the tumor. In fact, immunolocalization of TIMP-2 and MMP-2 in some tumors differs from findings using *in situ* hybridization, which demonstrated expression of both mRNAs in the stroma of various human carcinomas including breast (Poulsom *et al.*, 1993), cutaneous basal cell (Poulsom *et al.*, 1993), colon (Pyke *et al.*, 1993) and ovarian (Autio-Harmanen *et al.*, 1993). In another study (Davies *et al.*, 1993a), MMP-9 mRNA and protein in malignant breast tissue were both confined to the tumor stroma, as opposed to the results presented here where MMP-9 staining was present in the tumor cells in 83% of the cases, whereas only 17% showed very weak stromal MMP-9 staining. The cause for this discrepancy is unknown but may be related to the antibodies used (polyclonal vs. monoclonal) and

TABLE III - RELATIONSHIP BETWEEN MMP-2, MMP-9 AND COLLAGEN IV IMMUNOSTAINING

Enzymes	Collagen IV		
	Absent	Focal	Diffuse
Negative	6/6 <sup>1</sup>	14/20	3/4
Weak	5/10	13/11	8/7
Strong	20/15	14/10	0/0

$p < 0.001$  statistically significant associations (both enzymes), chi square test.<sup>1</sup>Number of cases in each group, epithelial staining only.

the epitopes recognized. Stromal staining for MMP-2 was present in 42% of the cases; however, the staining was always significantly weaker than the staining of MMP-2 in the tumor cells. Studies have shown that urokinase can localize in the plasma membrane of tumor cells *via* specific receptors following synthesis and secretion by stromal cells (Pyke *et al.*, 1991). Whether this is the case with MMP-2 and MMP-9 remains to be determined. In many tumor samples examined here, both enzymes were localized in the membrane of the neoplastic cells. Studies with a human breast cancer cell line have shown binding of MMP-2 to the cell surface (Emonard *et al.*, 1992). In addition, activation of pro-MMP-2 appears to be mediated by interaction of the enzyme precursor with the plasma membrane and this activation is inhibited by TIMP-2 (Strongin *et al.*, 1993). It is plausible that, *in vivo*, the differential tissue expression of TIMP-2 and 72-kDa enzyme may contribute to the existence of a pool of TIMP-2 free pro-MMP-2 capable of undergoing complete activation when associated with the tumor cell membrane, thus facilitating ECM degradation. If so, tumor invasion is not only a consequence of an imbalance production of proteinases and inhibitors but it may also due to a differential tissue and cellular localization of gelatinases and their inhibitors as shown here.

In summary, our data show that MMP-2 and MMP-9 are frequently co-over-expressed in breast carcinomas and further, expressed reciprocally in relation to collagen IV. Enzyme immunostaining failed to predict for adverse disease outcome. However, intense staining for TIMP-2 with our MAB defined an aggressive group of breast neoplasms. These results suggest a role for the gelatinases and TIMP-2 in breast carcinoma progression. The nature of this role, however, is likely complex and modulated by dynamic host-tumor interactions.

#### REFERENCES

- AUTIO-HARMAINEN, H., KARTTUNEN, T., HURSKAINEN, HÖYHTYÄ, M., KAUPPILA, A. and TRYGGVASON, K., Expression of 72-kDa type IV collagenase (gelatinase A) in benign and malignant ovarian tumors. *Lab. Invest.*, **69**, 312-321 (1993).
- BASSET, P., BELLOCO, J.P., WOLF, C., STOLL, I., HUTIN, P., LIMACHER, J.M., PODHAJEC, O.L., CHENARD, M.P., RIO, M.C. and CHAMBON, P., A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. *Nature (Lond.)*, **348**, 699-704 (1990).
- BOAG, A.H. and YOUNG, I.D., Increased expression of the 72-kDa type IV collagenase in prostatic adenocarcinoma. *Amer. J. Pathol.*, **144**, 585-591 (1994).
- BROWN, P.D., BLOXIDGE, R.E., ANDERSON, E. and HOWELL, A., Expression of activated gelatinase in human invasive breast carcinoma. *Clin. exp. Metastasis*, **11**, 183-189 (1993).
- DAVIES, B., MILES, D.W., HAPPERFIELD, L.C., NAYLOR, M.S., BOBROW, L.G., RUBENS, R.D. and BALKWILL, F.R., Activity of type IV collagenases in benign and malignant breast disease. *Brit. J. Cancer*, **67**, 1126-1131 (1993a).
- DAVIES, B., WAXMAN, J., WASAN, H., ABEL, P., WILLIAMS, G., KRAUSZ, T., NEAL, D., THOMAS, D., HANBY, A. and BALKWILL, F.R., Levels of matrix metalloproteinases in bladder cancer correlate with tumor grade and invasion. *Cancer Res.*, **53**, 5365-5369 (1993b).
- D'ERRICO, A., GARBISA, S., LIOTTA, L.A., CASTRANOVO, V., STETLER-STEVENSON, W.G. and GRIGIONI, W.F., Augmentation of collagenase IV, laminin receptor and Ki67 proliferation antigen associated with human colon, gastric and breast carcinoma progression. *Mod. Pathol.*, **4**, 239-246 (1991).
- EMONARD, H.P., REMACLE, A.G., NOEL, A.C., GRIMAUD, J.-A., STETLER-STEVENSON, W.G. and FOIDART, J.-M., Tumor cell surface-associated binding site for the Mr 72,000 collagenase IV. *Cancer Res.*, **52**, 5845-5848 (1992).
- FRIDMAN, R., BIRD, R.E., HÖYHTYÄ, M., OELKUCI, M., KOMAREK, D., LIANG, C.-M., BERMAN, M.L., LIOTTA, L.A., STETLER-STEVENSON, W.G. and FUERST, T.R., Expression of human recombinant 72-kDa gelatinase and tissue inhibitor of metalloproteinase-2 (TIMP-2): characterization of complex and free enzyme. *Biochem. J.*, **289**, 411-416 (1993).
- GOLDBERG, G.I., STRONGIN, A., COLLIER, I.E., GENRICH, L.T. and MARMER, B.L., Interaction of 92-kDa type IV collagenase with the tissue inhibitor of metalloproteinases prevents dimerization, complex formation with interstitial collagenase, and activation of the proenzyme with stromelysin. *J. Biol. Chem.*, **267**, 4583-4591 (1992).

# Activation of Progelatinase B (MMP-9) by Gelatinase A (MMP-2)<sup>1</sup>

Rafael Fridman,<sup>2</sup> Marta Toth, Daniel Peña, and Shahriar Mobashery

Departments of Pathology [R. F., M. T., D. P.] and Chemistry [S. M.], Wayne State University, Detroit, Michigan 48201

## ABSTRACT

The  $M_r$  72,000 (MMP-2; gelatinase A) and  $M_r$  92,000 (MMP-9; gelatinase B) gelatinases are two members of the family of matrix metalloproteinases (MMPs). These proteinases are thought to play a critical role in tumor cell invasion and are frequently coexpressed in human cancers. Gelatinases are secreted in a latent inactive form, and their conversion to the active species can be accomplished by other proteolytic enzymes, including other MMPs. We report herein that organomercurial or plasma membrane-activated  $M_r$  72,000 gelatinase A activates progelatinase B to an  $M_r$  82,000 active form in a process inhibited by tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2. Progelatinase B activation was accomplished by the two active species of gelatinase A, the  $M_r$  62,000 and  $M_r$  45,000 forms, generated after plasma membrane or organomercurial activation of TIMP-2-free progelatinase A. The  $M_r$  45,000 species of gelatinase A lacks both the  $\text{NH}_2$ -terminal profragment and the COOH-terminal domain known to play a role in plasma membrane activation and the regulation of TIMP-2 inhibition. These results suggest a novel mechanism of activation of progelatinase B mediated by gelatinase A species that may be localized in the surface of tumor cells and enhance matrix degradation during cancer metastasis.

## INTRODUCTION

Tumor metastasis requires proteolytic degradation of ECM<sup>3</sup> components to facilitate the invasion of basement membranes and connective tissue matrices by the malignant cancer cells. Several groups of proteases have been implicated in tumor cell invasion including MMPs (1, 2), serine proteases (3), and cysteine proteases (4). The  $M_r$  72,000 gelatinase A/type IV collagenase (MMP-2; Ref. 5) and the  $M_r$  92,000 gelatinase B/type IV collagenase (MMP-9; Ref. 6) are two members of the MMP family postulated to play a critical role in tumor invasion and angiogenesis (1, 7). Elevated levels of these enzymes were reported in human cancers (7-14), and the metastatic potential of tumor cells in experimental models of metastasis has been correlated with the expression and activity of these proteinases (reviewed in Ref. 7). The association of the gelatinases with malignancy may be related to their ability to degrade basement membrane collagen IV to yield 1/4  $\text{NH}_2$ -terminal and 3/4 COOH-terminal fragments (5, 6, 15). For that reason, these proteinases are also known as type IV collagenases (5, 6). In addition, they degrade gelatin and thus are named gelatinases (2, 16). Gelatinases degrade other ECM components *in vitro*, including collagens V, VII and XI; fibronectin; laminin (2, 5, 6, 16); elastin (17, 18); proteoglycans (18-20); and entactin (21), although with different efficiencies and probably involving different cleavage sites. They may also attack other biologically relevant molecules. For example, gelatinase A hydrolyzes the  $\text{Lys}^{16}$ - $\text{Leu}^{17}$  peptide bond of a synthetic decapeptide representing the soluble  $\beta$ -amyloid sequence of amino-acid residues 10-22 (22) and the  $\text{Lys}^{16}$ - $\text{Leu}^{17}$  and  $\text{Met}^{35}$ - $\text{Val}^{36}$

peptide bonds of  $\beta$ -amyloid peptides 1-40 and 1-42, respectively, isolated from brains of Alzheimer's disease patients (23). We have shown recently that the gelatinases can hydrolyze galectin-3, a cell surface lectin involved in cell-cell and cell-matrix interactions and metastasis (24). Thus, the spectrum of proteins that can be potentially cleaved by these MMPs suggests an important role for these proteinases in the regulation of various biological processes.

The progelatinases, like other members of the MMP family, are secreted in a latent form that requires activation (1, 2, 16). Thus, proenzyme activation is a critical event in the regulation of gelatinase activity and may be essential for ECM degradation during tumor cell invasion (1, 7). The physiological mechanisms responsible for activation of the progelatinases are not completely understood but may involve the action of other proteases, including other MMPs. Previous studies (25-27) have shown a plasma membrane-dependent activation specific for progelatinase A, possibly mediated by a recently identified MT-MMP (28). The plasma membrane-dependent activation of progelatinase A is induced in cultured cells by treatment with phorbol ester (26, 27), concanavalin A (25, 26, 29, 30), transforming growth factor  $\beta$  (26), or a collagen substrate (31). Plasma membrane activation of progelatinase A was shown to generate the reported  $M_r$  62,000 active species with the  $\text{NH}_2$ -terminal sequence starting at  $\text{Tyr}^{81}$  but also to generate an additional active species of  $M_r$  41,000-45,000 (27) with high specific activity (29, 30). A similar pattern of activation was observed after organomercurial activation of progelatinase A free of TIMP-2 (32, 33), a specific inhibitor of the  $M_r$  72,000 enzyme known to form a noncovalent complex with the proenzyme form (34, 35). We (32) have reported previously that the  $M_r$  45,000 species showed an electrophoretic mobility similar to a recombinant truncated gelatinase A lacking the COOH-terminal domain, suggesting that formation of the  $M_r$  45,000 form also involved a cleavage at the COOH-terminal region. Interestingly, the COOH-terminal domain of progelatinase A is the TIMP-2 binding domain (32, 36-38) in the proenzyme form. Removal of this domain does not impair catalytic activity but reduces the rate of TIMP-2 inhibition (32, 37, 38). Thus, the nature of the active species of gelatinase A, formed after plasma membrane activation, may regulate enzyme activity and inhibition by TIMP-2.

