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13. ABSTRACT *(Maximum 200*
The high fatality of breast cancer is mostly caused by metastases. Excessive secretion of a group of matrix metalloproteinases (MMPs) or decreased production of their tissue inhibitors (TIMPs) has been implicated in tumor invasion and metastasis. The hypothesis that an imbalance of MMPs over TIMPs may contribute to the invasive and metastatic processes of human breast tumors was tested by localizing and contrasting the expression of specific MMPs; gelatinases,and collagenases and their inhibitors (TIMP -1, -2, & -3) by immunohistochemical analysis of tissue sections of human breast tissues. MMPs and TIMPs were quantitated from the same tissue used for immunohistochemistry by gelatin substrate zymography (MMPs, gelatinases), reverse zymography (TIMPs) and substrate protein assays (collagenases, MMP-1). Gelatinases can be quantitated easily from small amount of tissue by zymography using an imager and GelBase Pro software. Zymography of tissue extracts showed that MMP-9 was exclusively found in breast carcinomas (18 specimens) in varying amounts and levels were 15-25 times higher than that found in normal and benign tissues. This finding was confirmed by immunostaining of tissue with specific MMP antibodies. Northern blot analysis of all MMPs in breast tissues is in progress. Tumor cell line, metastatic, MET-1 (murine) showed the secretion of mainly MMP-9 Immunofluorescence staining and confocal analyses of cells showed the presence MMP-9 and MMP-2 (not secreted), MT-MMP-1 and CD44v-III around the cell plasma membrane. Reverse zymography of tissue extracts showed the presence of TIMPs,but at this point quantitation was not acheived but feasible in future.

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Bernard Gunja-Smith
PI - Signature

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Date

(4).Table of Contents

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

(5) INTRODUCTION

BACKGROUND

5.A.1. Breast Cancer

The overall objective of this research is to explore the role of matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) in the invasive and metastatic processes of human breast tumors [1-4]. The hypothesis to be tested is that an imbalance arises through normal or elevated production of MMPs accompanied by a diminished production of inhibitory TIMPs. Metastasis of an initially localised tumor to vital organs is the dominant cause of cancer related deaths [5]. The mechanisms controlling the metastatic progression of a localized tumor are very complex, involving many biochemical and cellular events [6]; but recent evidence indicates that secreted matrix metalloproteases (MMPs) play a major role in the penetration of the basement membrane surrounding the tumor [7].

Metalloproteinases are needed for invasiveness of tumors. The most important traits of tumor cells are their ability to specifically attach to the extracellular matrix (ECM), degrade this extracellular barrier so as to escape out of the primary location into the circulation and establish themselves at the site of metastasis. The breakdown of the ECM can be traced to the action of one or more members of the family of zinc proteases named **matrix metalloproteinases (MMPs)** secreted as proenzymes [8] and are activated outside the cell by serine proteases such as plasmin [9-11] by the removal of the 9 kDa prosegment from the active site. Three enzymes are most likely responsible for the degradation of ECM [5, 11]: 72 kDa gelatinase (gelatinase A, type IV collagenase, MMP-2), 92 kDa gelatinase (gelatinase B, type V collagenase, MMP-9), and the 57 kDa stromelysin (MMP-3) [12-15]. 72 kDa gelatinase was implicated in human breast cancer where 36/40 cases of invasive tumors were positive by immunohistochemistry [16]. Stromelysin and also interstitial collagenase (MMP-1) have been implicated in human breast cancer by Polette [17] and Clavel [18]. Recently, stromelysin-3 has been found in breast cancers [19], but the proteolytic activity of this enzyme appears to be very weak [20].

