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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 specialized proteinases including the matrix metalloproteinases (MMPs) and cathepsins. We have studied the regulation of activation and plasma membrane localization of MMP-2 and MMP-9, two members of the MMP family, and cathepsin B in breast epithelial and fibroblast cells. For these studies it was necessary to isolate primary breast fibroblasts. In this report we show the isolation and characterization of primary breast fibroblasts derived from normal and tumor tissue and the expression of MMPs and cathepsin B. Studies were carried out to establish the role of tumor-stroma interactions and the extracellular matrix on the expression of MMPs, TIMPs and cathepsin B. We also report on the nature of the association of MMP-9 to the surface of breast epithelial cells. Experiments in progress will study the role of stromal fibroblasts on proteases (MMPs, cathepsin B) in cell surface localization and activation in breast cancer cells.

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Fridman 7/9/96

PI - Signature Date

Rafael A. Fridman, Ph.D.

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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 cells. 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 proteolitically 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-ß (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). *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? In this report, we now present data on the isolation of primary breast fibroblasts.

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 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). *In vitro*, expression of this enzyme can be induced in immortalized breast epithelial cells, MCF10A, in response to phorbol ester and tumor necrosis factor-α (TNF-α). 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 co-localization of the two gelatinases in breast tumors on the cell surface may facilitate the interaction of these two enzymes. Furthermore, cell surface localization of gelatinases may play a role in concentrating proteinases on the areas of cell-matrix contacts. Regarding cell surface association, little is known about the interactions of MMP-9 with the cell surface. Here we present evidence showing the localization of MMP-9 on breast MCF10A epithelial cells.

6. BODY OF REPORT

During this year we have concentrated on Tasks 1 and 2 of Years 1-2 and Tasks 1, 2 and 3 of Years 2 and 3.

Following is a description of the major accomplishments for each of the specific tasks and a response to the reviewer's comments to our previous report.

Years 1-2

Task 1. In our previous report we informed about the findings regarding the expression of MMPs and inhibitors in the various breast epithelial cell lines and fibroblast-derived from breast tissue. This has been accomplished.

Task 2. Here we present new results regarding the expression and localization of cathepsin B in breast epithelial cells and fibroblasts.

Years 2-3

Tasks 1 and 2. We present evidence on isolation and establishment of primary fibroblast and the initiation of the co-culture experiments.

Response to the Reviewer's Comments to the Previous Report (94-95)

Contractual Issues.

1. *"Incomplete Task 2 from Years 1-2."*

We now report progress in the studies described in Task 2 of the SOW.

Technical Issues.

1. *"In question are the assumptions when employing non-mammary cell lines to address breast cancer metastasis. The PI has made clear the difficulty in maintaining primary breast fibroblasts; however, more effort should be made in this area for future reports."*

We concur with this comment of the reviewer. During the last year we have made a considerable effort to isolate primary breast fibroblasts to address tumor- stroma interactions in breast cancer in a more relevant system. Initially, we obtained breast fibroblasts from Drs. Helene Smith and Dr. Derkie which helped us to start these studies. However, due to the short life span of these cells and the difficulty of obtaining a continuous supply, we were compelled to isolate these cells in our Institution. Here we report the isolation and characterization of five primary breast fibroblasts and the characterization of breast fibroblasts provided by Dr. Derkie. Unfortunately, the life span of Dr. Derkie's fibroblasts was very short and they are no longer available. That is the reason we have isolated our own fibroblasts.

2. "Can MMP-2 be activated if breast fibroblasts are exposed to conditioned media from MDA-435 cells." This an important and fundamental question that we are trying to answer. We have designed the appropriate experiments and the results have been disappointing. The details of these experiments are provided.

6.1 EXPERIMENTAL METHODS

NOTE: In the present report, we have only included methods that were not described in our previous report or in the original application.

6.1.a. Isolation of Primary Breast Fibroblasts

This project is in collaboration with Dr. Herb Soule and Steven Santner from the Karmanos Cancer Center (formerly the Michigan Cancer Foundation). Samples of breast tissue were obtained from the Department of Pathology, Wayne State University, under the supervision of Drs. Daniel Visscher and Wael Sakr. Breast tissue samples (0.2-1.0 g) were first washed thoroughly with phosphate buffered saline containing antibiotics. The tissue was then placed in a tissue culture dish and finely minced into small pieces (<1mm). Then, the tissue was placed in a 15 ml tube containing 150 units/ml of hyaluronidase (Sigma H4272) and 100 units/ml of collagenase (Sigma C2674) in DMEM/F12 containing 20% calf serum. The tube was mixed overnight at 37°C until the tissue dissagreggates into small organoids. The organoids were placed in tissue culture flasks in DMEM/F12 containing 20% calf serum until attachments and spreading of cells. After several days, the fibroblasts were removed from the flask by trypsinization for 2 min. The cells were then seeded and passaged in Waymouth's MB752-1 media supplemented with 15% fetal bovine serum. The use of Waymouth's media was determined empirically to yield mostly fibroblasts. After several days in culture, the fibroblasts can be seen migrating from the organoids. The fibroblasts are maintained in complete Waymouth's media and splitted at a ratio of 1:2. When sufficient cells are obtained, generally after 2-3 passages, a frozen stock is prepared for cryopreservation in liquid nitrogen using standard freezing techniques.

6. 1.b. Co-Culture Experiments.

These experiments were performed in two ways.

1. The primary breast fibroblasts were seeded in 6 well plates in complete Waymouth's media and allowed to grow until a 80-90% confluence. Then, the media was removed and various amounts (2 x 10^5-1 x 10^6 cells/well) of breast epithelial cells (benign or tumor) were added to the fibroblast

cells in 1.5 ml/well serum free Waymouth's media. As control, some wells received only Waymouth's media. In other plate, the breast epithelial cells were cultured alone. The cultures were then incubated for a period of 3 days in a CO2 incubator at 37 C. At the end of the incubation period, the media and cells were collected for analysis.

2. The primary breast fibroblasts were cultured in the bottom well of a 24 well plate to be used with the Transwell inserts. Simultaneously, the breast epithelial cells were cultured in the Transwell inserts which were previously coated with collagen I. When both the fibroblasts and the epithelial cells reached a 80% confluence, the inserts were placed on the wells containing the fibroblasts. The cultures were then incubated in serum-free Waymouth's media for 3 days as described above and then the media were analyzed for MMPs and TIMP-2 expression.

6.1.c. Analysis of Cell surface binding of proMMP-9 to MCF10A breast epithelial cells.