Progelatinase B, in contrast to the  $M_r$  72,000 enzyme, appears not to be activated by a plasma membrane-dependent mechanism and is usually detected in the culture media of normal and tumor cells in a latent form (25-27). Studies with purified enzymes, however, have shown the ability of several proteases to activate progelatinase B. These include stromelysin-1 (MMP-3; Refs. 39 and 40), plasmin (41), and tissue kallikrein (42). Activation of progelatinase B with stromelysin-1, which is most efficient (40), generates an  $M_r$  82,000 active species with enzymatic activity (39, 40). The coordinated regulation of progelatinase B and stromelysin-1 expression by cytokines in certain cells has been suggested to facilitate progelatinase B activation (39). However, in some tumors, these enzymes are not always coexpressed, in contrast to gelatinase A and B. For example, high levels of gelatinase A and B were detected in breast tumors (43-45), whereas stromelysin-1 mRNA was undetectable (43). We have also reported enhanced expression of both gelatinases in breast tumors by immunohistochemistry (46). High levels of both gelatinases were also reported in colon (10) and bladder (14) cancers. Due to the frequent

Received 1/13/95; accepted 4/17/95.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>1</sup> This work was supported in part by Department of Defense Grant DAMD17-94-J-4356 (to R. F.).

<sup>2</sup> To whom requests for reprints should be addressed, at Department of Pathology, School of Medicine, Wayne State University, 540 East Canfield Avenue, Detroit, MI 48201.

<sup>3</sup> The abbreviations used are: ECM, extracellular matrix; MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; TIMP, tissue inhibitor of metalloproteinase; APMA, *p*-aminophenylmercuric acetate.

coexpression of the  $M_r$  72,000 and  $M_r$  92,000 gelatinases in human tumors, we chose to investigate the ability of these enzymes to activate each other. Here we show that gelatinase A can activate progelatinase B to generate an  $M_r$  82,000 active form. This process was observed after plasma membrane activation of progelatinase A and could be mediated by either of the active species of gelatinase A. In addition, progelatinase B activation by gelatinase A was inhibited by TIMP-1 and TIMP-2. These results suggest a novel, but not exclusive, mechanism for progelatinase B activation that may enhance ECM degradation in certain tumors and possibly contribute to tumor cell invasion.

## MATERIALS AND METHODS

### Expression and Purification of Recombinant Enzymes and Inhibitors.

Human recombinant progelatinase A, progelatinase B, TIMP-2, and TIMP-1 were all expressed in mammalian cells using a recombinant vaccinia virus expression system (Vac/T7), as described previously (32, 47). The cDNA for human progelatinase B was a generous gift from Drs. K. Tryggvason and A. Tuuttila (University of Oulu, Oulu, Finland). TIMP-1 cDNA was kindly provided by Dr. Stetler-Stevenson (NIH, Bethesda, MD). Recombinant vaccinia viruses containing either the progelatinases or TIMP cDNAs were obtained by homologous recombination as described previously (32, 47).

Pregelatinase A and B were purified from the media (Opti-MEM I; GIBCO, Grand Island, NY) of HeLa cells infected with the appropriate recombinant viruses, as described previously (32, 33). TIMP-2 was purified by affinity chromatography using a mAb (CA-101) against human TIMP-2, as described previously (32, 33). TIMP-1 was purified from the media of infected HeLa cells using a lentil lectin-Sepharose 4B (Sigma Chemical Co.); the column was equilibrated with 20 mM HEPES (pH 7.5), 500 mM NaCl, 1 mM  $\text{CaCl}_2$ , 10% glycerol, 0.05% Brij-35, and 0.02%  $\text{NaN}_3$  (48). TIMP-1 was eluted with 500 mM methyl  $\alpha$ -D-mannopyranoside (Sigma) diluted in the same buffer, dialyzed against 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 5 mM  $\text{CaCl}_2$ , and 0.02% Brij-35 (collagenase buffer), concentrated in a Centricon-10 concentrator (Amicon, Beverly, MA), and purified by reverse phase-HPLC (48). The concentrations of the purified enzymes and TIMPs were determined by amino acid analysis (49).

**Purification of Gelatinase A Active Species.** To isolate the active forms of gelatinase A, 1–2 mg of progelatinase A in 20 mM Tris/HCl (pH 7.5), 5 mM  $\text{CaCl}_2$ , and 0.02% Brij-35 were activated with 1 mM APMA for 1 h at 37°C. The activated gelatinase A was applied to a red-agarose (Sigma) column (10 x 0.5 cm) equilibrated with the same buffer. A gradient of 0.05–3 M NaCl in equilibrating buffer was then applied to the column. The  $M_r$  45,000 species was recovered in the void volume, whereas the  $M_r$  62,000 species eluted with 3 M NaCl. To separate other activation fragments from the  $M_r$  62,000 species, the sample was dialyzed against collagenase buffer and was subjected to gelatin affinity chromatography. Three fragments of  $M_r$  32,000, 26,000, and 12,000 were recovered in the flow through, whereas the  $M_r$  62,000 species was eluted from the gelatin column with 10% DMSO in the collagenase buffer. The purified  $M_r$  62,000 and  $M_r$  45,000 species were concentrated with a Centricon-10 concentrator and analyzed by zymography and SDS-PAGE. Protein concentrations of the purified active species were determined by amino acid analysis (49).

**Microsequence Analysis.** The activation species of either gelatinase A or gelatinase B were separated by SDS-PAGE under reducing conditions and transferred to an Immobilon membrane (Millipore, Marlboro, MA). The transferred proteins were stained with 1% amido black in 20% isopropanol and 10% acetic acid, and the appropriate stained bands were cut out of the membrane. The  $\text{NH}_2$ -terminal sequence of the immobilized proteins was determined on an Applied Biosystems 475A gas-phase protein sequencer.

**Enzyme Assays.** Zymography was performed in 10% SDS-polyacrylamide gels containing 0.1% gelatin using precast mini-gels from Novex (Encinitas, CA), as described earlier (32, 33). Samples of the purified enzymes were resuspended in the Laemmli sample buffer without reducing agents and were not subsequently heated. Gelatinase activity was determined using heat-denatured rat  $^3\text{H}$ collagen type I (18,000 cpm/ $\mu\text{g}$ ; Dupont NEN, Wilmington, DE) as the substrate (~60,000 cpm/reaction) as described previously (32, 33).

**Immunoblots.** Purified samples were subjected to SDS-PAGE under reducing conditions. The separated proteins were transferred to nitrocellulose paper. After blocking with 3% BSA and 3% nonfat dry milk in 50 mM Tris/HCl (pH 7.5), the blots were incubated with the corresponding primary antibody diluted in 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween 20. The immunodetection of the antigen was performed using the immunoperoxidase ABC kit (Vector Laboratories). The characterization of the mAbs to the gelatinases and TIMP-2 was described previously (46, 50). A rabbit polyclonal antibody against a synthetic peptide comprising residues 513 to 530 of human progelatinase A was a generous gift from Dr. Steven Ledbetter (Upjohn Co., Kalamazoo, MI).

**Activation of Progelatinases.** Progelatinase A was activated with either APMA or plasma membrane. For APMA activation, progelatinase A in collagenase buffer was incubated (30 min at 37°C) with 1 mM APMA (final concentration) prepared from a 10X APMA solution in 50 mM NaOH. To remove APMA, the active gelatinase A was applied to a Quick-Spin column (Boehringer Mannheim, Indianapolis, IN) that was equilibrated previously with collagenase buffer. The concentration of active gelatinase A was then determined by titration with a known amount of TIMP-2, assuming a 1:1 stoichiometry for complete inhibition (32, 33, 51). Plasma membrane activation of progelatinase A (27) was performed using plasma membranes isolated from HT1080 cells treated with phorbol ester (a generous gift from Dr. Greg Goldberg, Washington University, St. Louis, MO). Briefly, progelatinase A (50–200 ng) diluted in 25 mM HEPES/KOH (pH 7.5) and 0.1 mM  $\text{CaCl}_2$  was incubated (1–3 h) with 10  $\mu\text{g}$  of isolated plasma membrane, as described previously (27). The presence of active gelatinase A species was determined by zymography or immunoblot analysis.

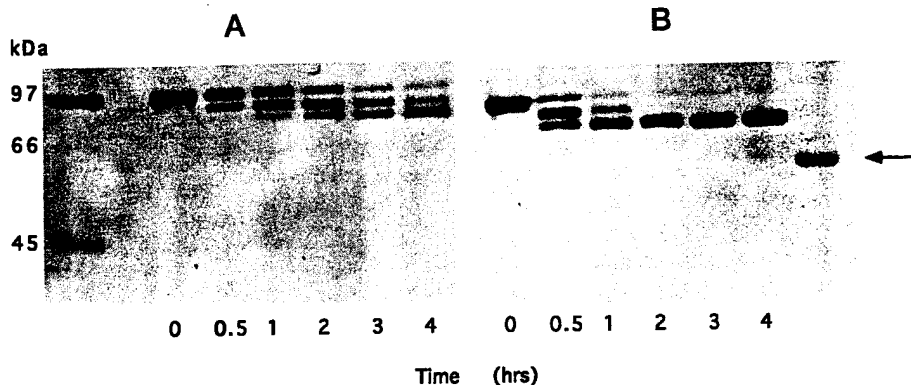
To activate progelatinase B with gelatinase A, progelatinase B was diluted in collagenase buffer, and each solution was individually incubated (37°C for various periods of time) with either activated gelatinase A (by APMA or plasma membranes) or purified  $M_r$  62,000 or  $M_r$  45,000 species. The reaction was then stopped by the addition of the Laemmli sample buffer containing 1%  $\beta$ -mercaptoethanol. Conversion to the active forms was determined after separation of the samples by SDS-PAGE and staining of the gels with 0.25% Coomassie Brilliant Blue R-250 in 10% methanol/5% acetic acid or by immunoblot analysis using a mAb (CA-209) to human progelatinase B. In some experiments, progelatinase B was incubated with stoichiometric amounts of TIMP-1 or TIMP-2 for 20 min at 22°C before activation with gelatinase A. Progelatinase B was also activated in concentrated conditioned media of HT1080 cells by adding various concentrations of exogenous recombinant gelatinase A species. To obtain conditioned medium, a confluent T75 tissue culture flask was incubated (16 h at 37°C) with 7 ml of serum-free DMEM in a  $\text{CO}_2$  incubator. The medium was then collected, clarified by centrifugation (10 min at 2000 rpm), and concentrated (10-fold) using a Centricon-10 concentrator.

## RESULTS

**Activation of Progelatinase B by Gelatinase A.** We examined the ability of gelatinase A to activate progelatinase B. Incubation of progelatinase B with APMA-activated gelatinase A resulted in a time- and dose-dependent conversion of the  $M_r$  92,000 zymogen to the  $M_r$  82,000 species (Fig. 1). An intermediary form of  $M_r$  86,000 could be observed as early as 30 min after the addition of gelatinase A. Full conversion to the  $M_r$  82,000 species was observed after a 2-h incubation period (Fig. 1B; 1:50 gelatinase A:progelatinase B molar ratio). To rule out the possibility that trace amounts of APMA remaining in the gelatinase A preparation were responsible for the activation of progelatinase B, the  $M_r$  92,000 zymogen was incubated with a 10-fold excess APMA (10 mM) for 1 h. Under these conditions, APMA failed to fully convert progelatinase B to the  $M_r$  82,000 form (data not shown). Gelatinase B did not activate progelatinase A (data not shown).

The  $\text{NH}_2$ -terminal sequences of the  $M_r$  86,000 and the  $M_r$  82,000 gelatinase B species formed after activation by gelatinase A were determined and found to be Met<sup>41</sup> for the  $M_r$  86,000 form and Phe<sup>88</sup> for the  $M_r$  82,000 form. These sequences are the same as

Fig. 1. SDS-PAGE analysis of the activation of progelatinase B by gelatinase A. Human recombinant progelatinase A was activated with 1 mM APMA (1 h at 37°C) and applied to a Quick Spin column to remove the APMA as described in "Materials and Methods." The activated gelatinase A was incubated (37°C) with human recombinant progelatinase B for various time periods at a 1:100 (A) or 1:50 (B) gelatinase A:progelatinase B molar ratio. At the end of the indicated incubation times, the samples (1 µg/lane of progelatinase B) were analyzed in 10% SDS-polyacrylamide gel under reducing conditions, followed by staining of the gel with 0.25% Coomassie Brilliant Blue. Left lane, molecular weight standards (Bio-Rad). Arrow on the right, the activated gelatinase A used in the experiment.



those reported previously for the activation of progelatinase B by stromelysin-1 (39).