5.A.2. Metalloproteinase action is limited by specific inhibitors

In addition to MMPs, cells also produce TIMPs. These are small proteins that neutralize the destructive activities of MMPs and play an important role in controlling ECM degradation. There are at least three species that may be implicated in tumors: TIMP-1, TIMP-2 and TIMP-3 of relative mass 28 kDa, 20 kDa, and 23 kDa respectively [9]. They bind to

activated MMPs in a 1:1 molar ratio and inhibit their activity. TIMP-2 and TIMP-3 share an amino acid homology with TIMP-1, although they are encoded by different genes [21]. Moreover, many cells produce MMP-2 in a 1:1 complex with TIMP-2; similarly, MMP-9 usually comes with an associated molecule of TIMP-1. The TIMP molecule in these cases is bound to the C-domain of the enzyme, but interferes with the activation of the latent gelatinases (MMP-2and MMP-9). The role of TIMP-3 is just begining to unravel. TIMP-3 binds to ECM and is difficult to solubilize and its role in cancer has not been identified. Although some reports [22] show that TIMP-3 is secreted during normal differentiation processes in normal mouse embryos and may play a role in the development of cancer *in vivo* and may be secreted in the early cancer development [23].

5.A.3. Role of MMPs and TIMPs in breast cancer

Since normal cells as well as non-invasive cells produce MMPs and also TIMPs, it seems likely that the extent of activation of MMPs and the levels of inhibitory TIMPs may be two key factors in the progression of normal to invasive cell type. Elevated levels of MMP-9 have been detected in the plasma of patients with breast cancer [24] although no such correlation was found for MMP-2 [25].

5.A.4. Summary of Background

In summary, there is compelling evidence of a role for metallo-proteases (and other proteases) and the TIMP inhibitors in tumor cell invasiveness. There are lines of evidence to support the concept that an increase in gelatinases and a decrease in TIMPs may be important in this process together with a greater conversion of latent to active forms of the MMPs. The resultant increased MMP activity would permit penetration of basement membrane surouding the tumor by cells which produce these enzymes or stimulate neighboring stromal cells to produce such enzymes. The same processes would also be important in metastasis of the tumor cells. What is generally missing from these literature reports is a unified study of the changes of multiple enzymes and inhibitors in the same tissue, bolstered by immunohistochemical data on enzyme localization, biochemical assay of latent and active enzyme activities and molecular biological determination of mRNA levels. It is the development of such a unified picture. We have begun this unified study and our first year research report attests to our ability to perform such studies.

5.b. PURPOSE OF THE PRESENT WORK:

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

In order to test the hypothesis, the following types of studies are planned. i) Identification and localization of various MMPs and TIMPs by immunohistochemistry, with qualitative grading and measurement of enzyme activity by the use of substrates for gelatinase and stromelysin activities. ii) Determination of the amounts and ratios of active and latent enzyme by substrate zymography and immuno-precipitation combined with zymography. Methods suitable for extracting and assaying enzymes directly from tumor tissue are available. iii) Determination of mRNA levels for the MMPs and TIMPs in the same tissue samples, based on Northern blotting. iv) Culture of epithelial cells growing out from explants of human mammary carcinomas and determination of their production of active and latent MMPs and TIMPs.

The research study takes advantage of unusual resource available to us - a repository or archive of tumor tissue collected for 4 years complete with sections and snap-frozen blocks of fresh tissue. Because of the existence of the Florida tumor registry, it is possible to return to these tissues, to examine them by immunohistochemistry and then to perform biochemical measurements on the frozen blocks of tissue. The MMPs and TIMPs are extremely stable, so there is no problem in measuring after prolonged storage. One can then use the results in prognostic fashion, since it is known if the patients subsequently developed metastasis, or died of the cancer.

5.C. METHODS OF APPROACH

Identify, localize and contrast the the expression of specific MMPs and TIMPs by immunohistochemical analysis of tissue sections of human breast carcinoma

Initially, the plan is to investigate tissues classified by pathological evaluation: i) Invasive carcinoma with no subsequent metastasis ii) invasive carcinoma with subsequent metastasis and iii) **in situ** carcinoma with no metastasis. These tissues will all be drawn from a group collected and stored as both paraffin blocks and frozen cubes of tissue (1 cm^3). The subsequent history of the patients can be determined from the registry. Sections will be stained with one of the 6 monospecific polyclonal rabbit antibodies to MMPs and TIMPs and examined for didstribution of reaction, relation to cell interior/exterior and cell type, intensity, etc. As the data collection continues, the groups expanded and make finer subdivisions to include

age, estrogen status, lobular versus ductal location, nuclear grade, etc.. This can be done in subsequent years. The tissue archives will, of course, continue to grow with each subsequent year. We do not anticipate many difficulties with this aim. The antibodies used will be checked periodically for reactivity by Western Blots.