Cell surface biotinylation and immunoprecipitations—Cell surface proteins were biotinylated with 0.5 mg/ml of the water-soluble, cell impermeable biotin analog sulfo-NHS-biotin (Pierce, Rockford, IL) for 30 min (twice) at 4°C in PBS containing calcium and magnesium. The reaction was then quenched with 50 mM NH_4Cl in PBS followed by two washes with cold PBS. Biotinylated cells were then solubilized with 1.5% Triton X-114 in Tris-buffered saline (TBS), 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM PMSF and 5 mM EDTA. The extracts were briefly (5 min) centrifuged (14,000 rpm, 4°C), and the supernatants were warmed (2 min., 37°C) and centrifuged (14,000 rpm, 22°C) to separate the detergent and aqueous phases. The aqueous phase was then incubated (1 hr, 4°C) with gelatin-agarose beads followed by a brief centrifugation. The beads were then incubated with TBS containing 0.02% Brij-35, protease inhibitors and 10% DMSO, centrifuged and the supernatant was then 5-fold diluted with immunoprecipitation buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Brij-35 and 1 mM PMSF). The samples were then incubated with various anti-MMP-9 antibodies or mouse or rabbit IgGs, as controls. The immunoprecipitates were then subjected to SDS-PAGE under reducing conditions followed by transfer to a nitrocellulose membrane. Detection of the biotinylated proteins was accomplished using a streptavidin-HRP conjugate provided in the enhanced chemiluminescence (ECL) kit (Amersham, Arlington Heights, IL) according to the manufacturer instructions. The specificity of the avidin detection was determined in non-biotinylated cells extracted and immunoprecipitated in a similar way.

Pulse-chase Analysis—MCF10A cells were grown to 80% confluence in 60 mm dishes and then treated with 100 mM TPA for 12 hrs in complete media. After TPA treatment, the media was removed and the cell layer was washed twice with warm PBS followed by incubation (30 min) with 1 ml/dish of DMEM without methionine supplemented with 25 mM Hepes (starve media). The cells were then pulsed with 500 µCi/ml of ^{35}S-methionine (Label Express, Amersham) in 0.6 ml/dish of starve media for 15 min at 22°C. After the pulse, the dishes were placed on ice, the media was removed and the cells washed gently with PBS before addition of 1 ml/dish of DMEM supplemented with 10% FBS and 240 mM methionine (chase media). At the end of the chase period (ranging from 0-180 min incubation times at 37°C), the cells and media were harvested with 60 mM Tris-HCl pH 7.5 containing 0.5% SDS, 2 mM EDTA and 1 mM methionine (final concentrations). The samples (media and cell lysates) were then boiled (100°C, 5 min) and received 5 mM iodoacetamide, 2.5 % Triton X-100 and 20 µg/ml aprotinin (final concentration). For immunoprecipitation, the samples were then incubated (16 hrs., 4°C) with 5 µg/ml of CA-209 mAb followed by addition of 30 µl Fast-flow Protein G beads (Pharmacia) and incubation for 3

additional hrs. at 4°C. The samples then centrifuged to obtain the pellet which was subsequently washed (5 times) with 50 mM Tris-HCl pH 7.5 containing 150 mM NaCl, 0.1% NP-40 and 10% glycerol. The pellets were then resuspended in 15 µl Laemmli sample buffer with dithiotreitol followed by boiling (100°C, 5 min) and loading into 8-16% SDS-polyacrylamide gels. Detection of the proteins was performed by autoradiography.

Endoglycosidase H digestion—The beads of the immunoprecipitations from the biotinylated or pulse chase samples were washed with 50 mM Tris-HCl pH 7.5 containing 150 mM NaCl, centrifuged and then resuspended in 25 µl of 100 mM sodium phosphate pH 5.5 containing 50 mM 2-mercaptoethanol, 0.05% SDS. The samples were then boiled (100°C, 5 min) and cooled followed by the addition of 2 µl of Endo H (25 U/ml) (Glyko, Novato, CA). After incubation (2 hrs, 37°C), the samples received Laemmli sample buffer, boiled and subjected to SDS-PAGE in 7.5% polyacrylamide gels followed by either immunoblot analysis (biotinylated samples) or autoradiography (pulse chase).

Immunoblots—Samples were subjected to SDS-PAGE under reducing conditions. The separated proteins were then transferred to BA-S 85 nitrocellulose paper (Schleicher & Schuell, Keene, NH). After blocking with 3% bovine serum albumin and 3% non-fat dry milk in 100 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.02 NaN_3 (blotto), the blots were incubated with the corresponding primary antibody diluted (1:5000) in 20 mM Tris-HCl pH 7.5, 137 mM NaCl and 0.1% Tween-20. The immunodetection of the antigen was performed using the ECL kit (Amersham) according to the manufacturer's instructions.

6.2 RESULTS.

6.2.a. Tumor-derived and Benign Breast Fibroblasts obtained from Dr. Derkie.

We obtained the following primary fibroblast isolates from Dr. Derkie:

Benign: 998, 987, 1042, G125, G61, G332, G351, and G347
Tumor: G344, G346, S176, and S177.

Expression of MMP-2 and MMP-9. These fibroblasts all express MMP-2 at similar levels (**Figure 1**) when tested by gelatin zymography. No differences were found between the tumor derived or the benign fibroblasts. In some cells, we identified a gelatinolytic band of ≈120 kDa which its identity remains unknown.

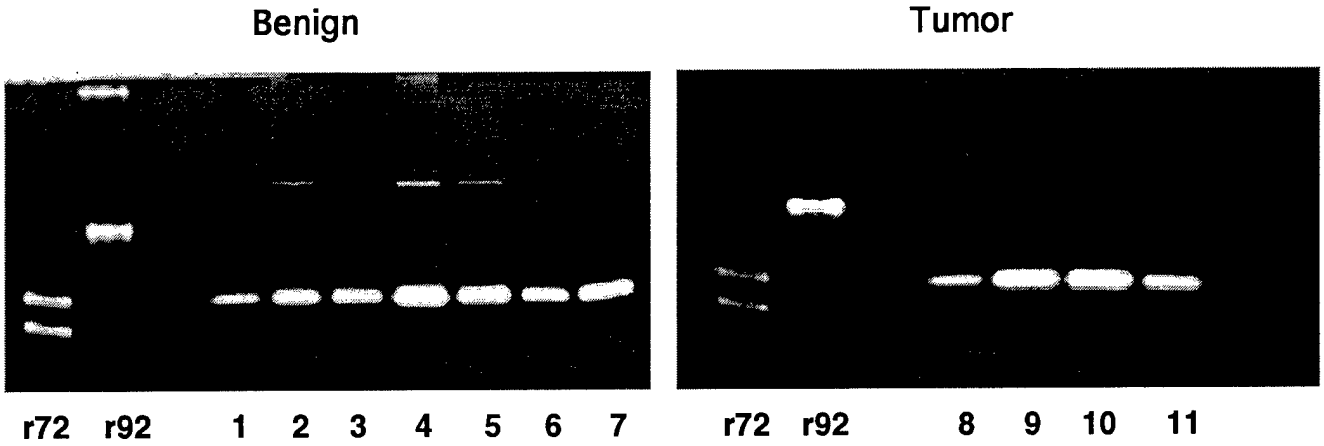

Figure 1. Gelatin zymogram of conditioned media from primary breast fibroblasts obtained from Dr. Derkie. Lane 1, 998; lane 2, 987; lane 3, 1042; lane 4, G125; lane 5, G61; lane 6, G332; lane 7, G351; lane 8, S176; lane 9, G344; lane 10, G346; and lane 11, S177. r72, human recombinant MMP-2 showing the 72 and 62-kDa forma; r92, human recombinant MMP-9 showing the dimer and monomer forms. Note that all the fibroblast cells express MMP-2 and no differences exist between benign and tumor fibroblasts.