**Plasma Membrane-activated Gelatinase A Activates Progelatinase B.** Progelatinase A can be activated by APMA (5) or by plasma membrane containing MT-MMP (25-28). We reported previously that APMA activation of TIMP-2-free progelatinase A generated two active species of  $M_r$  62,000 and  $M_r$  45,000 (32, 33). We compared the pattern of activation of progelatinase A by APMA and plasma membrane of HT1080 cells treated with phorbol ester. As shown in Fig. 2,

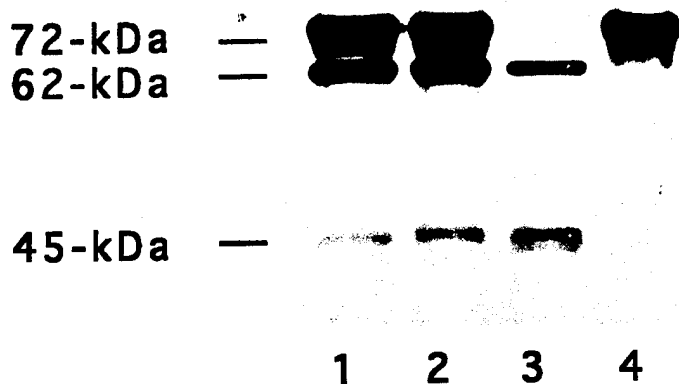
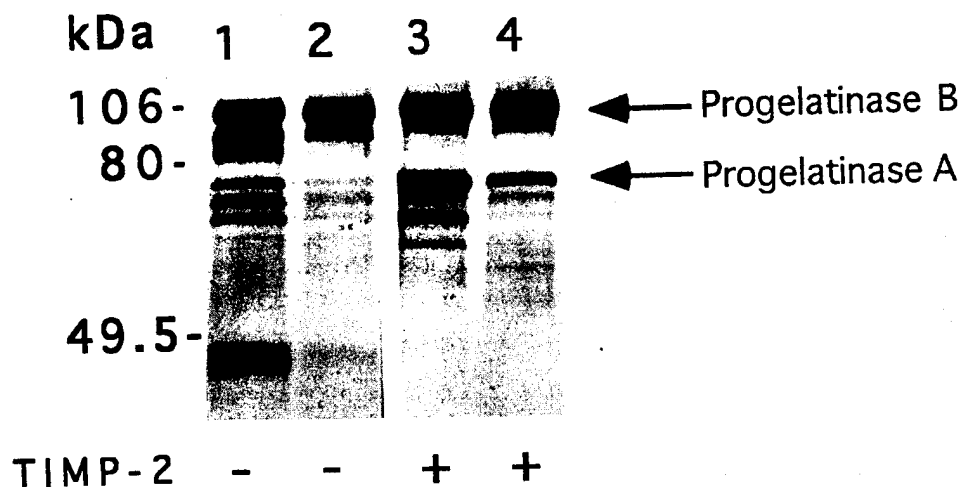


Fig. 2. Activation of progelatinase A by plasma membranes or APMA. Progelatinase A (200 ng/lane) in 25 mM HEPES/KOH (pH 7.5)-0.1 mM  $CaCl_2$  was incubated (37°C) with either 10 µg of HT1080 plasma membranes (Lane 1, 1 h; Lane 2, 2 h) or with 1 mM APMA for 1 h (Lane 3) or with buffer alone (Lane 4). The samples were then separated by electrophoresis in a 10% SDS-polyacrylamide gel under reducing conditions, followed by transfer to nitrocellulose paper. The blot was developed using a mAb to progelatinase A as described in "Materials and Methods." Molecular weights on the left indicate the relative mass of the gelatinase A species.

plasma membrane- (Fig. 2, Lanes 1 and 2) or APMA- (Fig. 2, Lane 3) activated progelatinase A into a  $M_r$  62,000 and a  $M_r$  45,000 species. An intermediary form of  $M_r$  64,000 to 66,000 could also be observed (not visible in Fig. 2). Thus, both APMA and plasma membrane activation of progelatinase A generate the  $M_r$  62,000 and  $M_r$  45,000 species.

Since APMA activated-gelatinase A caused activation of progelatinase B, we wished to examine the effect of plasma membrane-activated gelatinase A on progelatinase B activation. To this end, the plasma membranes from phorbol ester treated-HT1080 cells were incubated (3 h at 37°C) with progelatinase A, and then progelatinase B was added to the reaction mixture for an additional 3-h incubation period. The reaction mixture was then analyzed by immunoblot using a mAb (CA-209) against progelatinase B and a mAb (CA-801) against progelatinase A. These studies (Fig. 3) showed that plasma membrane activated-gelatinase A caused the conversion of progelatinase B to the  $M_r$  86,000 and  $M_r$  82,000 species (Fig. 3, Lanes 1 and 2). The immunoblot also showed the bands corresponding to the plasma membrane-activated gelatinase A, including a  $M_r$  64,000, 62,000, and a 45,000 form. When progelatinase A was incubated (3 h at 37°C) with plasma membranes in the presence of recombinant TIMP-2, there was a significant reduction in the formation of the  $M_r$  62,000 and  $M_r$  45,000 species, and most of the  $M_r$  72,000 enzyme remained in the latent form (Fig. 3, Lanes 3 and 4). Consistently, the addition of progelatinase B to the mixture of plasma membranes, progelatinase A, and TIMP-2, followed by another 3-h incubation period, had no effect on the activation of progelatinase B (Fig. 3, Lanes 3 and 4). Also, progelatinase B incubated with plasma membranes in the absence of progelatinase A was not activated (data not shown), as reported previously (25-27). Taken together, these studies demonstrate that

Fig. 3. Progelatinase B activation by plasma membrane-activated gelatinase A and inhibition by TIMP-2. Plasma membranes (10 µg/reaction) were incubated (3 h at 37°C) with progelatinase A (200 ng/reaction) in 25 mM HEPES/KOH (pH 7.5)-0.1 mM  $CaCl_2$  in the absence (Lanes 1 and 2) or presence (Lanes 3 and 4) of stoichiometric amounts (60 ng/reaction) of TIMP-2 to progelatinase A. An aliquot of the reaction mixture was then incubated with progelatinase B (200 ng/lane) at 1:10 (Lanes 1 and 3) and 1:50 (lanes 2 and 4) molar ratios (gelatinase A:progelatinase B) for another 3 h at 37°C. The mixtures were then separated by electrophoresis in a 10% SDS-polyacrylamide gel under reducing conditions, followed by transfer to nitrocellulose paper. The blot was developed using a mixture of mAbs to progelatinase A and B, as described in "Materials and Methods." Molecular weights on the left represent the prestained molecular weight standards (low range; Bio-Rad).



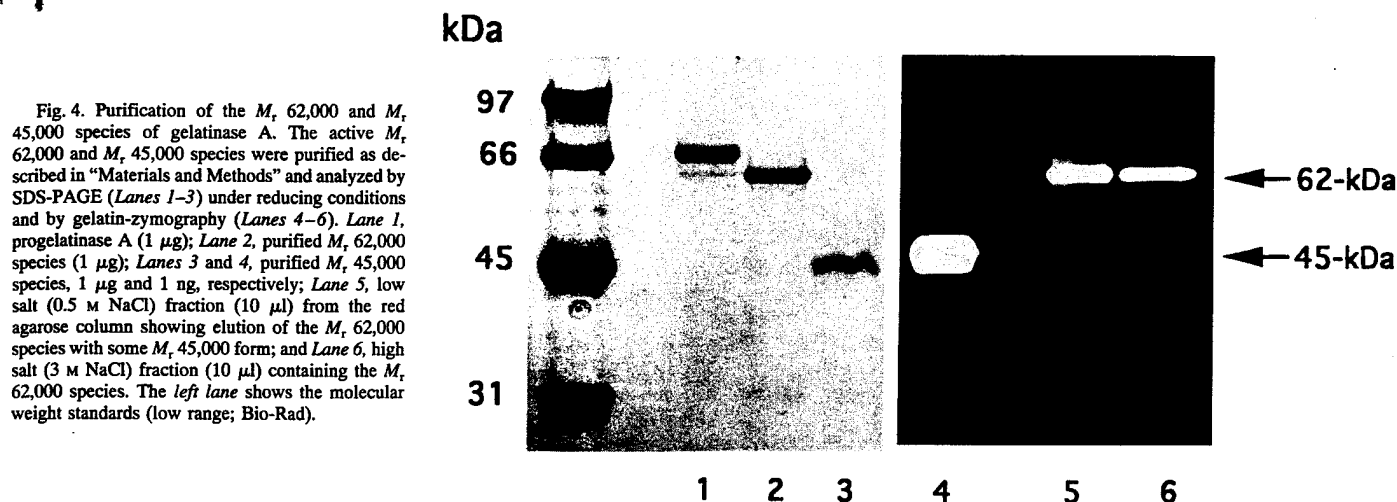


Fig. 4. Purification of the  $M_r$  62,000 and  $M_r$  45,000 species of gelatinase A. The active  $M_r$  62,000 and  $M_r$  45,000 species were purified as described in "Materials and Methods" and analyzed by SDS-PAGE (Lanes 1-3) under reducing conditions and by gelatin-zymography (Lanes 4-6). Lane 1, progelatinase A (1  $\mu$ g); Lane 2, purified  $M_r$  62,000 species (1  $\mu$ g); Lanes 3 and 4, purified  $M_r$  45,000 species, 1  $\mu$ g and 1 ng, respectively; Lane 5, low salt (0.5 M NaCl) fraction (10  $\mu$ l) from the red agarose column showing elution of the  $M_r$  62,000 species with some  $M_r$  45,000 form; and Lane 6, high salt (3 M NaCl) fraction (10  $\mu$ l) containing the  $M_r$  62,000 species. The left lane shows the molecular weight standards (low range; Bio-Rad).

both APMA and plasma membrane-activated gelatinase A activate progelatinase B.

**Isolation of Gelatinase A Species.** To demonstrate the ability of the gelatinase A species to activate progelatinase B, it was necessary to isolate the  $M_r$  62,000 and the  $M_r$  45,000 forms. To this end, progelatinase A was activated with APMA, and the active species were separated by red-agarose chromatography using a NaCl gradient. SDS-PAGE analysis and zymography of the purified  $M_r$  45,000 species, which eluted in the void volume, revealed a single active band of  $M_r$  45,000 (Fig. 4, Lanes 3 and 4). Increasing concentrations of NaCl resulted in a stepwise elution of the  $M_r$  62,000 active species (Fig. 4, Lane 5, 0.5 M NaCl; both the  $M_r$  45,000 and the  $M_r$  62,000 forms were observed). At high salt concentrations (3 M NaCl), a single active form of  $M_r$  62,000 was detected (Fig. 4, Lane 6). The high-salt fraction containing the  $M_r$  62,000 enzyme also showed three additional inactive fragments of  $M_r$  32,000, 26,000, and 12,000 that separated as three distinct bands in SDS-PAGE under reducing conditions (data not shown). To remove these fragments from the  $M_r$  62,000 active species, the dialyzed sample was applied to a gelatin agarose column, and a single protein of  $M_r$  62,000 kDa was eluted with DMSO (Fig. 4, Lane 2); the three smaller fragments did not bind to the gelatin column.  $NH_2$ -terminal sequence of the  $M_r$  62,000 and the  $M_r$  45,000 species revealed the same  $NH_2$  terminus, which started from Tyr<sup>81</sup>. The  $M_r$  32,000 fragment revealed a major and a minor

component. The  $NH_2$  terminus of the former started from Tyr<sup>416</sup> (YGASPDIDLG) and that of the latter from Gly<sup>417</sup> (GASPDIDLG). The  $NH_2$  terminus of the  $M_r$  26,000 fragment also started from Tyr<sup>416</sup>. Thus, the  $M_r$  32,000 and 26,000 fragments may represent the COOH-terminal domain of progelatinase A.

Immunoblot analysis using a polyclonal antibody directed against the COOH-terminal end (residues 513-530) of progelatinase A showed that the antibody failed to react with the purified  $M_r$  45,000 species (Fig. 5, Lane 6), consistent with the deletion of the COOH-terminal domain in this species. In contrast, this antibody reacted with the proenzyme form and the  $M_r$  62,000 species of activated gelatinase A (Fig. 5, Lanes 4 and 5, respectively). As expected, a mAb (CA-801) to human progelatinase A, known to bind to the COOH-terminally truncated recombinant  $M_r$  72,000 enzymes (32), reacted with the three enzymes forms (Fig. 5, Lane 1, progelatinase A; Lane 2, activated gelatinase A; and Lane 3,  $M_r$  45,000 species). Similar results were obtained when plasma membrane-activated  $M_r$  72,000 enzyme was used as antigen (data not shown).