Identify, characterize and quantitate the MMPs and TIMPs and their mRNA from the same tissue used for immunohistichemistry: Enzymes, inhibitors and their mRNAs are extracted from small frozen blocks of tissue, corresponding to sections examined for enzymes and immunohistochemistry. We have developed methods for protein extraction that are quantitative. We also have developed assay methods over many years that are quite sensitive, detecting subnanogram amounts of MMPs and TIMPs. These methods are detailed in the next section. In addition to enzyme assays, extensive use will also be made of zymography for detecting MMPs and of reverse zymography for detecting TIMPs. These methods permit estimation of the active and latent forms of MMPs (since both forms display activity upon refolding from SDS) and distinction of TIMP-1 and -2. The gelatinases present a special problem in zymography (overlapping bands) which can be solved by a new method of immunoprecipitation followed by zymography. Finally, the mRNA levels are to be determined by Northern analysis using specific cDNA probes. If necessary RT-PCR methods can be used These various approaches should enable us to test our hypothesis about enzyme/inhibitor imbalance in several ways.

Isolation and culture of primary human mammary epithelial cells from breast cancer tissue explants.

Using methods by which we successfully isolated the primary epithelial cells from prostate tissues [26], we will do the same for human carcinomas. We cannot use the archival tissue in this study, but will obtain fresh benign and invasive tumor tissues. The resultant cells growing out in the epithelial cell selection medium will be compared for production of TIMPs and latent and active MMPs. The conditioned media will be analyzed by zymography, reverse zymography, quantitative enzyme assays, and ELISAs. Enzyme and inhibitor activities will be corelated to protein content of the media or to the cell numbers.

(6). BODY

6.A EXPERIMENTAL METHODS

6 A.1.Tissue Samples: The tissues (~ 1 cm^3, snap frozen, stored at -70^{o} C) diagnosed as displaying breast carcinoma, normal or begnin (fibrosarcoma) are obtained from the Tissue Procurement laboratory, Pathology Department, University of Miami Medical School, Miami Florida. Corresponding to each frozen sample is a paraffin-fixed block of adjacent tissue which are used for immunohistochemistry. It is possible that some blocks may be used up or lost from the collection. In each subsequent year, a new series of tissues are available. The tissues chosen for present study were chosen randomly that is tissues from years 1991-95 with or without follow-ups (history) and were not classified into subtypes.

In the third year onwards the tissues will be chosen : 1) Specimens showing tumors histo-pathologically evaluated as invasive with the donor patients showing no subsequent metastatic disease. 2) Specimens showing tumors histo-pathologically evaluated as invasive with the follow up of the patients showing development of metastatic disease. 3) Specimens showing tumors histo-pathologically evaluated as noninvasive **in situ** tumors showing no subsequent metastatic disease.

The Florida tumor registry can provide the history of patients in intervening years regarding the development of metastasis, survival rate of the patients up to five years and beyond. The snap frozen tissues kept for five years will be suitable for Northerns (mRNA estimation) as long as care is taken during the preparation of total tissue RNA [27,17].

Informed consent forms are not needed for this study. In accordance with 21 CFR 56.111(a)(3), expedited review (#96/480) has been approved by the University of Miami's Human Subjects Institutional Review Board for use of residual tissues.

6.A.2. Explant cultures: Tissues either fresh or frozen in 5% dimethyl sulfoxide are obtained according to State and Federal regulations from the Tissue Procurrement Laboratories, Sylvester Cancer Center, University of Miami. Neoplastic tissues are obtained as chips or pieces and classified for tumor grades by the University of Miami Pathology Reference Service.

Tissues are collected under sterile conditions, minced into pieces, rinsed (PBS), seeded [26] in T25 flasks and cultured in MEGM - a serum free epithelial growth medium (Clonetics Corp., San Diego, CA). The conditioned medium(CM) is collected, filtered and stored in aliquots at -70^{o}C until used for enzyme and TIMP analyses.