Expression of TIMPs. As shown in **Figure 2**, the breast fibroblasts all secrete TIMP-1 albeit at different levels. Similar data was obtained with TIMP-2 (not shown). As with MMP-2 expression, we could not establish a solid difference between benign and tumor-derived fibroblasts. Furthermore, the reasons for the variable expression of TIMPs among the different isolation cannot be explained.

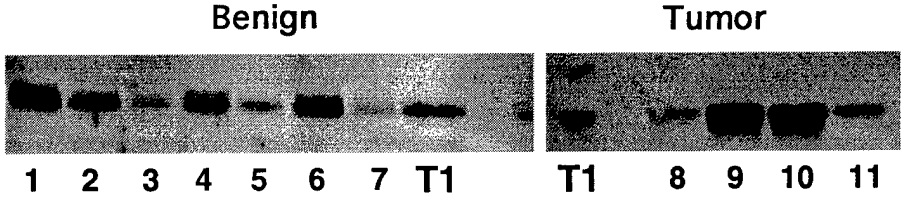

Figure 2. Immunoblot analysis of TIMP-1 expression in primary breast fibroblasts obtained from Dr. Derkie. Lane 1, G351; lane 2, G332; lane 3, G61; lane 4, G125; lane 5, 1042; lane 6, 987; lane 7, 998, lane 8, S176, lane 9, G346; lane 10, G344; and lane 11, S176. T1 refers to recombinant TIMP-1, as control. TIMP-1 was detected in concentrated serum-free conditioned media. Variable levels of TIMP-1 expression can be seen but they are not related to the origin of the fibroblasts.

6.2.b. Isolation and Characterization of Primary Breast Fibroblasts from Benign and Tumor Breast Tissue.

The fibroblasts obtained from Dr. Derkie were mostly late passage (passage 10-13) and we believed that this is the reason for our inability to maintain them in culture. In addition frozen stocks of these cells were, unfortunately, non viable. To obtain meaningful data it was necessary to

obtain new breast fibroblasts at early passages so that viable frozen stocks could be established. To this end, we attempted to isolate our own breast fibroblasts from tissue obtained from the Department of Pathology. The tissue was macroscopically classified by Dr. Visscher as tumor tissue or benign tissue from where the fibroblasts were isolated as described above. The isolated fibroblasts were designated as tumor tissue-derived or benign tissue -derived fibroblasts. It should be noted that this is an arbitrary designation and it does not imply the existence of intrinsic differences between these fibroblasts. In fact, we are not aware of a marker capable of distinguishing normal vs tumor fibroblasts. Therefore, these cells may be essentially similar and their behavior is altered by their intimate association with the tumor cells.

Figure 3 shows three different stages in the process of fibroblast isolation including the typical organoid (**A**), the emigrating cells from the organoid (**B**) and the isolated fibroblasts with the characteristic spindle shape morphology (**C**).

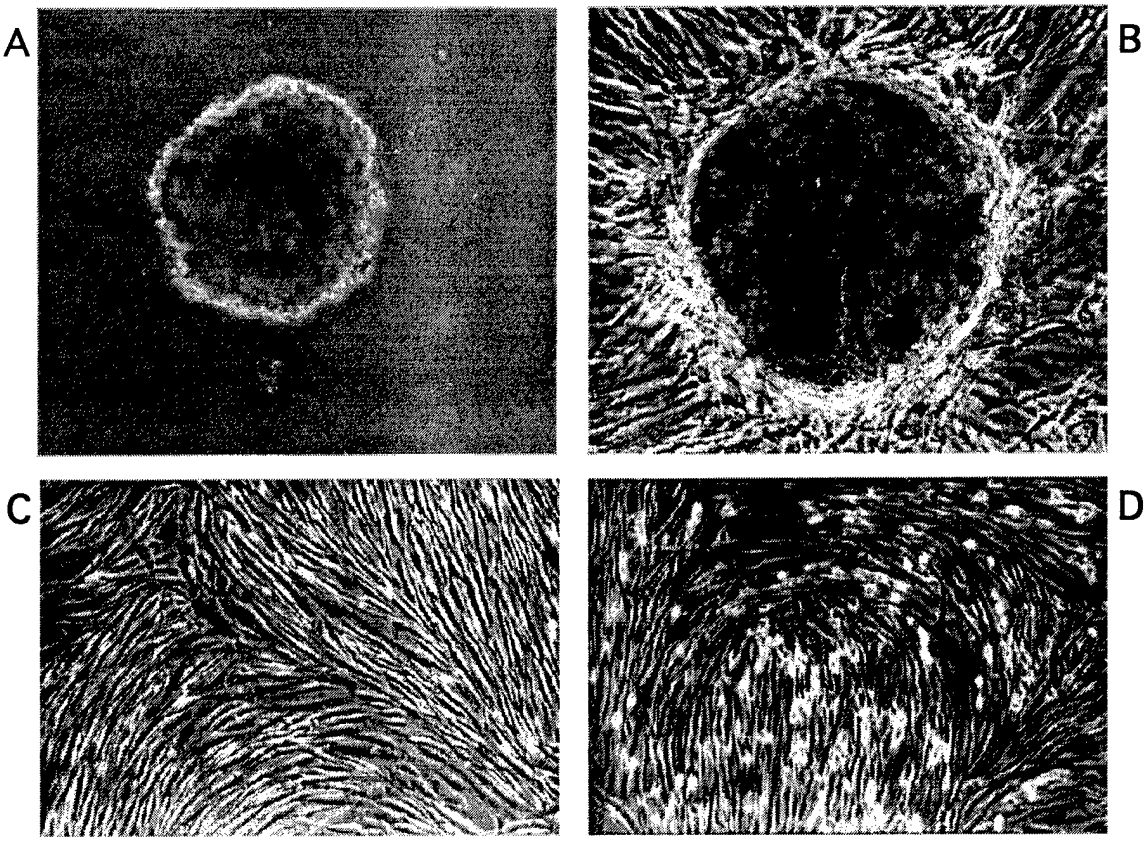

Figure 3. Morphology of cultured primary breast fibroblasts. **A**. Organoid after 5 days in culture. **B**. Organoid attached to the plate with emerging fibroblast-like cells. **C**. Isolated breast fibroblasts at passage 2. **D**. Primary breast fibroblasts co-cultured with breast cancer MDA-MD-231 cells (round cells).