**Activation of Progelatinase B by the Active Species of Gelatinase A.** The purified active species of gelatinase A were used to evaluate their ability to activate progelatinase B. Fig. 6 shows that the purified  $M_r$  45,000 species activated progelatinase B (Fig. 6) to the  $M_r$  86,000 and 82,000 forms in a time- and dose-dependent manner. A similar pattern of activation was obtained when purified  $M_r$  62,000 species was used as activator of progelatinase B (results not shown). Thus, both gelatinase A species activate progelatinase B.

We wished to examine the ability of the gelatinase A species to activate the progelatinase B present in the conditioned media of human fibrosarcoma HT1080 cells. A 100- $\mu$ l portion of the 10-fold concentrated conditioned media were incubated (1 h at 37°C) with 0.1 and 1  $\mu$ g of purified  $M_r$  45,000 species. The medium was then analyzed for  $M_r$  92,000 enzyme activation by immunoblot using a mAb against human progelatinase B (46). As shown in Fig. 7 (Lanes 3 and 4), the  $M_r$  45,000 species activated the endogenous progelatinase B to the  $M_r$  86,000 and  $M_r$  82,000 species as shown with recombinant progelatinase B. Conditioned medium incubated under the same conditions, but without gelatinase A species, showed no evidence of spontaneous progelatinase B activation (Fig. 7, Lane 2). This experiment suggests that natural progelatinase B can be activated by the gelatinase A active species.

**Gelatinase Activity of Gelatinase B and Effects of TIMPs.** We measured the activity of gelatinase B against soluble [<sup>3</sup>H]gelatin after treatment with the  $M_r$  45,000 species. As shown in Fig. 8, incubation of progelatinase B with the  $M_r$  45,000 species generated an enzyme

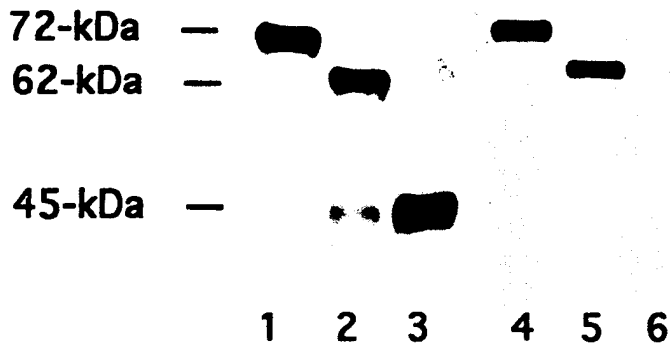


Fig. 5. Immunoblot analysis of activated gelatinase A and the  $M_r$  45,000 species. Samples (200 ng/lane) of progelatinase A (Lanes 1 and 4), APMA-activated gelatinase A (Lanes 2 and 5) and purified  $M_r$  45,000 species (Lanes 3 and 6) were subjected to SDS-PAGE under reducing conditions, followed by blotting to nitrocellulose paper. After blocking, the blots were incubated with a mouse mAb (CA-801) against human progelatinase A (Lanes 1-3) or with a rabbit polyclonal antibody against a synthetic peptide comprising residues 513-530 of human progelatinase A (Lanes 4-6). Molecular weights on the left indicate the relative mass of the purified proteins.

PROGELATINASE B ACTIVATION

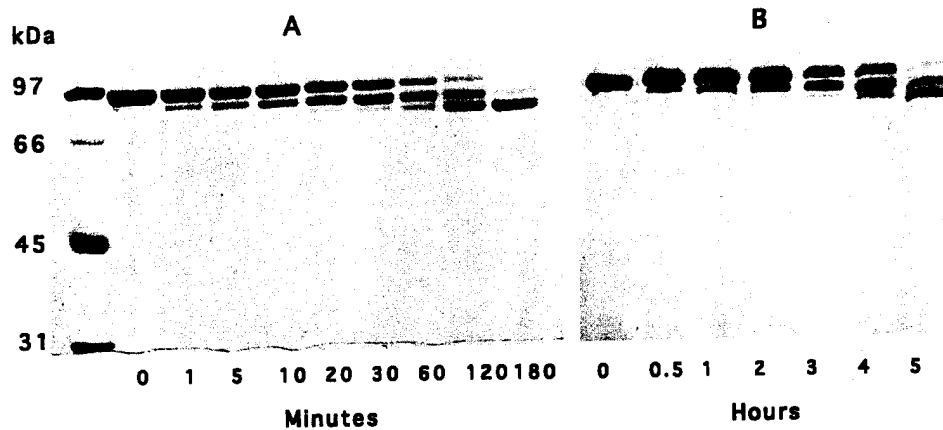


Fig. 6. SDS-PAGE analysis of the activation of progelatinase B by the  $M_r$  45,000 species of gelatinase A. Samples of purified progelatinase B (1  $\mu$ g/lane) were incubated (37°C) with purified  $M_r$  45,000 species at 1:10 (A) or 1:100 (B) molar ratios of  $M_r$  45,000:progelatinase B for various periods of time and then subjected to SDS-PAGE analysis under reducing conditions and staining of the gels with 0.025% Coomassie blue. Left lane, the molecular weight standards (low range; Bio-Rad).

with gelatinase activity. The  $M_r$  45,000 species alone also showed activity against [ $^3$ H]gelatin that was 3-fold lower than the activity of the gelatinase B/ $M_r$  45,000 species. In contrast, progelatinase B showed virtually no enzymatic activity.

The effect of TIMP-1 and TIMP-2 on the activation of progelatinase B by gelatinase A species was examined by measuring the activity against [ $^3$ H]gelatin in the presence or absence of inhibitor (Table 1). To this end, progelatinase B was incubated with stoichiometric amounts of either TIMP-1 or TIMP-2 before the addition of the gelatinase A species. As controls, we measured the activity of progelatinase B (0.1  $\mu$ M) and the  $M_r$  45,000 species alone (0.01  $\mu$ M). As shown in Table 1, the purified  $M_r$  45,000 species alone showed a significantly higher (27% more) activity than progelatinase B, as expected. Incubation of progelatinase B with one-tenth molar concentrations of the  $M_r$  45,000 species generated high enzymatic activity (considered 100%). When progelatinase B was incubated with either TIMP-1 or TIMP-2 prior to the addition of the  $M_r$  45,000 species, the presence of the inhibitors caused a complete inhibition of enzymatic

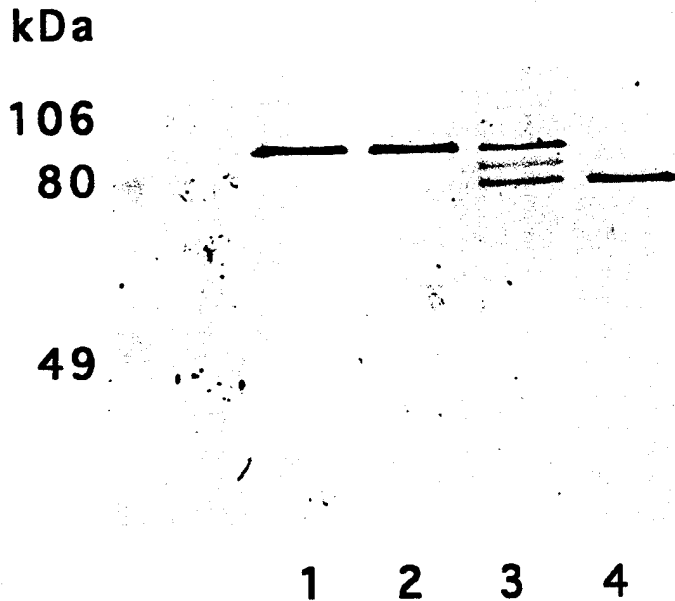


Fig. 7. Activation of progelatinase B in the conditioned media of HT1080 cells by the  $M_r$  45,000 species of gelatinase A. Concentrated (100  $\mu$ l/lane) serum-free conditioned medium of HT1080 cells prepared as described in "Materials and Methods" was incubated (1 h at 37°C) with either 0.1 (Lane 3) or 1  $\mu$ g of purified  $M_r$  45,000 species or alone (Lane 2). The samples were then subjected to SDS-PAGE under reducing conditions and immunoblot analysis using a mAb (CA-209) against human progelatinase B. Lane 1, the conditioned media not incubated at 37°C. Left lane, the molecular weight of prestained standards (low range; Bio-Rad).

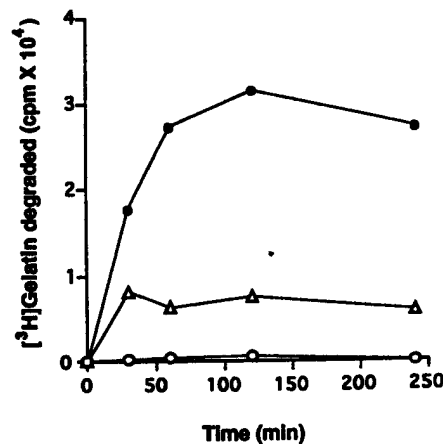


Fig. 8. Enzymatic activity of gelatinase B activated by the  $M_r$  45,000 species. Progelatinase B (0.1  $\mu$ M) was incubated with (●) or without (○) the  $M_r$  45,000 species at a 1:10 molar ratio of  $M_r$  45,000:progelatinase B for various time periods and analyzed for activity against soluble [ $^3$ H]gelatin, as described in "Materials and Methods." As control, samples of the  $M_r$  45,000 species (0.01  $\mu$ M) were incubated alone (Δ) and tested for activity. Soluble degraded [ $^3$ H]gelatin was quantified by scintillation spectrometry. Points, duplicate samples that varied by less than 15% (cpm) from each other. Similar results were obtained in three independent experiments.

Table 1 Effect of TIMP-1 and TIMP-2 on the activation of progelatinase B by the  $M_r$  45,000 species

Progelatinase B (0.1  $\mu$ M) was incubated (20 min at 22°C) in the presence (+) or absence (-) of equimolar concentrations of either TIMP-1 or TIMP-2 and then incubated (2 h at 37°C) in the presence or absence of the  $M_r$  45,000 species (0.01  $\mu$ M). The mixtures were then tested for activity against soluble [ $^3$ H]gelatin, as described in "Materials and Methods." Data are expressed as the percentage of activity (solubilized counts) remaining relative to the activity of  $M_r$  45,000-activated gelatinase B (100%).

Enzyme	TIMP-1	TIMP-2	% activity remaining
Progelatinase B	-	-	1
$M_r$ 45,000	-	-	27
Progelatinase B/ $M_r$ 45,000	-	-	100
Progelatinase B/ $M_r$ 45,000	+	-	1
Progelatinase B/ $M_r$ 45,000	-	+	0

activity. Similar results were obtained when progelatinase B was incubated with or without TIMPs with the purified  $M_r$  62,000 species.

DISCUSSION

In this study, we show that gelatinase A can activate progelatinase B to a  $M_r$  82,000 form with activity against gelatin. Activation of progelatinase B was observed with both plasma membrane or APMA-activated,  $M_r$  72,000 enzyme and with both recombinant and natural progelatinase B. Activation of progelatinase B by gelatinase A is

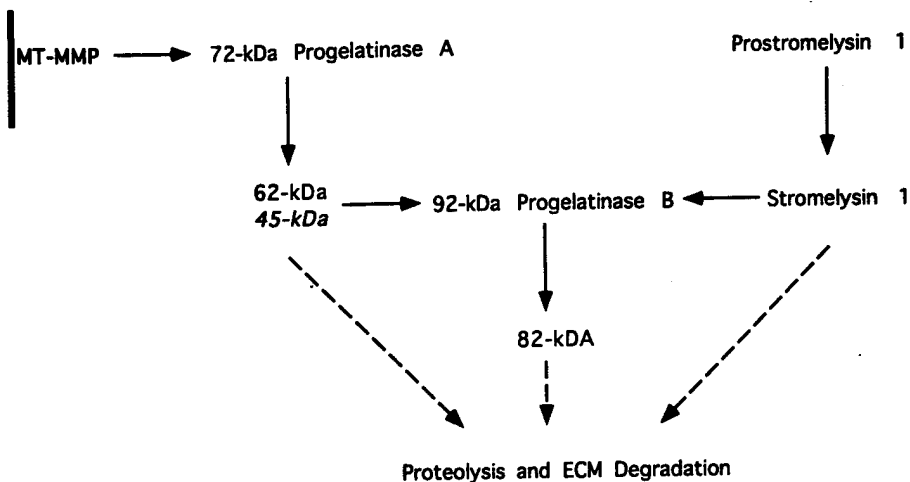


Fig. 9. Schematic model of progelatinase B activation.