The epithelial nature of the cell monolayer is confirmed by immunocytochemical staining, using an anti-cytokeratin monoclonal antibody specific to cytokeratin 8 and 18 (CAM 5.2, Becton-Dickinson Immunocytometry Systems, San Jose, CA). Greater than 90% of the cells

should stain positive in randomly selected microscopic fields under observation [26].

6.A.3. Cell culture model and Cell lines: A number of breast carcinoma, prostate carcinoma and other carcinoma cell lines are screened for their ability to synthesize and secrete MMPs and TIMPs in culture.Collection of **MCF-7 , DA-3, MDA-MB-231, HT 101 (supplied by Goodwin Institute, Florida), PC-3 and PPC-1 provided by Dr. Gina Bai, Univ. of Miami** or other mammary tumor cell supernatants. Tumorigenic epithelial cell line **MCF-7** (human breast carcinoma) and non tumoregenic cell line **CV-1** (isolated from normal monkey kidney) are provided by **Dr. T.J. Lampidis**, Dept. of Cell Biology & Anatomy, Univ. of Miami Med. Sch. **DA-3** cell suspensions will be provided by **Dr. Diana Lopez**, Dept of Micro. & Immunology, Univ. of Miami. Cell membrane fractions of **MET-1 and PM-1**, murine metastic cell lines are provided by **Dr. Lily Bourguignon**, Dept. of Anatomy and Cell Biology, U. of Miami Medical School.

The cell lines are maintained as cell suspensions in DMEM-F12 supplemented media (5 - 10% FCS), passaged as necessary and after confluency, the cells are maintained in serum free media for 1, 2, 4, 24 or 48 h when media is separated and frozen at -70^{o}C for various biochemical analysis. The secretion pattern of MMPs and TIMPs of these established cell lines are compared with the secretion pattern from the tumor tissue extracts. The cell lines are also treated with phorbol 12-myristate 13-acetate (PMA), cytokines, growth factors and inhibitors.

6.A.4.Generation of antibodies (IgGs) and western blotting: Antibodies were raised in rabbits against the whole molecule of enzyme stromelysin (MMP-3 from human cartilage), TIMP-1 from human cartilage chondrocytes, TIMP-3 from ECM of MCF-7 cultures and TIMP-2 from skin fibroblasts. Pro-segment peptides for 92 kDa, 72 kDa, and stromelysin (MMP-1), metal binding region peptides of 92 kDa and 72 kDa and N-terminal peptides of active enzyme, MMP-1 (interstitial collagenase) containing 15-17 amino acids were prepared by the Peptide Laboratories of Department of Biochemistry and Molecular Biology using Advanced Chemtech automatic synthesizer model ACT350. These purified peptide preparations were conjugated to bovine serum albumin, ovalbumin or hemocyanin, dialyzed, and injected into rabbits to raise the antibodies [28].

The polyclonal antibodies were found to be monospecific. They were further characterized for their reactivity to other MMPs with the use of Western blot analysis [29] and ELISA method [28].

6.A.5. Immunohistochemistry: These analyses are carried out by Dr. Zeenat Gunja-Smith, Principal Investigator and Dr. Mehrdad Nadji, Co-Investigator, Department of Pathology, University of Miami Medical School. Paraffin sections (3 microns) are cleared of paraffin, blocked for endogenous peroxidase, washed in water, PBS, blocked with normal horse serum and then are treated with drops of specific primary antibodies in a humidity chamber (1-3 h). The tissues are washed, and treated with a biotinylated secondary antibodies (1-3h), followed by avidin-biotin-peroxidase complex [30,4]. They are then washed and treated with the chromogen (DAB, 3-3'-Diaminobenzidene). The slides are counterstained with hematoxylin, washed, dehydrated and evaluated for the localization of various MMPs and TIMPs in specific cells in the tissue. Recently, immunohistochemical analyses are also carried out in my laboratory using Histostain™ SP Kit (Zymed Laboratories, CA). The kit incorporates HorseRadish Peroxidase (HRP), streptavidin, and affinity-purified antibodies into the Labeled-[strept]Avidin-Biotin (LAB-SA) method [31]. The chromogen/substrate system [aminoethyl carbazole (AEC) for a red signal or diaminobenzidine (DAB) for a brown signal] creates an intense color deposit around the antigen/antibody/enzyme complex in the tissue or cell sample.