The isolated fibroblasts were designated **WS** for Wayne State University and a letter **B** for benign

or **T** for tumor-derived. Some isolates of benign and tumor fibroblasts were obtained from the same breast. In other isolation, only one type survived. The following isolates are now available: WS8B, WS8T, WS9T, WS10T, WS11B. WS11T, WS12B and WS12T.

Morphology and Expression of Cell Specific Markers. The isolated fibroblasts were initially characterized by immunohistochemistry using commercially available antibodies to several markers of epithelial cells, fibroblasts, and myoepithelial cells. To this end, cells between passage 1 and 3 were grown in glass slides (Labtech) chambers, fixed with a solution of 60% methanol, chloroform 30% and acetic acid 10% for 10 min. and then the cells were treated for immunostaining using routine procedures. **Table 1** summarizes the results of the expression of the cell markers.

	BENIGN-DERIVED	TUMOR-DERIVED
MARKER		
Vimentin	+++	+++
[1]α-Smooth muscle actin	++	++
[2]Pancytokeratin	-	-
[3]Cytokeratin 18	-	-
[4]Cytokeratin 14	-	-

Table 1. Expression of cell type specific markers in isolated human primary fibroblasts. [1]Staining for α-smooth muscle actin in the benign-derived fibroblasts showed in 10-50% of the cells and in the tumor-derived 40-100%. [2]Pancytokerastin is an antibody recognizing a mixture of cytokeratins, markers of epithelial cells. [3]Cytokeratin 18 is a marker for breast luminal epithelial cells. [4]Cytokeratin 14 is a marker for myoepithelial cells.

These studies demonstrates that the cells isolated from the breast tissues are fibroblastic. It should be mentioned that the analysis of the cell type specific markers will be performed again in latter passages to confirm the stability of the cultures. We will also analyze the karyotype of the cells using conventional techniques of karyotyping available in the Department of Pathology.

MMP and TIMP Expression. We investigated the expression of MMP-2 and MMP-9 and TIMPs (TIMP-1 and TIMP-2) in these human breast fibroblasts using gelatin zymography as previously described (32). These data demonstrated that WS9T, WS10T, WS8B, WS8T, WS11B, and WS11T secrete the latent form of MMP-2 (**data not shown**). No differences were observed whether the cells were derived from the benign or the tumor tissue. A faint gelatinolytic band of ≈87 kDa was also detected in all these fibroblasts. We tested the fibroblasts for the presence of MT-MMP-1 expression in the detergent phase after extraction with Triton X-114 and found that all the isolates produce MT-MMP-1 (**Figure 4, next page**).

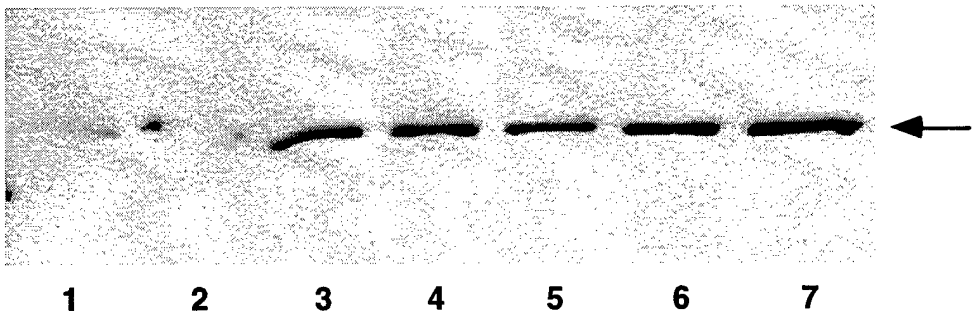

Figure 4. Immunoblot analysis of MT-MMP-1 expression in primary WS human breast fibroblasts. Cell were lysed with Triton X-114 and the detergent phase analyzed by immunoblot using a polyclonal antibody against a synthetic peptide from MT-MMP-1. Lane 2, WS9; lane 3, WS10T; lane 4, WS8B; lane 5, WS8T; lane 6, WS11B; and lane 7, WS11T. Lane 1, plasma membranes of HT1080 known to contain MT-MMP-1, as control.

The expression of TIMP-1 and TIMP-2 in the WS fibroblasts was tested by immunoblot analysis of concentrated conditioned media using specific antibodies. These studies demonstrated that the WS fibroblasts secrete both TIMP-2 and TIMP-1 (**Figure 5**).

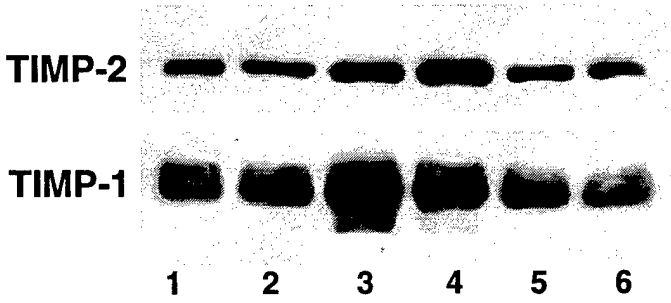

Figure 5. Immunoblot analysis of TIMP-1 and TIMP-2 expression in primary WS human breast fibroblasts. Concentrated conditioned media was analyzed using a monoclonal antibody against TIMP-2 and a polyclonal antibody against TIMP-1. Lane 1, WS9T; lane 2, WS10T; lane 3, WS8B; lane 4, WS8T; lane 5, WS11B; and lane 6, WS11T.

Effect of ConA and TPA. In other cell systems, ConA and TPA were shown to induce proMMP-2 activation, possibly mediated by MT-MMP-1. To determine the ability of the primary breast fibroblast to respond to ConA or TPA, the cells (WS9T, G332, G347, and G351) were treated with 10 μg/ml ConA (**Figure 6, lanes 2 and 5, next page**) or 100 nM TPA (**Figure 6, lanes 1 and 4**) for 16 hrs. in serum free media. Then, the media was analyzed for MMP-2 activation by zymography. As shown in **Figure 6**, G351 (**panel A, lane 2**), G347 (**panel A, lane 5**), G344 (**panel B, lane 2**), G346 (**panel B, lane 5**), G332 (**panel 6, lane 2**) and WS9T (**panel C, lane 5**)

all responded to ConA by activating MMP-2 to generate the 62- and 59-kDa active forms. TPA, also caused the induction of proMMP-2 activation in the same fibroblasts however less efficient than ConA. In addition, TPA induced expression of proMMP-9 in all the breast fibroblasts with the exception of G332 (**panel C, lane 1**).