accomplished by cleavage at the Glu<sup>40</sup>-Met<sup>41</sup> amide bond to generate a  $M_r$  86,000 form and cleavage at the Arg<sup>87</sup>-Phe<sup>88</sup> amide bond to generate a  $M_r$  82,000 species in a process similar to the activation of progelatinase B by stromelysin-1 (39). The formation of these species is probably due to the action of gelatinase A alone and does not involve an autocatalytic process, since conversion of the  $M_r$  86,000 species to the  $M_r$  82,000 form was not observed without stromelysin-1 (39). The activation of progelatinase B by gelatinase A observed here is in contrast to the findings of Ogata *et al.* (39), who did not observe any activation of progelatinase B by gelatinase A. This discrepancy may be due to the source of the  $M_r$  72,000 enzyme used for activation of progelatinase B in experiments by Ogata *et al.* (39). Progelatinase A isolated from HT1080 cell culture media [as described in the study of Ogata *et al.* (39)] is expected to be in complex with TIMP-2 (34, 52) and would possess diminished enzymatic activity when compared to either the recombinant (32, 33) or natural (53) gelatinase A, which are devoid of TIMP-2. In this study, we showed that preincubation of progelatinase B with equal molar amounts of either TIMP-1 or TIMP-2 inhibits activation by the gelatinase A active species, as previously reported for activation of progelatinase B with stromelysin-1 (54). The effect of TIMP-1 may be due to formation of a complex with progelatinase B (6), whereas that of TIMP-2 may be caused by an inhibition of gelatinase A (32, 33), as shown in Table 1 with the  $M_r$  45,000 species. Although the  $M_r$  45,000 form of gelatinase A is less susceptible to inhibition by TIMP-2, compared to the  $M_r$  62,000 species,<sup>4</sup> the amount of TIMP-2 used in the experiment described in Table 1 was 10-fold higher than that of the  $M_r$  45,000 species. At this concentration, TIMP-2 inhibits the activity of the  $M_r$  45,000 species completely, as reported previously for the truncated forms of gelatinase A lacking the COOH-terminal domain (32). The inhibitory effect of TIMP-2 on progelatinase B activation was also demonstrated by inhibiting the plasma membrane and APMA activation of progelatinase A with TIMP-2. This is consistent with the ability of this inhibitor to form a complex with progelatinase A that prevents activation (33, 51, 55).

We have shown in this report that activation of human recombinant progelatinase A, with either plasma membrane or APMA, generates a  $M_r$  62,000 and a  $M_r$  45,000 species, as reported previously (25, 27, 32, 33, 55). Howard *et al.* (56) also reported the formation of a  $M_r$  42,500 active gelatinase A fragment after separation of TIMP-2 from progelatinase A by acid treatment and reverse phase-HPLC. The presence of these species was also reported in the medium of fibroblast cells

treated with concanavalin A (25, 29, 30). The formation of the  $M_r$  45,000 species, as shown in the present report, involves cleavage of both the NH<sub>2</sub>-terminal profragment and the COOH-terminal domain. This was demonstrated by NH<sub>2</sub>-terminal sequencing of the purified  $M_r$  45,000 species that revealed an NH<sub>2</sub> terminus starting from Tyr<sup>81</sup>. The same residue was reported as the NH<sub>2</sub> terminus for the  $M_r$  62,000 species after APMA treatment (56, 57) or plasma membrane activation of progelatinase A (27). In addition, a polyclonal antibody generated against the COOH-terminal end of progelatinase A failed to react with the  $M_r$  45,000 species but reacted with the  $M_r$  62,000 form, further demonstrating the absence of the COOH-terminal region in the  $M_r$  45,000 species.

We isolated the active species of gelatinase A and showed that both the  $M_r$  62,000 and the  $M_r$  45,000 species activated purified progelatinase B. The ability of the 45-kDa species to activate progelatinase B is consistent with previous studies that showed that the COOH-terminal domain of gelatinase A is not required for catalytic activity (32, 37, 38). The purified  $M_r$  62,000 and  $M_r$  45,000 species were also able to activate progelatinase B present in the conditioned media of HT1080 cells. However, high levels of the exogenous gelatinase A species were required, possibly due to the presence of TIMPs in the media. Previous studies showed that activation of progelatinase A in the media of HT1080 cells that had been treated with phorbol ester did not cause the activation of the  $M_r$  92,000 enzyme (26, 27). The lack of progelatinase B activation under these conditions may be due to the presence of TIMP-1, which is induced by the treatment with phorbol ester.<sup>4</sup> Thus, the high levels of TIMP-1 results in inhibition of progelatinase B activation, and by consequence, a general inhibition of MMP activity ensues. This is consistent with our previous data showing the inhibitory effect of phorbol ester on the *in vitro* invasion of HT1080 cells (58).

The activation of progelatinase B by gelatinase A may be significant in tissues where stromelysin-1 may not be present (43). Recent *in situ* hybridization studies in 17 cases of breast cancer showed high levels of gelatinase A and B mRNAs in 60–80% of the cases, whereas low levels of stromelysin-1 mRNA were detected in only 30% of the cases.<sup>5</sup> Also, our previous immunohistochemical studies showed a colocalization of gelatinase A and B in the malignant cells in 72% of 83 cases of breast carcinoma tissue (46). Other studies also showed coexpression of gelatinases in breast (44, 45), colon (10), and bladder cancers (14). Therefore, it is highly plausible that colocalization of

<sup>4</sup> Unpublished results.

<sup>5</sup> L. Matrisian, personal communication.

these enzymes in tumor tissue would facilitate their interaction for activation, leading to degradation of ECM.

Fig. 9 depicts a model for the interaction of several members of the MMP family, leading to the activation of progelatinase B. The membrane enzyme, MT-MMP, of tumor cells activates progelatinase A to generate the  $M_r$  62,000 and  $M_r$  45,000 species. This process has been recently shown to involve the binding of TIMP-2 to MT-MMP, followed by binding of progelatinase A to the TIMP-2-MT-MMP complex (55). The activation process and substrate specificity of MT-MMP remain to be defined. The active gelatinase A species may in turn activate progelatinase B, as shown in this study, and degrade ECM. Although the physiological significance of the  $M_r$  45,000 species of gelatinase A (Fig. 9, *italics*) remains presently obscure, it is interesting that the rate of TIMP-2 inhibition of active gelatinase A species lacking the COOH-terminal domain is significantly lower than that of the  $M_r$  62,000 form (32, 37, 38, 59). Thus, cleavage of the COOH-terminal domain, should it occur *in vivo*, would result in the formation of gelatinase A species that are less susceptible to TIMP-2 inhibition but remain catalytically competent and capable of activation of progelatinase B. Recently, activation of progelatinase B in fibroblast monolayers supplemented with plasminogen or prostromelysin-1 was described (40). Thus, stromelysin-1 would efficiently activate progelatinase B (39, 40, 54), if it were available; however, its expression in breast cancer remains unclear at the present (43). Plasmin, on the other hand, is a poor activator of progelatinase B (40, 41, 54). Nevertheless, the role of each proteinase in activation of progelatinase B would be determined by its abundance and accessibility, the state of activation, the rate of formation of the active species, and the presence of specific inhibitors (29). Although the role of TIMP-1 and TIMP-2 is not depicted in this model (Fig. 9), these inhibitors are critical regulators of the activation process and modulators of the enzymatic activity of the MMPs. As such, they play a crucial role in determining the extent of ECM degradation during tumor cell invasion. This and previous studies have shown that both TIMP-1 and TIMP-2 can inhibit the activation of progelatinase A and B and the respective proteolytic activity of the resultant enzymes by forming a complex with the latent enzymes and/or binding to the active enzyme forms. Further studies on the relationship between these MMPs and TIMPs will provide a clearer understanding of the regulation of MMP activity and the role of these proteases in tumor cell invasion.

#### ACKNOWLEDGMENTS

We are indebted to Dr. Greg Goldberg for providing us with the plasma membrane preparation and for his useful comments on the manuscript and Dr. Scott Wilhelm for his valuable suggestions.

#### REFERENCES

- Liotta, L. A., Steeg, P. A., and Stetler-Stevenson, W. G. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell*, 64: 327-336, 1991.
- Matrisian, L. M. Metalloproteinases and their inhibitors in tissue remodelling. *Trends Genet.*, 6: 121-125, 1990.
- Testa, J. E., and Quigley, J. P. The role of urokinase-type plasminogen activator in aggressive tumor cell behavior. *Cancer Metastasis Rev.*, 9: 353-367, 1990.
- Sloane, B. F., Moin, K., and Lah, T. T. Regulation of lysosomal endopeptidases in malignant neoplasia. In: T. G. Pretlow and T. P. Pretlow (eds.), *Biochemical and Molecular Aspects of Selected Cancers*, Vol. 2, pp. 411-466. New York: Academic Press, 1994.
- Collier, I. E., Wilhelm, S. M., Eisen, A. Z., Marmer, B. L., Grant, G. A., Seltzer, J. L., Kronberger, A., He, C., Bauer, E. A., and Goldberg, G. I. H-ras oncogene-transfected human bronchial epithelial cells (TBE-1) secrete a single metalloprotease capable of degrading basement membrane collagen. *J. Biol. Chem.*, 263: 6579-6587, 1988.
- Wilhelm, S. M., Collier, I. E., Marmer, B. L., Eisen, A. Z., Grant, G. A., and Goldberg, G. I. SV40-transformed human lung fibroblasts secrete a 92 kDa type IV collagenase which is identical to that secreted by normal human macrophages. *J. Biol. Chem.*, 264: 17213-17221, 1989.
- Stetler-Stevenson, W. G. Type IV collagenases in tumor invasion and metastasis. *Cancer Metastasis Rev.*, 9: 289-303, 1990.
- D'Errico, A., Garbisa, S., Liotta, L. A., Castranovo, V., Stetler-Stevenson, W. G., and Grigioni, W. F. Augmentation of type IV collagenase, laminin receptor and Ki62 proliferation antigen associated with human colon, gastric and breast carcinoma progression. *Mod. Pathol.*, 4: 239-246, 1991.
- Poulsom, R., Hanby, A. M., Pignatelli, M., Jeffrey, R. E., Longcroft, J. M., Rogers, L., and Stamp, G. W. Expression of gelatinase A and TIMP-2 mRNAs in desmoplastic fibroblasts in both mammary carcinomas and basal cell carcinomas. *J. Clin. Pathol.*, 46: 429-436, 1993.
- Pyke, C., Ralfkiaer, E., Tryggvason, K., and Dano, K. Messenger RNA for two type IV collagenases is located in stromal cells in human colon cancer. *Am. J. Pathol.*, 142: 359-365, 1993.
- Poulsom, R., Pignatelli, M., Stetler-Stevenson, W. G., Liotta, L. A., Wright, P. A., Jeffrey, R. E., Longcroft, J. A., Rogers, L., and Stamp, G. W. H. Stromal expression of 72 kDa type IV collagenase (MMP-2) and TIMP-2 mRNAs in colorectal neoplasia. *Am. J. Pathol.*, 141: 389-396, 1992.
- Autio-Harmanen, H., Karttunen, T., Hurskainen, T., Hoyhtya, M., Kaupilla, A., and Tryggvason, K. Expression of 72 kDa type IV collagenase (gelatinase A) in benign and malignant ovarian tumors. *Lab. Invest.*, 69: 312-321, 1993.
- Zucker, S., Lysik, R. M., Zarrabi, M. H., and Moll, U. M. 92,000 type IV collagenase is increased in plasma of patients with colon cancer and breast cancer. *Cancer Res.*, 53: 140-146, 1993.
- Davies, B., Waxman, J., Wasan, H., Abel, P., Williams, G., Krausz, T., Neal, D., Thomas, D., Hanby, A., and Balkwill, F. Levels of matrix metalloproteinases in bladder cancer correlate with tumor grade and invasion. *Cancer Res.*, 53: 5365-5369, 1993.
- Fessler, L., Duncan, K., and Tryggvason, K. Identification of the procollagen IV cleavage products produced by a specific tumor collagenase. *J. Biol. Chem.*, 259: 9783-9789, 1984.
- Woessner, J. F. Matrix metalloproteinases and their inhibitors in connective tissue remodelling. *FASEB J.*, 5: 2145-2154, 1991.
- Senior, R. M., Griffin, G. L., Fliszar, C. J., Shapiro, S. D., Goldberg, G. I., and Welgus, H. G. Human 92- and 72 kDa type IV collagenases are elastases. *J. Biol. Chem.*, 266: 7870-7875, 1991.
- Murphy, G., Cockett, M. I., Ward, R. V., and Docherty, A. J. Matrix metalloproteinase degradation of elastin, type IV collagen and proteoglycan. A quantitative comparison of the activities of 95 kDa and 72 kDa gelatinases, stromelysins-1 and -2 and punctuated metalloproteinase (PUMP). *Biochem. J.*, 277: 277-279, 1991.
- Nguyen, Q., Murphy, G., Hughes, C. E., Mort, J. S., and Roughley, P. J. Matrix metalloproteinases cleave at two distinct sites on human cartilage link protein. *Biochem. J.*, 295: 595-598, 1993.
- Fosang, A. J., Last, K., Knauper, V., Neame, P. J., Murphy, G., Hardingham, T. E., Tschesche, H., and Hamilton, J. A. Fibroblast and neutrophil collagenases cleave at two distinct sites in the cartilage aggrecan interglobular domain. *Biochem. J.*, 295: 273-276, 1993.
- Sires, U. I., Griffin, G. L., Broekelman, T. J., Mecham, R. P., Murphy, G., Chung, A. E., Welgus, H. G., and Senior, R. M. Degradation of entactin by matrix metalloproteinases: susceptibility to matrilysin and identification of cleavage sites. *J. Biol. Chem.*, 268: 2069-2074, 1993.
- Miyazaki, K., Hasegawa, M., Funahashi, K., and Umeda, M. A metalloproteinase inhibitor domain in Alzheimer amyloid protein precursor. *Nature (Lond.)*, 362: 839-841, 1993.
- Roher, A. E., Kasunic, T. C., Woods, A. S., Cotter, R. J., Ball, M. J., and Fridman, R. Proteolysis of A $\beta$  peptide from Alzheimer disease brain by gelatinase A. *Biochem. Biophys. Res. Commun.*, 205: 1755-1761, 1994.
- Ochieng, J., Fridman, R., Nangia-Makher, P., Liotta, L. A., Stetler-Stevenson, W. G., and Raz, A. Galectin-3 is a novel substrate for human matrix metalloproteinases 2 and 9. *Biochemistry*, 33: 14109-14114, 1994.
- Ward, R. V., Atkinson, S. J., Slocombe, P. M., Docherty, A. J. P., Reynolds, J. J., and Murphy, G. Tissue inhibitor of metalloproteinases-2 inhibits the activation of 72 kDa progelatinase by fibroblasts membranes. *Biochim. Biophys. Acta*, 1079: 242-246, 1991.
- Brown, P. D., Kleiner, D. E., Unsworth, E. J., and Stetler-Stevenson, W. G. Cellular activation of the 72 kDa type IV procollagenase/TIMP-2 complex. *Kidney Int.*, 43: 163-170, 1993.
- Strongin, A. Y., Marmer, B. L., Grant, G. A., and Goldberg, G. I. Plasma membrane-dependent activation of the 72 kDa type IV collagenase is prevented by complex formation with TIMP-2. *J. Biol. Chem.*, 268: 14033-14039, 1993.
- Sato, H., Takino, T., Okada, Y., Cao, J., Shinagawa, A., Yamamoto, E., and Seiki, M. A matrix metalloproteinase expressed in the surface of tumor cells. *Nature (Lond.)*, 370: 61-65, 1994.
- Overall, C. M., and Dodek, J. Concavalin-A produces a matrix-degradative phenotype in human fibroblasts. Induction/endogenous activation of collagenase, 72 kDa gelatinase and pump-1 is accompanied by suppression of tissue inhibitor of metalloproteinases. *J. Biol. Chem.*, 265: 21141-21151, 1990.
- Overall, C. M. Regulation of tissue inhibitor of matrix metalloproteinase expression. *Ann. NY Acad. Sci.*, 732: 51-64, 1994.
- Azzam, H. S., and Thompson, E. W. Collagen-induced activation of 72,000 type IV collagenase in normal and malignant fibroblastoid cells. *Cancer Res.*, 52: 4540-4544, 1992.
- Fridman, R., Fuerst, T. R., Bird, R. E., Hoyhtya, M., Oelkuct, M., Kraus, S., Komarek, D., Liotta, L. A., Berman, M. L., and Stetler-Stevenson, W. G. Domain structure of human 72-kDa gelatinase/type IV collagenase. *J. Biol. Chem.*, 267: 15398-15405, 1992.
- Fridman, R., Bird, R., Hoyhtya, M., Oelkuct, M., Komarek, D., Liang, C.-M., Berman, M. L., Liotta, L. A., Stetler-Stevenson, W. G., and Fuerst, T. R. Expression of human