6.A.6.Extraction of Metalloproteinases. Human breast tissues are weighed (50-200 mg), minced (finely) and homogenized (in the hood) in 7.5 volumes extraction buffer (**0.25% Triton-X 100 or 1 M GuHCL or 0.5 - 1% SDS in 50 mM Tris/HCl buffer, pH 7.5**) using Polytron homogenizer, centrifuged and the pellet reextracted with 2.5 volumes of appropriate extraction buffer, centrifuged and supernatants combined [32]. All steps are carried out at about 4°C. Extracts are dialyzed and stored in aliquots at -70°C. The extracts can be fractionated by column chromatography using ACA54 molecular seive resin [29] or directly used for zymography and other techniques.

Most tissues contain inhibitory activity which appears to be TIMP (tissue inhibitor of metalloproteinases). These can be destroyed, without affecting the metalloproteinases, by reduction (2 mM DTT) and alkylation [33]. This step also destroys any alpha-2-macroglobulin.

DNA content of tissue homogenates are measured by the method of LaBarca and Paigen [34]. Protein estimation of homogenates or concentrated media are by the use of BioRad protein estimation kit.

6.A.7. Enzyme and inhibitor assays: Tissue extracts (with or without reduction and alkylation) and column fractions (molecular seive or affinity chromatograaphy) are assayed and quantitated for various MMPs using (1) ^{3}H-acetylated Type I gelatin to estimate the MMP-2 and MMP-9 gelatinases [35] (2) ^{3}H-acetylated Type I rat skin collagen for MMP-1 (interstitial collagenase) [36]. This assay also quantitates MMP-13 (collagenase-3) and neutrophil collagenase. and ^{3}H-carboxymethylated transferrin [37]; ^{3}H-acetylated proteoglycan monomer bead assay [29] for stromelysin (MMP-3) and MMP-7. Blanks are set up with 1,10-

phenanthroline and p-aminophenylmercuric acetate (APMA) are used to activate latent enzymes.

Measurement of TIMPs in tissue extracts, media or column fractions are achieved by the inhibition of uterine MMP-7 and remaining activity assayed using Azocoll as substrate [33]. After centrifugation of undigested Azocoll, the absorbance of dye-released supernatant is measured. One mole of MMP-7 is assumed to bind with one mole of TIMPs. Quantitation of the latent and active forms of various MMPs and TIMPs are also done by immunoassay (EIA) [28] using monospecific monoclonal and polyclonal antibodies made against the whole enzyme molecules or against specific peptides for each MMPs. I have generated several polyclonal antibodies against most MMPs and the TIMPs.

6.A.8.Zymography and Immunoprecipitation. Gelatin zymography follows a modified procedure of Herron et al. [38] for detecting picograms of MMP-2 & -9 and nanograms of other MMPs and proteases. SDS-PAGE is performed in 7.5% or 10% polyacrylamide (39) containing 0.33 mg/ml gelatin (or other substrates i.e. casein or transferrin or soluble elastin). The gels are then rinsed twice in 0.25% Triton X-100, and incubated (18 h, 37^{o} C in Tris-NaCl-$ZnCl_2$, 3 mM phenylmethylsulfonyl fluoride (PMSF) assay buffer). Gels are stained with Coomassie blue R 250. Both latent and active forms of gelatinases or other MMPs produce clear areas in the gel. The relative amounts of enzymes are also quantitated by densitometry of gels (and also dried gels in membranes).The imager used is supplied by Department of Biochemistry, University of Miami Medical School to scan and quantitate 250-500 gels generated per year. The monies allocated ($7,500) in the first year were spent to purchase a Pentium computer and printer to use the software compatible with the imager. The software was purchased from Ultra Violet Products (UVP) as GelBase/GelBlot Pro Software. The image from the imager is recorded on discs and each lane quantitated for enzyme spots.