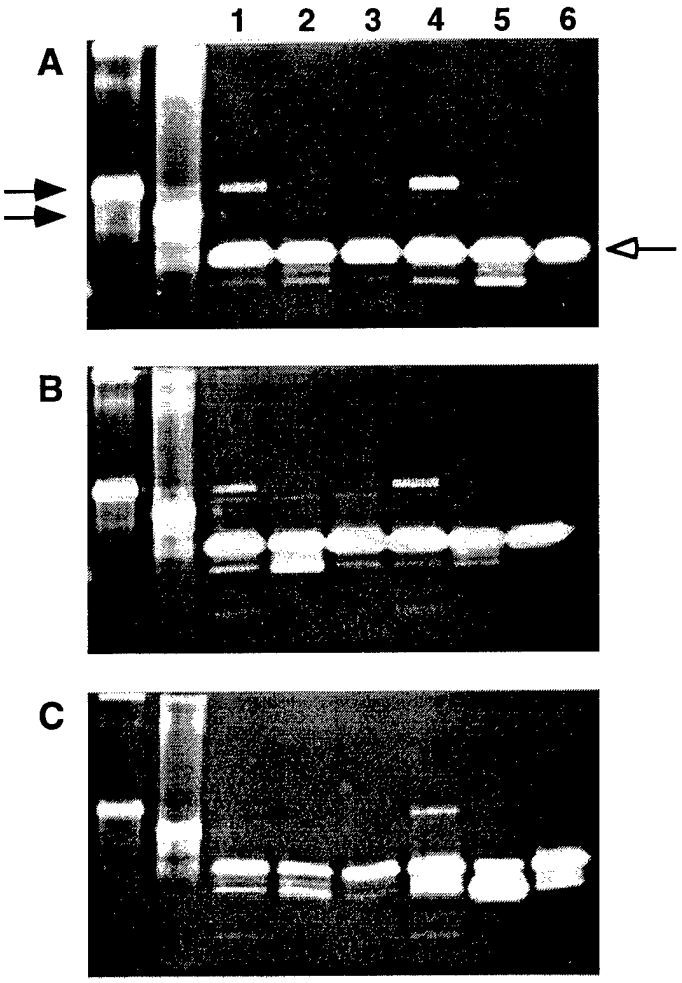

Figure 5. Effects of ConA and TPA on human primary breast fibroblasts. ConA (lanes 2 and 5) and TPA (lanes 1 and 4) induce activation of proMMP-2 compared to untreated cells (lanes 3 and 6). TPA also induces expression of MMP-9. **A**, G351 (lanes 1-3) and G347 (lanes 4-6). **B**, G344 (lanes 1-3) and G346 (lanes 4-6). **C**, G332 (lanes 1-3) and WS9T (lanes 4-6). The two arrows in the left side of A represent latent (upper arrow) and activated (lower arrow) recombinant MMP-9. The open arrow in the right side of A points to the latent MMP-2 (72 kDa) produced by the fibroblasts.

Breast Fibroblasts-Breast Tumor Cell Interactions. We initiated studies as proposed in the SOW to address the role of tumor-stroma interactions on the expression of proteases and protease inhibitors. These studies are particularly difficult when using primary cells due to the slow growth

and short life span of these cells in culture. These facts affect the amount of experiments that can be carried out with a particular set of breast fibroblasts. Nevertheless we were able to set up different types of experiments. Two major type of experiments were performed: 1. Effect of conditioned media. This experiment was designed to address the question as to whether there is a factor in the tumor cells that stimulates expression or activation of MMPs or expression of TIMPs. Unfortunately, we can report at this time of a significant and reproducible effect/s of conditioned media on any of the parameters studied, except for a slight increase in TIMP-2 expression. The reason/s for these negative results are presently unknown. However, several explanations can be provided. (i) The cells used (MCF10A, MDA-435, MDA-231, MCF7) do not produce *in vitro* putative stimulators of MMP/TIMP regulation. It should be noted that a few natural factors are known to induce MMP-2 expression and/or activation including TGF-ß and collagen type I. (ii) The factor is produced at very low levels and/or its production is mediated by the action of another factor. Thus, the breast epithelial would require prior stimulation to produce it. (iii) The response of the fibroblasts is dependent on the substrate that the cells are grown. (iv) Primary fibroblasts lose the response *in vitro*. Other explanations are possible but we are making a major effort to address these issues in order to provide meaningful and reproducible new information. In terms of TIMP expression, we examined the effects of conditioned media on TIMP-2 and no positive results were obtained. However, we are not surprised from these negative results since there are no indications of a known modulator of TIMP-2 expression (Dr. Ives DeClerck, personal communication).

We decided to carry out co-culture experiments by culturing the breast tumor cells (2 x 10^5 cell/dish) in the presence of the WS9T (2 x 10^5 cell/dish) primary fibroblasts (**see Figure 3D**) for 3 days. The media of the cultures were then examined for expression of gelatinases and TIMP-2. These preliminary studies, as for today, failed to show any significant effect on gelatinase expression and/or activation (not shown). In the case of TIMP-2 (**Figure 6**), we only observed a slight increase when the WS9T cells were co-cultured with MDA-MD-435 breast cancer cells that, at equal protein loading, was higher than the TIMP-2 produced by WS9T and MDA-MD-435 breast cancer cells, alone. The significance and reproducibility of this finding is yet unknown. However, we will continue our research in this direction.

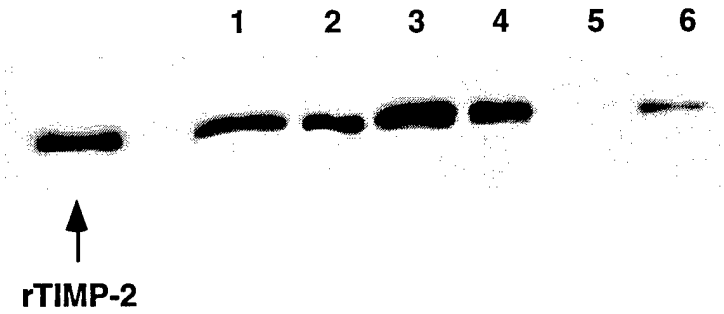

Figure 6. Immunoblot analysis of TIMP-2 expression in co-cultures of human primary breast fibroblasts with non-malignant and malignant breast epithelial cell lines. WS9T fibroblasts were cultured alone (lane 4) or with either MCF10A (lane 1), MDA-MD-231 (lane 2) or MDA-MD-435 (lane 3). Lane 5 and 6, MCF10A and MDA-MD-231 cells alone, respectively. MDA-MD-435 alone produce very low levels of TIMP-2 (not shown). Note the slight increase in TIMP-2 in the co-culture of WS9T with MDA-MD-435 breast cancer cells (lane 3).