- recombinant 72 kDa gelatinase/type IV collagenase and TIMP-2: characterization of complex and free enzyme. *Biochem. J.*, 289: 411-416, 1993.
34. Goldberg, G. I., Marmer, B. L., Grant, G. A., Eisen, A. Z., Wilhelm, S. M., and He, C. Human 72-kilodalton type IV collagenase forms a complex with tissue inhibitor of metalloproteinase designated TIMP-2. *Proc. Natl. Acad. Sci. USA*, 86: 8207-8211, 1989.
  35. Stetler-Stevenson, W. G., Kruttsch, H. C., and Liotta, L. A. Tissue inhibitor of metalloproteinase-2 (TIMP-2). *J. Biol. Chem.*, 264: 17374-17378, 1989.
  36. Howard, E. W., and Banda, M. J. Binding of tissue inhibitor of metalloproteinases-2 to two distinct sites on human 72 kDa gelatinase. *J. Biol. Chem.*, 266: 17972-17977, 1991.
  37. Murphy, G., Willenbrock, F., Ward, R. V., Cockett, M. I., Eaton, D., and Docherty, A. J. P. The C-terminal domain of 72 kDa gelatinase is not required for catalysis, but is essential for membrane activation and modulates interactions with tissue inhibitors of metalloproteinases. *Biochem. J.*, 283: 637-641, 1992.
  38. Willenbrock, F., Crabbe, T., Slockcombe, P. M., Sutton, C. W., Docherty, A. J. P., Cockett, M. I., O'Shea, M., Blockehurst, K., Phillips, I. R., and Murphy, G. The activity of the tissue inhibitors of metalloproteinases is regulated by C-terminal domain interactions: a kinetic analysis of the inhibition of gelatinase A. *Biochemistry*, 32: 4330-4337, 1993.
  39. Ogata, Y., Enghild, J. J., and Nagase, H. Matrix metalloproteinase 3 (stromelysin) activates the precursor for the human matrix metalloproteinase 9. *J. Biol. Chem.*, 267: 3581-3584, 1992.
  40. O'Connell, J. P., Willenbrock, F., Docherty, A. J. P., Eaton, D., and Murphy, G. Analysis of the role of the COOH-terminal domain in the activation, proteolytic activity and tissue inhibitor of metalloproteinase interactions of gelatinase B. *J. Biol. Chem.*, 269: 14967-14973, 1994.
  41. Okada, Y., Gonoji, Y., Naka, K., Tomita, K., Nakanishi, I., Iwata, K., Yamashita, K., and Hayakawa, T. Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) from HT1080 human fibrosarcoma cells. *J. Biol. Chem.*, 267: 21712-21719, 1992.
  42. Menashi, S., Fridman, R., Desrevieres, S., Lu, H., Legrand, Y., and Soria, C. Regulation of 92-kDa gelatinase B activity in the extracellular matrix by tissue kallikrein. *Ann. NY Acad. Sci.*, 732: 466-468, 1994.
  43. Polette, M., Clavel, C., Cockett, M., Bentzmann, S. G., Murphy, G., and Birembaut, P. Detection and localization of mRNAs encoding matrix metalloproteinases and their inhibitors in human breast pathology. *Invasion Metastasis*, 13: 31-37, 1993.
  44. Brown, P. D., Bloxidge, R. E., Anderson, E., and Howell, A. Expression of activated gelatinase in human invasive breast carcinoma. *Clin. Exp. Metastasis*, 11: 183-189, 1993.
  45. Davies, B., Miles, D. W., Happerfield, L. C., Naylor, M. S., Bobrow, L. G., Rubens, R. D., and Balkwill, F. R. Activity of type IV collagenases in benign and malignant breast disease. *Br. J. Cancer*, 67: 1126-1131, 1993.
  46. Visscher, D. W., Hoyhtya, M., Ottosen, S. K., Liang, C-M., Sarkar, F. H., Crissman, J. D., and Fridman, R. Enhanced expression of tissue inhibitor of metalloproteinase-2 (TIMP-2) in the stroma of breast carcinomas correlates with tumor recurrence. *Int. J. Cancer*, 59: 339-344, 1994.
  47. Fuerst, T. M., Earl, P. L., and Moss, B. Use of a hybrid vaccinia virus-T7 RNA polymerase system for expression of target genes. *Mol. Cell. Biol.*, 7: 2538-2544, 1987.
  48. Howard, E. W., Bullen, E. C., and Banda, M. J. Preferential inhibition of 72- and 92-kDa gelatinases by tissue inhibitor of metalloproteinases-2. *J. Biol. Chem.*, 266: 13070-13075, 1991.
  49. Heinrikson, L., and Meredith, S. C. Amino acid analysis by reverse-phase high-performance liquid chromatography: precolumn derivatization with phenylisothiocyanate. *Anal. Biochem.*, 136: 65-74, 1984.
  50. Hoyhtya, M., Fridman, R., Komarek, D., Porter-Jordan, K., Stetler-Stevenson, W. G., Liotta, L. A., and Liang, C-M. Immunohistochemical localization of matrix metalloproteinase 2 and its specific inhibitor TIMP-2 in neoplastic tissues with monoclonal antibodies. *Int. J. Cancer*, 56: 500-505, 1994.
  51. Kleiner, D. E., Tuuttila, A., Tryggvason, K., and Stetler-Stevenson, W. G. Stability analysis of latent and active 72-kDa type IV collagenase: the role of issue inhibitor of metalloproteinases-2 (TIMP-2). *Biochemistry*, 32: 1583-1592, 1993.
  52. Moll, U. M., Youngleib, G. L., Rosinski, K. B., and Quigley, J. P. Tumor promoter-stimulated  $M_r$  92,000 gelatinase secreted by normal and malignant human cells: isolation and characterization of the enzyme from HT1080 tumor cells. *Cancer Res.*, 50: 6162-6170, 1990.
  53. Kolkenberg, H., Orgell, D., Hecker-Kia, A., Noack, W., and Ulbrich, N. The complex between a tissue inhibitor of metalloproteinases-2 (TIMP-2) and 72-kDa gelatinase is a metalloproteinase inhibitor. *Eur. J. Biochem.*, 198: 775-781, 1991.
  54. Goldberg, G. I., Strongin, A., Collier, I. E., Genrich, L. T., and Marmer, B. L. Interaction of 92-kDa type IV collagenase with the tissue inhibitor of metalloproteinases prevents dimerization, complex formation with interstitial collagenase, and activation of the proenzyme with stromelysin. *J. Biol. Chem.*, 267: 4583-4591, 1992.
  55. Strongin, A. Y., Collier, I., Bannikov, G., Marmer, B. L., Grant, G. A., and Goldberg, G. I. Mechanism of cell surface activation of 72 kDa type collagenase: isolation of the activated form of the membrane metalloprotease. *J. Biol. Chem.*, 270: 5331-5338, 1995.
  56. Howard, E. W., Bullen, E. C., and Banda, M. J. Regulation of the autoactivation of human 72-kDa progelatinase by the tissue inhibitor of metalloproteinases-2. *J. Biol. Chem.*, 266: 13064-13069, 1991.
  57. Stetler-Stevenson, W. G., Kruttsch, H. C., Wachter, M. P., Margulies, I. M. K., and Liotta, L. A. The activation of human type IV collagenase proenzyme: sequence identification of the major conversion product following organomercurial activation. *J. Biol. Chem.*, 264: 1353-1356, 1989.
  58. Fridman, R., Lical, J. C., Reich, R., Bonfil, R. D., and Ahn, C. Differential effect of phorbol ester on the *in vitro* invasiveness of malignant and non-malignant human fibroblast cells. *J. Cell. Physiol.*, 142: 55-60, 1990.
  59. Nguyen, Q., Willenbrock, F., Cockett, M. I., O'Shea, M., Docherty, A. J., and Murphy, G. Different domain interactions are involved in the binding of tissue inhibitor of metalloproteinases to stromelysin-1 and gelatinase A. *Biochemistry*, 33: 2089-2095, 1994.