Tissue extracts or concentrated filtered media from cell lines are immunoprecipitated with rabbit anti-MMP(s)or anti-TIMP(s) IgG(s) using protein A-agarose suspensions. Blanks are prepared with specific IgG alone, preimmune serum with enzyme fraction, and enzyme with protein A gels but no IgG. After the reacted agarose gels are washed, the immune complexes dissolved in sample buffer and analyzed by zymography for specific enzyme activity or for TIMPs by reverse zymography or for proteins by SDS-PAGE [40]. By this method, 90-95% of the antigen present is immunoprecipitated.

6.A.9. Reverse zymography: A modified method of Heron et al [38] is used to detect the TIMPs. Recently, a kit is availble to perform reverse zymography of tissue extracts and conditioned media from University Technologies International Inc., Alberta, Canada. The kit provides standards of TIMP-1, -2 and -3 and the media containing the enzymes. Conditioned media (or extract) is fractionated by SDS-PAGE [39] electrophoresis using 12,5% acrylamide , 0.75 mg/ml gelatin solution and the supplied media (0.1 ml) containing enzymes. After washing with 2.5%

Triton X-100, the gel is incubated in Tris buffer (37^{O} C, and minimum gentle shaking) and stained with Coomassie blue solution to reveal cleared and uncleared area of the gelatin in the gel. Uncleared blue staining areas are revealed only if TIMPs are present. This is a very sensitive method revealing as little as 2 ng of TIMPs. This kit does not provide calibrated TIMP standards. We have calibrated pure TIMP-1 and -2 solutions by protein estimations.

6.A.10. Enzyme Linked Immunoassays, ELISAs: This method will be used to quantitate MMPs and TIMPs in tissue extracts, serum free media of cell lines and column fractions of the tissue extracts or media. The method is based on an inhibition immunoassay (under nonequilibrium conditions) [28], uses purified polyclonal antiserum and detects 5-500 pmoles of MMPs and TIMPs . The antibodies generated for each MMPs and TIMPs are monospecific and do not crossreact. The sample values are read from standard curves produced by several commercially available computer programs. Total MMPs or TIMPs are calculated as ng/mg tissue or ng/ml media. The ELISA method is useful as a quick guide to the amount of MMPs and TIMPs in a sample, so that appropriate amounts of samples can be applied for quantitation of MMPs by zymography. This method will be useful for quantitating total TIMPs because TIMPs-enzyme complex is not quantitated by the enzyme-based inhibitor assay. The ELISA method, however, is not suitable for distinguishing active and latent enzyme forms.

6.A 11.. RNA extraction from tissue cell samples and Northern Blot Analysis:

Tissues aliquots or confluent cells that were fresh rapidly frozen in liquid nitrogen and stored at -70^{O}C are used. Total cellular RNA is prepared by the method of Chomczynski and Sacchi (guanidinium-acid-phenol extraction)[41] or Use of RNA Stat 60 kit (tel-Test Inc. Friendsville, Tx). Standard Northern blot technique is used [41]. Briefly, total RNA (3-10 μg) is electrophoresed through agarose-formaldehyde gels, transferred onto nylon membranes Nytran, Schleicher & Schuell) by capillary electrophoresis, followed by prehybridization of the membrane, and hybridization with the appropriate radiolabeled probe. Labelled probes are obtained using Prime-a-Gene random hexamer kit (Promega, Madison, WI and NEN [alpha-^{32}P]dCTP. Quantification of bands are performed using a Molecular Dynamics PhoshmoImager. Normalization of the amount of RNA loaded in each lane, cDNA probe for constitutively expressed actin or Glyceraldehyde phophate dehydrogenase are hybridized on the same blot.