6.2.c Cell Surface localization of MMP-9. 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. MMP-9 in breast tumor is produced by inflammatory cells, tumor cells and fibroblasts. Regardless of the source of the enzyme, immunohistochemical data demonstrated that MMP-9 is localized on the cell surface of the tumor cells. Although the mechanisms of cell surface association of MMP-2 were investigated, little is known about the interactions of MMP-9 with the cell surface.

Cell Surface Localization of MMP-9 in MCF10A Cells. In our previous report we have shown for the first time the presence of MMP-9 in the cell surface of immortalized human breast epithelial MCF10A cells after induction with TPA. We have shown this using extractions with Triton X-114 which demonstrated the presence of two form of proMMP-9 in the aqueous phase. Since Triton X-114 solubilizes the cells, this procedure cannot distinguish between intracellular and cell surface proteins. Thus, one cannot relay on this technique to establish the cell surface association of a given protein. To definitely establish the presence of MMP-9 in MCF10A cells we decided to use cell surface biotinylation. This technique makes use of a cell impermeable biotin analogue that forms a covalent bond only with cell surface proteins. To determine whether proMMP-9 associates with the cell surface, control and TPA-treated MCF10A cells were subjected to cell surface biotinylation, extraction with Triton X-114 and immunoprecipitation with two different anti-MMP-9 antibodies. **Figure 7 (next page)** shows that the CA-209 mAb precipitates a major 92-kDa protein only from the biotinylated TPA-treated MCF10A cells. In addition, CA-209 specifically precipitated a minor biotinylated protein of approximately 83-85 kDa (**Figure 7, lane 2**). Same results were obtained when the immunoprecipitations were performed with a rabbit polyclonal antibody (ab109) directed against the N-terminal propeptide of proMMP-9 (**Figure 7, lane 4**) and known to recognize only latent MMP-9 (92 kDa). Biotinylation and immunoprecipitation signals were specific since immunoprecipitation of non-biotinylated cells with the same antibodies (**Figure 7, lanes 3 and 5**) or immunopreciptations using preimmune IgGs (not shown) showed no signals. Thus, these studies conclusively demonstrates the cell surface association of proMMP-9 in breast epithelial MCF10A cells

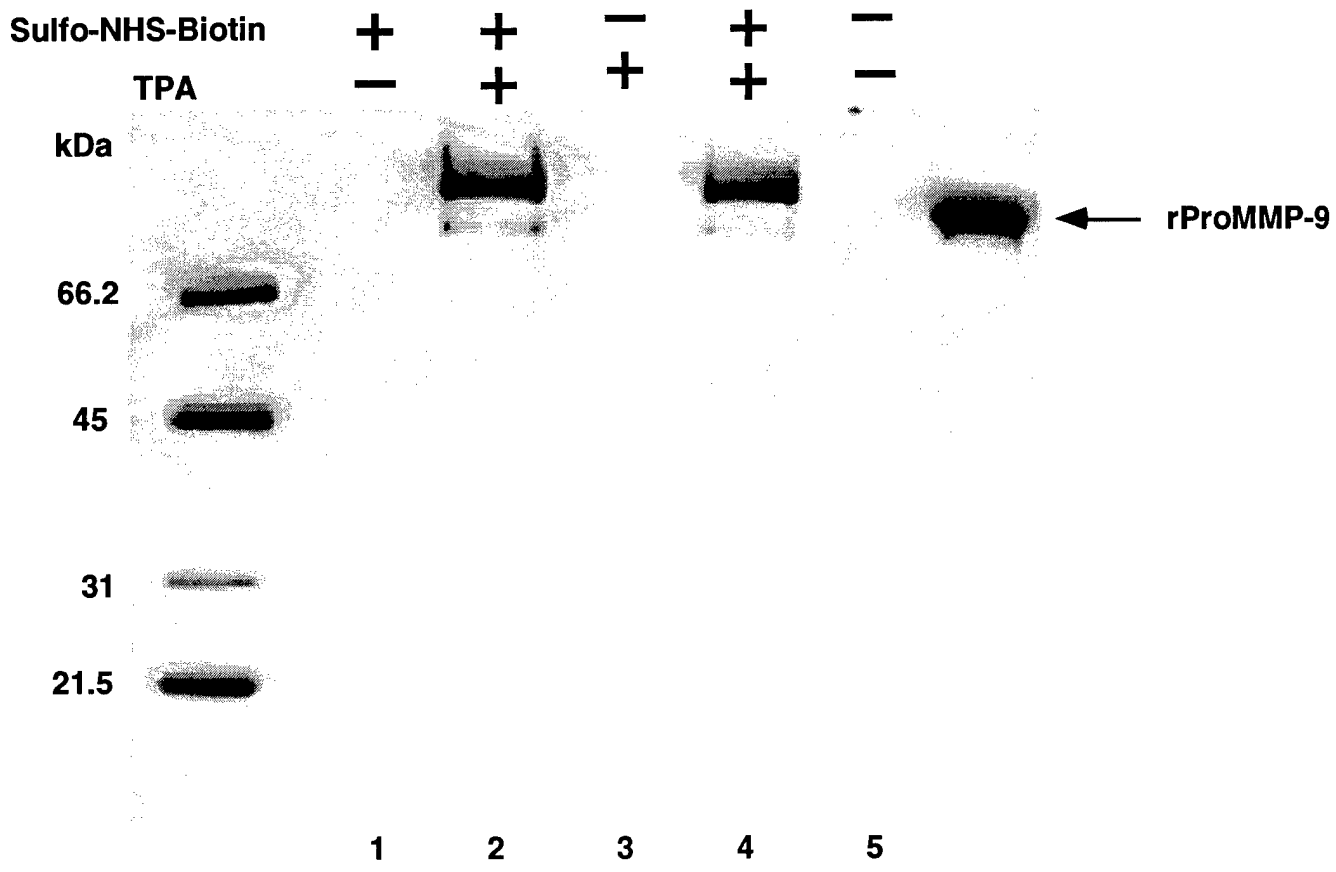

Figure 7. Immunoprecipitation of cell surface biotinylated MMP-9 from MCF10A cells. MCF10A cells treated (lanes 2, 3 and 4) or not (lanes 1 and 5) with 100 mM TPA were surface biotinylated (lanes 1, 2 and 4) and then immunoprecipitated with either mAb CA-209 (lanes 1-3) or ab109 (lane 4) or without antibody (lane 5). Purified recombinant biotinylated proMMP-9 (rProMMP-9) was used as control.