## THE EFFECT OF PLATELETS ON INVASIVENESS AND PROTEASE PRODUCTION OF HUMAN MAMMARY TUMOR CELLS

Catherine BELLOC<sup>1</sup>, He LU<sup>1</sup>, Claudine SORIA<sup>1</sup>, Rafael FRIDMAN<sup>2</sup>, Yves LEGRAND<sup>1</sup> and Suzanne MENASHI<sup>1,3</sup>

<sup>1</sup>Unité 353 INSERM, Hôpital Saint-Louis, 1 Ave Claude Vellefaux, 75010 Paris, France; <sup>2</sup>Department of Pathology, Wayne State University, Detroit, MI USA.

**Interaction of tumor cells with platelets facilitates metastasis of tumor cells. It has been proposed that platelets protect tumor cells against the host's immune defense and enhance tumor-cell extravasation. In the present work we show that platelets increase the invasiveness of 3 mammalian cell lines (MCF-7, ZR-51 and MDA-MB231) through extracellular matrix, and propose this as an additional mechanism by which platelets facilitate metastasis. Since gelatinase and urokinase have both been implicated in degradation of the extracellular matrix and cell migration, and therefore in tumor invasion, we have also analyzed whether the interaction of platelets with tumor cells can modify the secretion of these proteases by tumor cells. MDA-MB231, which was the most invasive cell line among the 3 tested and was the most potent in inducing platelet aggregation, secreted the highest level of urokinase and was the only one in which gelatinase was detected. While platelets had no significant effect on the urokinase activity expressed by these cells, they induced in MDA-MB231 an important increase in the secretion of gelatinase, which can be reproduced by both platelet membrane and platelet releasate of activated platelets. This increase in gelatinase could be responsible, at least in part, for the increased invasiveness of these cells, since added TIMP-1 significantly reduced the number of cells which traversed matrigel.**

© 1995 Wiley-Liss, Inc.

The relationship between malignancy and thrombotic disorder has long been recognized and thrombo-embolic disorders are an important cause of morbidity and mortality in malignant disease. Drugs that interfere with platelet function have been shown to limit metastasis production (Honn *et al.*, 1983, 1992). Platelets could essentially facilitate all steps, from initial tumor cell lodgement in the microvasculature to tumor-cell proliferation after arrest, interaction with the subendothelial matrix and extravasation. These diverse effects may result from direct platelet bonding with tumor cells or from the release reaction products following platelet aggregation and activation (for review, see Honn *et al.*, 1992).

Numerous studies have shown that tumor cells *in vitro* can induce platelet aggregation and this ability to aggregate platelets was correlated with their ability to cause metastasis *in vivo* (Pearlstein *et al.*, 1980). The mechanisms of induction of platelet activation appear to vary in the different tumor cells. The generation of thrombin could be responsible in most cases as tumor cells can express an activator of factor Xa and/or tissue-factor-like material (for review, see Gordon, 1992). Other mechanisms may also contribute, such as the activation of platelets by ADP produced by the tumor cells themselves or released by platelets in contact with tumor cells (Bastida *et al.*, 1982), and the production of 12 hydroxyeicosatetraenoic acid (12 HETE), which facilitates the expression of the glycoprotein IIb-IIIa receptor (Steinert *et al.*, 1993).

Platelet activation by tumor cells is thought to protect tumor cells from immune surveillance, to enhance tumor-cell adhesion to vascular endothelium—the first step of extravasation—and to provide permeability factors and tumor-growth factors which would facilitate extravasation and development of tumor metastasis (for review, see Gasic, 1984; Honn *et al.*, 1992). In this work, we studied the effect of platelets on the production, by tumor cells, of proteases which are implicated

in the degradation and invasion of the extracellular matrix (ECM). Both urokinase-type plasminogen activator (u-PA) and gelatinases have been implicated. u-PA activates plasminogen into plasmin, a wide-range serine protease, which degrades some of the components of the ECM either directly or indirectly by activating prometalloproteases (He *et al.*, 1989). Numerous studies have shown a correlation between tumor aggressiveness and expression of u-PA (for review, see Schmitt *et al.*, 1992). Gelatinase A (MMP-2) and gelatinase B (MMP-9) are metalloproteases of 72 and 92 kDa respectively, and numerous studies have implicated both of these in the digestion of basement membrane type-IV collagen in cancer (Ura *et al.*, 1989) and angiogenesis (Gross *et al.*, 1983). A correlation between tumor secretion of gelatinases (Ura *et al.*, 1989; Liotta and Stetler-Stevenson, 1989) and experimental metastasis has been reported. Transfection of gelatinase-B expression vector conferred metastatic capacity upon non-metastatic rat cells (Bernhard *et al.*, 1994). We have therefore compared the potential of 3 different mammary tumor cells (MCF-7, ZR 51 and MDA-MB231) to aggregate platelets, and the effect of platelets on the secretion of both u-PA and gelatinases by the 3 different types of tumor cells as well as their invasiveness through an ECM.

### MATERIAL AND METHODS

#### Preparation of platelets

Platelets from human blood freshly collected in the anticoagulant acid-citrate dextrose were washed under sterile conditions as described by Patscheke and Worner (1978), in the presence of 100 ng/ml prostaglandin E<sub>1</sub>. The platelets were finally resuspended in DMEM/F12 culture medium (GIBCO, Paisley, UK).

#### Activation of platelets

Platelet suspensions were incubated at 37°C for 15 min before thrombin was added at 0.2 U/ml and the suspension was agitated gently for 3 min to allow the aggregation to proceed. Thrombin was neutralized by the addition of 10 IU/ml hirudin (Sigma, Saint Louis, MO) and the aggregated platelets were then pelleted by centrifugation at 2,500 g for 10 min. The supernatant was used as a source of platelet-secreted factors and the pellet containing the degranulated platelets was resuspended in DMEM/F12 culture medium (GIBCO).

#### Cell lines and culture conditions

The human adenocarcinoma cell lines MDA-MB231 (Keydar *et al.*, 1978), MCF-7 (Soule *et al.*, 1973) and ZR-75-1

<sup>3</sup>To whom correspondence and reprint requests should be sent, at Unité 353 INSERM, Hôpital Saint-Louis, 1 Ave Claude Vellefaux, 75010 Paris, France. Fax: 33 1 42490733.

**Abbreviations:** TIMP, tissue inhibitor of metalloproteinases; PA, plasminogen activator; u-PA, urokinase-type plasminogen activator; PAI, plasminogen activator inhibitor; ECM, extracellular matrix.

Received: August 15, 1994 and in revised form October 3, 1994.

### 2. Production of gelatinases by the 3 mammary-cancer cell lines: effect of platelets

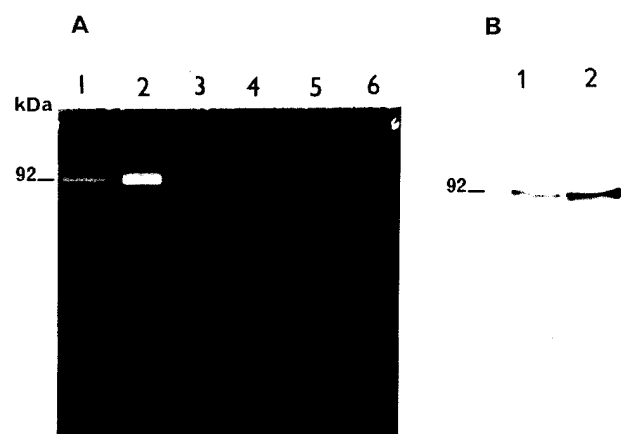
Zymographic analysis of the 3 cell lines (MDA-MB231, ZR 75-1 and MCF-7) showed that only MDA-MB231 cells secreted gelatinase activity. This activity at 92 kDa corresponding to gelatinase B was only detected in the conditioned medium and none was found in the cell lysate. No activity corresponding to the 72-kDa gelatinase A was detected. When MDA-MB231 cells were incubated with platelets for 24 hr, the intensity of the gelatinase-B lysis band greatly increased in the conditioned medium, whereas platelets did not induce any visible effect in the 2 other cell lines which did not secrete detectable gelatinase (Fig. 2a). No lysis band was observed when platelets alone were tested. The increase in the area of lysis of MDA-MB231 cells induced by platelets was not accompanied by a change in the molecular weight of the gelatinase, suggesting that this increase in lysis is related to an increased secretion rather than to an activation of the proenzyme. This increase in gelatinase secretion was confirmed by Western blot (Fig. 2b).

The secretion of gelatinase B by MDA-MB231 was studied using different matrix components as cell support, including collagen types I and IV, gelatin, fibronectin and laminin. While some variations were found in the basal secretion of gelatinase by the MDA-MB231 cells, depending on the matrix on which they were cultured, a similar increase in gelatinase activity was induced by platelets, regardless of the matrix which was used (results not shown).

In order to evaluate whether the effect of platelets is due to a soluble factor released into the supernatant during platelet activation or to the activated platelets themselves, we have compared the gelatinase secretion by MDA-MB231 cells incubated with either the platelet releasate or with the remaining degranulated platelets. As shown in Figure 3, the lysis band corresponding to the gelatinase was increased both by the supernatants of activated platelets and by the degranulated platelets.

### 3. Production of plasminogen activators by the 3 mammary-cancer cell lines: effect of platelets

All 3 cell lines expressed amidolytic activity which was only detected in the presence of plasminogen and therefore repre-



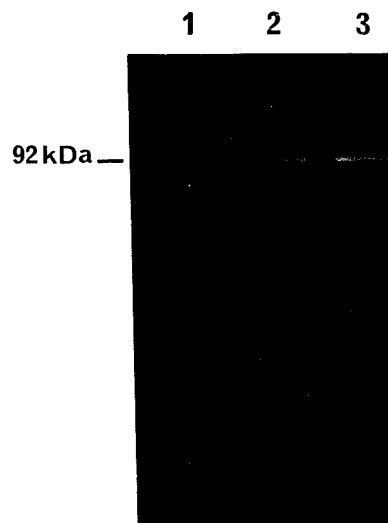
**FIGURE 2** – Effect of platelets on the secretion of gelatinase B by tumor cells. After 24 hr incubation of the 3 different cell lines in serum-free medium without or with platelet suspension ( $2 \times 10^8$ /ml), conditioned medium was tested at cell confluence by: (a) gelatin zymography. Lanes 1–2: MDA-MB231; lanes 3–4: ZR 75-1; lanes 5–6: MCF-7. Lanes 1, 3, 5: without platelet and lanes 2, 4, 6 in the presence of platelets. (b) Immunoblot of gelatinase B produced by MDA-MB231. Lane 1 without platelets, and lane 2 in the presence of platelets.

sented a true PA activity. This PA activity was of the urokinase type (u-PA) since it was totally inhibited by the presence of 1 mM amiloride, a selective inhibitor of u-PA (Sappino *et al.*, 1993). Therefore, the results were expressed as u-PA units. As shown in Table I, the highest u-PA activity was found in MDA-MB231 cells.

When the cells were treated for 24 hr with platelets, a small increase in u-PA activity was noted for MDA-MB231 cells and MCF-7. However, this increase was not statistically significant.

A very low level of u-PA activity was detected in the conditioned medium (less than 5% of the cell-associated u-PA), indicating that the u-PA secreted by these cells remained associated with the cell membrane. Platelets did not have a significant effect. Plasminogen-dependent amidolytic activity was not increased by the addition of fibrin monomers to the medium (results not shown), indicating that these cells did not secrete t-PA.

As platelets contain the u-PA inhibitor PAI-1, the possibility that this PAI-1 masks an increase in u-PA secretion brought about by platelets was investigated by zymography, which reveals both free u-PA and the complex u-PA/PAI. Figure 4 shows the zymography of PA activity obtained with the MDA-MB231 cell line. A major lysis band was observed at the region of 55 kDa which migrated to the same position as the high-molecular-weight urokinase, while the lysis band of 95 kDa corresponding to the u-PA/PAI-1 complex was very faint. The addition of platelets had no noticeable effect on the free u-PA band, but a small increase in the complex was observed. This increase in the u-PA/PAI complex was only observed with

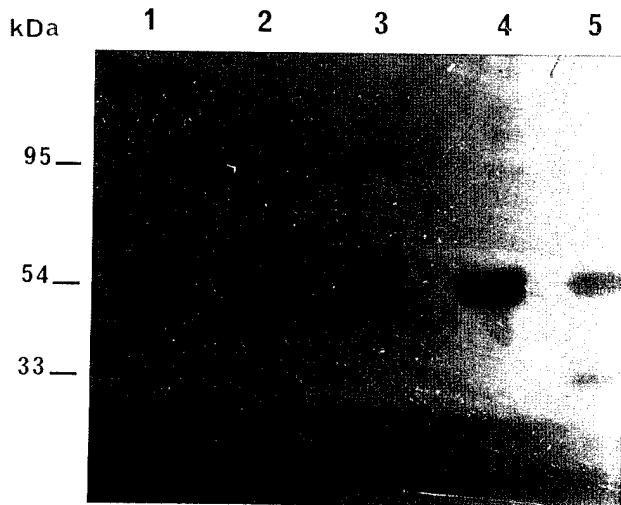


**FIGURE 3** – Gelatin zymography: comparison between the effect of releasate of activated platelets and degranulated platelets on the secretion of gelatinase B by MDA-MB231. MDA-MB231 cells at confluence were incubated for 24 hr: Lane 1 in serum-free medium; lane 2 with supernatants of thrombin-activated platelets and lane 3 with platelet pellets obtained after thrombin activation and centrifugation at 15,000 g for 10 min. Thrombin was used at 0.2 U/ml and neutralized after aggregation by 10 IU/ml hirudin.

**TABLE I** – U-PA ACTIVITY (U/10<sup>5</sup> CELLS) EXPRESSED BY THE 3 DIFFERENT MAMMARY CELL LINES: EFFECT OF PLATELETS

	MDA-MB231	MCF-7	ZR 75-1
Without platelets	21.9 ± 4.1	2.89 ± 0.8	1.19 ± 0.35
With platelets	27.7 ± 5.2	3.35 ± 0.5	1.20 ± 0.20

Results are expressed as mean ± SD of 4 different experiments.



**FIGURE 4**—Fibrin zymographic detection of the activity of cellular plasminogen activators (PA) on MDA-MB231: effect of platelets. MDA-MB231 at confluence were incubated for 24 hr. Lane 1, in serum-free medium; lane 2, with intact platelets; lane 3, with supernatants of thrombin-activated platelets; and lane 4, with platelet pellets obtained after thrombin activation and centrifugation at 15,000 g for 10 min (thrombin was used at 0.2 U/ml and was neutralized after aggregation by 10 IU/ml hirudin). Lane 5: purified u-PA.

the platelet releasate which contained the PAI and not with the degranulated platelets. Since this complex was not evidenced when purified uPA was added to platelet lysate which contains inactive PAI-1 (results not shown), we can only suggest that platelet PAI was activated by some factors secreted by the cells during the incubation thus able to form a complex with uPA.

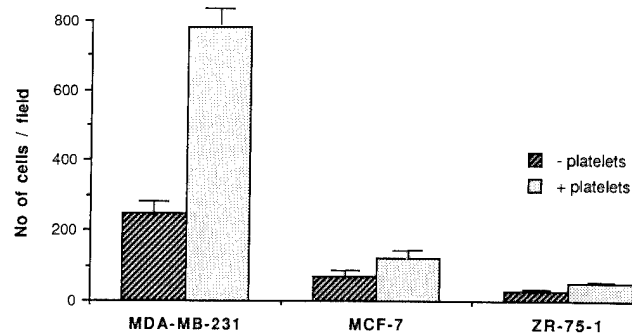
The u-PA activity in the 2 other cell lines was very low and not modified by platelet addition.

#### *Invasiveness of the 3 cell lines through the matrigel*

MDA-MB231 cells, which secreted the highest levels of both u-PA and gelatinase, showed the greatest capacity to traverse the matrigel in the Boyden chamber (Fig. 5). When platelet releasate was added to the cells, the capacity to traverse the matrigel was greatly increased ( $p < 0.001$ ). Since platelets also increased gelatinase secretion in MDA-MB231 cells, the participation of this protease in matrigel invasion was evaluated by the addition of the metalloproteinases inhibitor TIMP-1 (2  $\mu$ g/ml). An inhibition of 60% was observed, suggesting a possible role for gelatinase in the platelet-induced invasiveness of the MDA-MB231 cell line, although the participation of other metalloproteinases, such as stromelysin, cannot be ruled out. The fact that the matrigel invasion by the 2 cell lines, ZR 75-1 and MCF-7, which do not secrete detectable gelatinase, was also increased in the presence of platelets, indicates that other factors are involved.

#### DISCUSSION

Tumor cells in the vasculature are frequently observed in association with platelets, and this association has been postulated to be essential for the accomplishment of metastasis. The interaction of tumor cells with the platelets is thought to protect the cells against immune host defense mechanisms in the blood and to favor tumor-cell extravasation both by enhancing cell adhesion to the endothelium and by inducing



**FIGURE 5**—Invasiveness of the tumor cells: effect of platelets. Tumor cells ( $2 \times 10^5$ ) were introduced into the upper compartment of the Boyden chamber without or with the supernatant of  $3 \times 10^7$  thrombin-activated platelets in the presence of 0.1 mM APMA (4-aminophenylmercuric acetate), activator of metalloproteinases. After incubation at 37°C for 18 hr, the cells were counted on the lower side of the filter, after the cells remaining on the upper side had been scraped off. Results were expressed as the number of cells per microscopic field. Ten fields were counted in each of 3 different experiments.

endothelial cell retraction (Honn *et al.*, 1992). This present work suggests an additional role for platelets in the development of metastasis by facilitating the invasiveness of tumor cells through the ECM. Platelets significantly increased the invasiveness of the 3 types of mammalian tumor cell tested (MDA-MB231, MCF-7 and ZR-51). This increased invasiveness is due, at least partially, to an increase in gelatinase secretion. This was shown in the case of MDA-MB231 where the basal level of gelatinase was greatly stimulated by platelets. The stimulation of gelatinase shown by zymography corresponded to an increase in the protein level, demonstrated by Western blot, thus indicating increased secretion of the protease and not activation of the proenzyme. However, platelet-induced gelatinase could not be shown with the other 2 cell lines which did not produce detectable basal levels of gelatinase. This suggests that, in addition to gelatinase, platelets increase invasiveness of tumor cells by some other mechanisms. This is also supported by the fact that TIMP-1, a specific inhibitor of metalloproteinase, did not totally block the platelet-induced invasiveness (60% inhibition) of MDA-MB231. We can exclude a role for cell-associated u-PA as a mechanism of platelet-induced invasiveness, since u-PA was not significantly modified by platelets in any of the 3 cell lines, whatever the basal value.

The mechanisms proposed so far for the role of platelets in the metastatic process all refer to events occurring during the passage of tumor cells into the circulation. The increased invasiveness shown in this study suggests an additional role for platelets once tumor cells have traversed the endothelium into the ECM. We propose, therefore, a new mechanism by which the effect of platelets renders tumor cells more invasive, partially due to gelatinase secretion. We have already shown that platelets induce the secretion by endothelial cells of both gelatinase and u-PA (Menashi *et al.*, 1991), both implicated in angiogenesis. Taken together, these results suggest that platelets may act by favoring both tumor invasiveness and angiogenesis which is required for tumor progression and extravasation.

#### ACKNOWLEDGEMENTS

This work was supported by the Institut National de la Santé et de la Recherche Médicale (INSERM) and by the Ligue Nationale Contre le Cancer.

## REFERENCES

- BASTIDA, E., ORDINAS, A., GIARDINA, S.L. and JAMIESON, G.A., Differentiation of platelet aggregating effects of human tumor cell lines based on inhibition studies with apyrase, hirudin and phospholipase. *Cancer Res.*, **42**, 4348-4352 (1982).
- BERNHARD, E.J., GRUBER, S.B. and MUSCHEL, R.J., Direct evidence linking expression of matrix metalloproteinase 9 (92 kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells. *Proc. nat. Acad. Sci. (Wash.)*, **91**, 4293-4297 (1994).
- ENGEL, L.W. and YOUNG, N.A., Human breast carcinoma cells in continuous culture: a review. *Cancer Res.*, **38**, 4327-4339 (1978).
- FRANDSEN, T.L., BOYSEN, B.E., JURUS, S., ZWIEBEL, J., SPANG-THOMSEN, M., THOMPSON, E.W. and BRUNNER, N., Assays for the study of human cancer cell invasion and metastasis. *Fibrinolysis*, **6**, 71-76 (1992).
- GASIC, G.J., Role of plasma, platelets and endothelial cells in tumor metastasis. *Cancer Metast. Rev.*, **3**, 99-116 (1984).
- GORDON, S.G., Cancer cell procoagulants and their role in malignant disease. *Sem. Thromb. Haemost.*, **18**, 424-433 (1992).
- GRANELLI-PIPERNO, A. and REICH, E., A study of protease and protease inhibitor complexes in biological fluids. *J. exp. Med.*, **146**, 223-234 (1978).
- GROSS, J.L., MOSCATELLI, D. and RIFKIN, D.B., Increased capillary endothelial cell protease activity in response to angiogenic stimuli *in vitro*. *Proc. nat. Acad. Sci. (Wash.)*, **80**, 2623-2627 (1983).
- HE, C., WILHELM, S.M., PENTLAND, A.P., MARMER, B.L., GRANT, G.A., EISEN, A.Z. and GOLDBERG, G.I., Tissue cooperation in a proteolytic cascade activating human interstitial collagenase. *Proc. nat. Acad. Sci. (Wash.)*, **86**, 2632-2636 (1989).
- HERRON, G.S., WERB, Z., DWYER, K. and BANDA, M.J., Secretion of metalloproteinases by stimulated capillary endothelial cells. *J. biol. Chem.*, **261**, 2810-2813 (1986).
- HONN, K.V., BUSSE, W.D. and SLOAN, B.F., Prostacyclin and thromboxanes: implications for their role in tumor cell metastasis. *Biochem. Pharmacol.*, **32**, 1-11 (1983).
- HONN, K.V., TANG, D.G. and CHEN, Y.Q., Platelets and cancer metastasis: more than an epiphenomenon. *Sem. Thromb. Haemost.*, **18**, 392-415 (1992).
- KEYDAR, I., KARBY, C.S., WEISS, F.R., DELAREA, RADU, M., CHAITCIK, S. and BRENNER, H.J., Establishment and characterization of a cell line of human breast carcinoma origin. *Europ. J. Cancer*, **15**, 659-670 (1978).
- LIOTTA, L.A. and STETLER-STEVENSON, W., Metalloproteinases and malignant conversion: does correlation imply causality? *J. nat. Cancer Inst.*, **81**, 556-557 (1989).
- MENASHI, S., HE, L., SORIA, C., SORIA, J., THOMAIDIS, A. and LEGRAND, Y., Modulation of endothelial cell fibrinolytic activity by platelets. *Thromb. Haemost.*, **65**, 77-81 (1991).
- PATSCHEKE, H. and WORNER, P., Platelet activation detected by a turbidimetric shape-change analysis. *Thromb. Res.*, **12**, 485-496 (1978).
- PEARLSTEIN, E., SALK, P.L., YOGESWARAN, S. and KARPATKIN, S., Correlation between spontaneous metastatic potential, platelet-aggregating activity of cell surface extracts, and cell surface sialylation in 10 metastatic-variant derivatives of a rat renal sarcoma cell line. *Proc. nat. Acad. Sci. (Wash.)*, **77**, 4336-4339 (1980).
- SAPPINO, A.P., MADANI, R., HUARTE, J., BELIN, D., KISS, J.Z., WOHLWEND, A. and VASSALI, J.D., Extracellular proteolysis in the adult murine brain. *J. clin. Invest.*, **92**, 679-685 (1993).
- SCHMITT, M., JANICKE, F. and GRAEFF, H., Tumor associated proteases. *Fibrinolysis*, **6**, 3-26 (1992).
- SOULE, H.D., VASQUEZ, J., LONG, A., ALBERT, S. and BRENNAN, M., A human cell line from a pleural effusion derived from a breast carcinoma. *J. nat. Cancer Inst.*, **51**, 1409-1413 (1973).
- STEINERT, B.W., TANG, D.G., GROSSI, I.M., UMBARGER, L.A. and HONN, K.V., Studies on the role of platelet eicosanoid metabolism and integrin  $\alpha_{IIb} \beta_3$  in tumor-cell-induced platelet aggregation. *Int. J. Cancer*, **54**, 92-101 (1993).
- URA, H., BONFIL, D., REICH, R., REDDEL, R., PFEIFER, A. and HARRIS, C.C., Expression of type IV collagenase and procollagen genes and its correlation with tumorigenic, invasive and metastatic abilities of oncogene-transformed human bronchial epithelial cells. *Cancer Res.*, **49**, 4615-4621 (1989).