Synthesis of proMMP-9 in MCF10A cells. Since two proMMP-9 forms were found on the cell surface while only the 92-kDa zymogen form was in the media, we investigated the biosynthetic pathway of proMMP-9 in TPA-treated MCF10A cells by pulse-chase analysis (**Figure 8, next page**). After a 15 min pulse, the cells (intracellular) and media (extracellular) were harvested at various times and the fractions immunoprecipitated with CA209. As shown in Figure 2, an 83-85-kDa protein was detected at 0 min chase in the cell lysate. After 15 min. chase, the 83-85-kDa precursor form was gradually converted to the mature form of proMMP-9 consistent with processing of complex oligosaccharide chains. Both forms were clearly detectable intracellularly even after a 120 min chase. Analysis of the media from the same experiment showed a gradual secretion of the 92-kDa form starting after the 30 min. chase, in agreement with the time (15 min) of processing of the precursor form into the mature proenzyme. We could not detect in the media secretion of low molecular weight forms of proMMP-9.

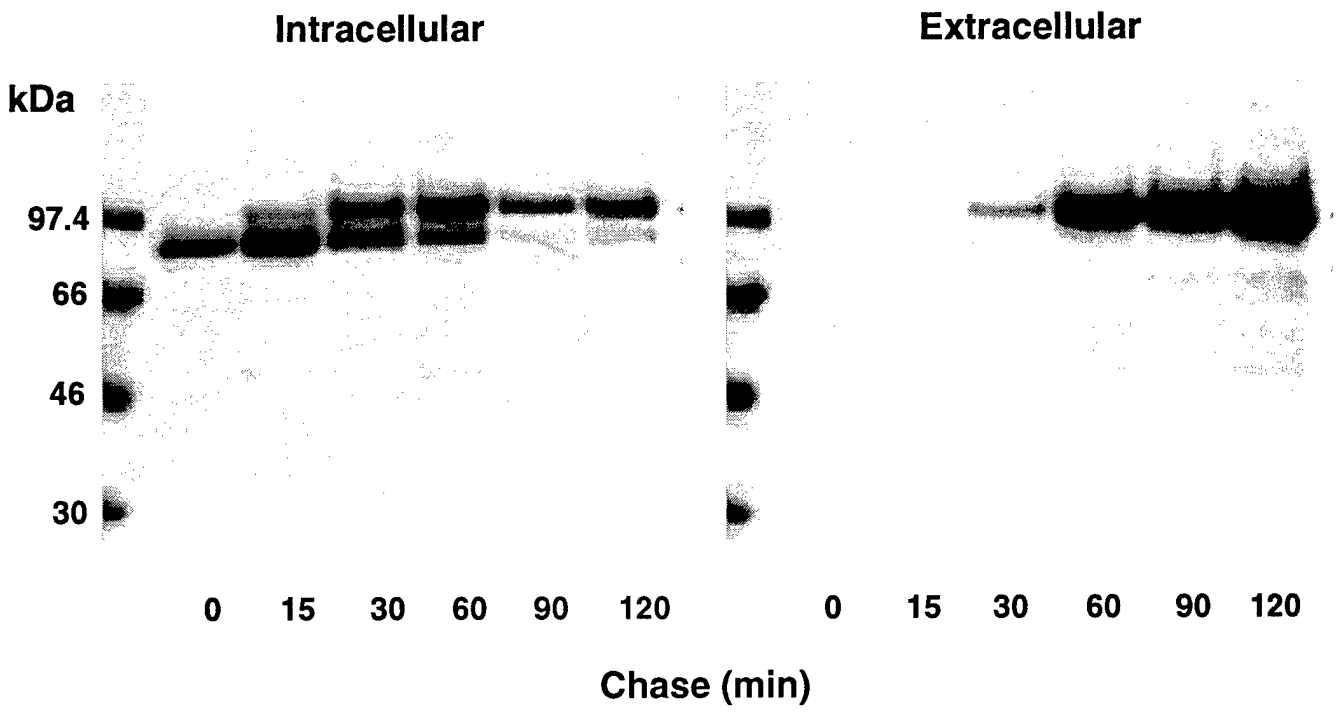

Figure 8. Pulse chase analysis of MMP-9 in MCF10A cells. Cells (intracellular) and media (extracellular) of ^{35}S-labeled MCF10A cells were chased after various times and immunoprecipitated with mAb CA-209.

Since proMMP-9 is a glycosylated enzyme, we examined the glycosylation pattern of the cell surface associated and intracellular forms of proMMP-9 by endoglycosidase H digestion. This enzyme specifically cleaves oligosaccharides of the high mannose and hybrid type and helps to distinguish complex from high mannose oligosaccharides. Using endoglycosidase H we wished to determine the similarities and differences in glycosylation between the cell surface 83-85-kDa form detected after biotinylation and the intracellular 85-kDa precursor form. To this end, the immunoprecipitates of the biotinylated cells (cell surface) and of the pulse chase experiment (intracellular) were subjected to endoglycosidase H treatment. **Figure 9 (next page)** shows that the intracellular 85-kDa precursor form was endoglycosidase H-sensitive (**Figure 9, lanes 2 and 4**) whereas the intracellular 92-kDa form was resistant to endoglycosidase H cleavage (Figure 3, lane 4) consistent with the addition of complex carbohydrates to the mature 92-kDa form in the Golgi complex. Endoglycosidase H digestion of the cell surface biotinylated forms revealed that the 83-85-kDa enzyme was endoglycosidase H-sensitive and that the 92-kDa form was endoglycosidase H resistant (**Figure 9, lane 6**).

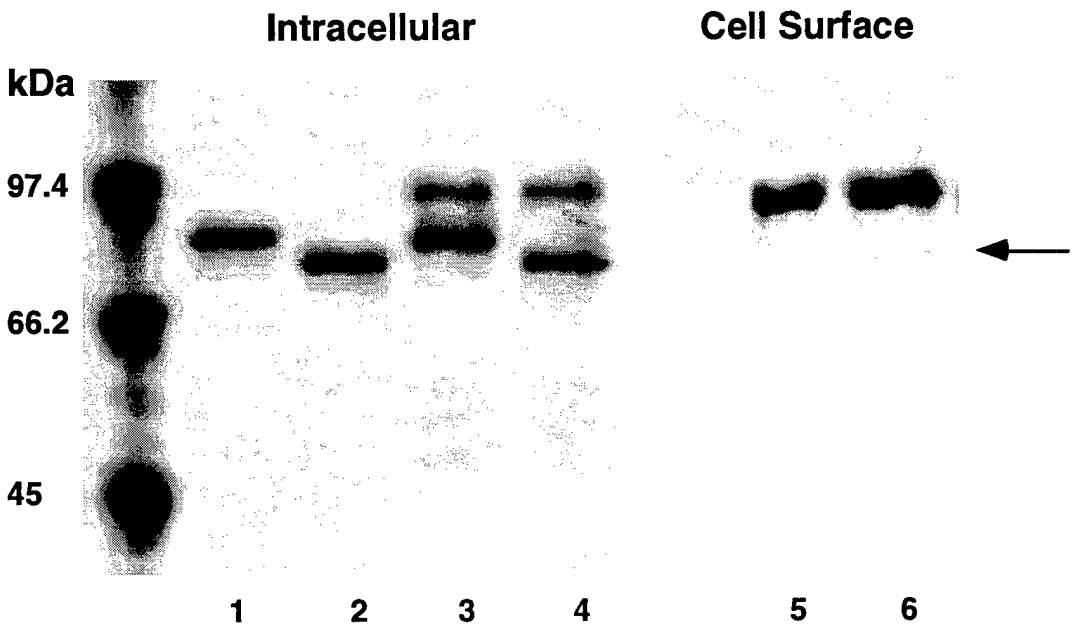

Figure 9. Endoglycosidase H digestion of intracellular and c ell surface proMMP-9. Immunoprecipitates from lysates of the pulse chase experiment collected at 15 (lanes 1 and 2) and 30 min (lanes 3 and 4) and from the biotinylated cells (lanes 5 and 6) were treated (lanes 2, 4, and 6) or not (lanes 1, 3 and 5) with endoglycosidase H. Note the shift in molecular weight of the 83-85-kDa form (left arrow) after treatment with endo H.

Taken together, these results demonstrate that in TPA-treated MCF10A cells proMMP-9 (92 kDa) is intracellularly processed and secreted as a classic glycoprotein. In addition, these data suggest that the minor cell surface-associated 83-85-kDa form of proMMP-9 possesses a glycosylation pattern similar to that of the intracellular 85-kDa precursor form suggestive of a unique processing and targeting of the precursor form. Although the precise function and fate of the precursor form of proMMP-9 on the cell surface are presently unknown, our data suggests that in TPA-treated MCF10A cells a small fraction of the proMMP-9 precursor enzyme is targeted to the cell surface in an endoglycosidase H sensitive form without being processed in the Golgi complex. The alteration/s in intracellular transport resulting in the cell surface association of the MMP-9 precursor form are unknown. However, they do not appear to be related to TPA treatment since the two proMMP-9 forms were also detected on the cell surface of biotinylated PC3 prostate carcinoma cells which constitutively produce MMP-9 (unpublished observation). Also, an enriched plasma membrane fraction isolated from TPA-treated MCF10A cells contains the two proMMP-9 forms and no detectable TIMP-1, as determined by gelatin zymography and immunoblot analysis (not shown). It is of interest that we were unable to detect TIMP-1 on the surface of MCF10A cells using various methods. Thus, it possible that TIMP-1, in contrast to proMMP-9, does not associate with the cell surface of MCF10A cells. In contrast, high amount of TIMP-1 were readily detected in the supernatant where could be identified in complex with proMMP-9. Therefore, while in the extracellular space the fate of proMMP-9 activation would be determined by its interactions with TIMP-1, on the cell surface the lack of available inhibitor would be expected to alter the activation and catalytic properties of proMMP-9. Although breast epithelial can synthesize MMP-9, the studies with the primary fibroblasts showing induction of MMP-9 secretion by TPA (**Figure 5**)

suggest that upon secretion the enzyme may bind to the breast tumor cells were it can be localized on the cell surface.

6.2.d. Expression of cathepsin B in breast epithelial and fibroblast cells.

The extracellular matrix has been shown to regulate gene expression in mammary epithelial cells. For example, expression of α-casein is induced by growth on laminin (43). Therefore, we have compared the expression and distribution of cathepsin B in human breast epithelial and carcinoma cells grown on laminin coated and uncoated cell culture inserts. In addition, we have compared the expression and distribution of cathepsin B in human breast fibroblasts grown within a collagen I gel such as that found *in vivo*.

Breast epithelial cells. Growth on laminin did not affect cathepsin B expression in MCF10A cells, either in parental cells or cells transfected with oncogenic c-Ha-ras. We have reported previously that the distribution of cathepsin B is altered by ras transfection so that cathepsin B is present in perinuclear vesicles in the parental MCF10A cells, but in both perinuclear and peripheral vesicles in the ras-transfected cells (44,45). This altered localization of cathepsin B was not affected by growth on laminin coated cell culture filters.

Breast cancer cell lines. In three breast carcinoma cell lines (BT20, SKBR3 and MCF-7), growth on laminin did not alter the expression of cathepsin B at the protein level, but did affect the distribution of cathepsin B. Cathepsin B in these cells becomes concentrated in focal adhesions, i.e., sites at which cells are attached to the matrix as well as sites at which motile and invasive cells disattach from the matrix. Proteases in the focal adhesions are believed to participate in this disattachment.

Breast fibroblasts. For these experiments we used the benign-derived breast fibroblast cells 998 obtained from Dr. Derkie. To this end, the cells were grown on uncoated coverslips or in three-dimensional collagen I gels. We have found that expression of cathepsin B was increased when the 998 fibroblast cells were grown in the 3-dimensional collagen I gel, suggesting that either type I collagen and/or cytoskeletal rearrangements can induce expression of cathepsin B. In order to determine whether collagen I is inducing an increase in expression of cathepsin B at the transcript or protein level, we are in the process of optimizing protocols for *in situ* hybridization. Digoxigenin labeled antisense and control sense cathepsin B riboprobes corresponding to exons 7 through 11 of the cathepsin B gene have been generated. The labeling efficiency of incorporation into the transcripts has been measured and northern blots have confirmed that only the antisense probe reacts with the 2.2 kb and 4.0 kb cathepsin B transcripts.

In conclusion, these results suggest that expression and distribution of cathepsin B in breast epithelial and fibroblasts cells can be regulated by interactions with the stroma.

7. CONCLUSIONS

1. We have isolated and characterized primary breast fibroblasts from normal and malignant tissue. A good stock of frozen cells in early passages has been established and it is now available for the studies proposed in the SOW.

2. The fibroblasts cells all express MMP-2, MT-MMP-1, TIMP-2 and TIMP-1 although at various levels. No significant differences were observed between benign and tumor-derived fibroblasts.

3. MMP-2 is activated in the breast fibroblasts by exposure to ConA or TPA as reported in other cell system indicating that the cellular machinery involved in proMMP-2 activation is functional in these primary cells. TPA induces MMP-9 expression.

4.. Co-cultures of breast fibroblasts and breast cancer cells do not show, under the conditions tested, any significant effect on MMP-2 expression and/or activation. However, a slight increase in TIMP-2 expression was observed in the presence of MDA-MD-435 breast cancer cells.

5. ProMMP-9 is associated with the cell surface of MCF10A cells and is present in two forms. A major 92-kDa latent form and a minor 85-kDa latent form. The 85-kDa form represent the endo H-sensitive intracellular precursor form.

6. Breast fibroblasts express cathepsin B and its expression is regulated by collagen I.

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