cDNA clones are obtained from American Type Culture Collection, Rockville, Maryland. ATCC has listed human fibroblast collagenase in vector pSP64 by Rahmsdorf, collagenase IVA in vector pBR322 by Tryggvason, stromelysin 1 (pUN121, by Matrisian), stromelysin (MMP-3, transin, in pUN121 by Matrisian), stromelysin 2 (transin-2) in pUn121 by Matrisian), TIMP-1, tissue inhibitor of metalloproteinase 1 in pTZ by Willard and TIMP-2 (two clones) in pBluescript SK^{-} by Venter. cDNA

clone for PUMP-1 is available from rat uterus (UMP, MMP-7) in our laboratory.The message of the various MMPs and TIMPs may be difficult to analyze due to low amounts. It may be necessary to use reverse transcriptase-polymerase chain reaction (RT-PCR) in some cases; if so, specific primers are used to cover distinctive regions of the message The RT-PCR method [42] although difficult may be ultimate method of choice. cDNA clones for MMP-2, MMP-9, MT-MMP-1 are kindly provided by Dr. G. Goldberg, Washington University, St. Louis.

6.B.RESULTS:

The second year (**September 1995-September 1996**) of the four year granting period shows meaningful data in certain aspects of the grant proposal. Considerable portion of the second year was spent in establishing molecular biology and immunohistostaining techniques in my laboratory.

We have further established that the MMPs specifically individual gelatinases (72 kDa - active and latent and 92 kDa - active and latent can be identified and quantitated by substrate zymography using the Imager and the software GelBase/GelBlot Pro in small amounts of breast tissue samples. TIMPs in tissue samples have been identified by reverse zymography but as yet not successful in quantitating them satisfactorily. For the time being we have quantitated TIMP proteins before and after reduction and alkylation of extracts to give us some measure of the amounts present in breast tissue. More breast tissue sections were stained with anti-MMPs and anti-TIMPs IgGs to corelate with the biochemical findings (zymographic evaluations).

As stated in the first year report, collaboration with Dr. L. Bourguignon, Department of Cell Biology and Anatomy , Univ. of Miami Medical School and also a recipient of DOD breast cancer grant continues. Another approach to the problem was also to show the spatial location of various MMPs using confocal microscope. The preliminary studies show that there may be a link between isoforms of CD44 (transmembrane gycoprotein) and the secretion pattern of MMPs. Implication of CD44 in breast and prostate cancer has been established [42,43]. Colocation of CD44 (transmembrane glycoprotein) variants and MMP-9 has been established in human (**MCF-7**) and a mouse mammary tumor cell line (MET). One approach was to immunoprecipitate the membrane fraction with anti-MMP-9 IgGs, anti MMP-2 IgGs and anti-CD44 variants V3, V7, V6 IgGs and locate each antigens that co-precipitated by zymography (for MMPs) and western Blot analysis (Avidin-Biotin Chemiluminescense, for CD44 [42, 43].These findings have been submitted for publication to Journal of Biological Chemistry.

This CD44-MMPs study has been tested first in the cell lines. Using the breast cancer cell line **MCF-7**, we show a correlation of MMP-2 and CD44 in the cell membrane. Staining with anti-MMP-9 reveals enzyme inside the cell in granules and around the nucleus and some around the cell membrane. In contrast staining with anti-MMP-2 enzyme is seen around the membrane. Rat anti-CD44 also shows staining around the edge of the cell membrane. This findings are observed in both the resting and PMA stimulated state in the cells .

However, when stimulated (PMA, phorbol 12-myristate 13-acetate) cells are stained with anti-MMP-2, the staining is observed in a broad visible around the membrane. Rat anti-CD44 staining also shows the stimulation of CD44 by PMA. Why is MMP-2 not secreted into the media similar to MMP-9, is it associated with CD44? and cannot be secreted. These results were presented in the last year's report.

Another exciting observation with CD44 studies was that addition of anti-CD44v-III (CD44 isoform) in the media with and without stimulation with PMA showed that antiserum had caused and initiated the activation of the secreted 92 kDa MMP. This finding was not observed with anti-CD44 (standard form) or with anti-CD44v-VI another isoform) These findings are exciting and requires further research to evaluate the role of MMPs in the transmembrane association. CD44v-III [42] has been implicated in breast cancer. This study will therefore be a part of the ongoing research in the coming year.

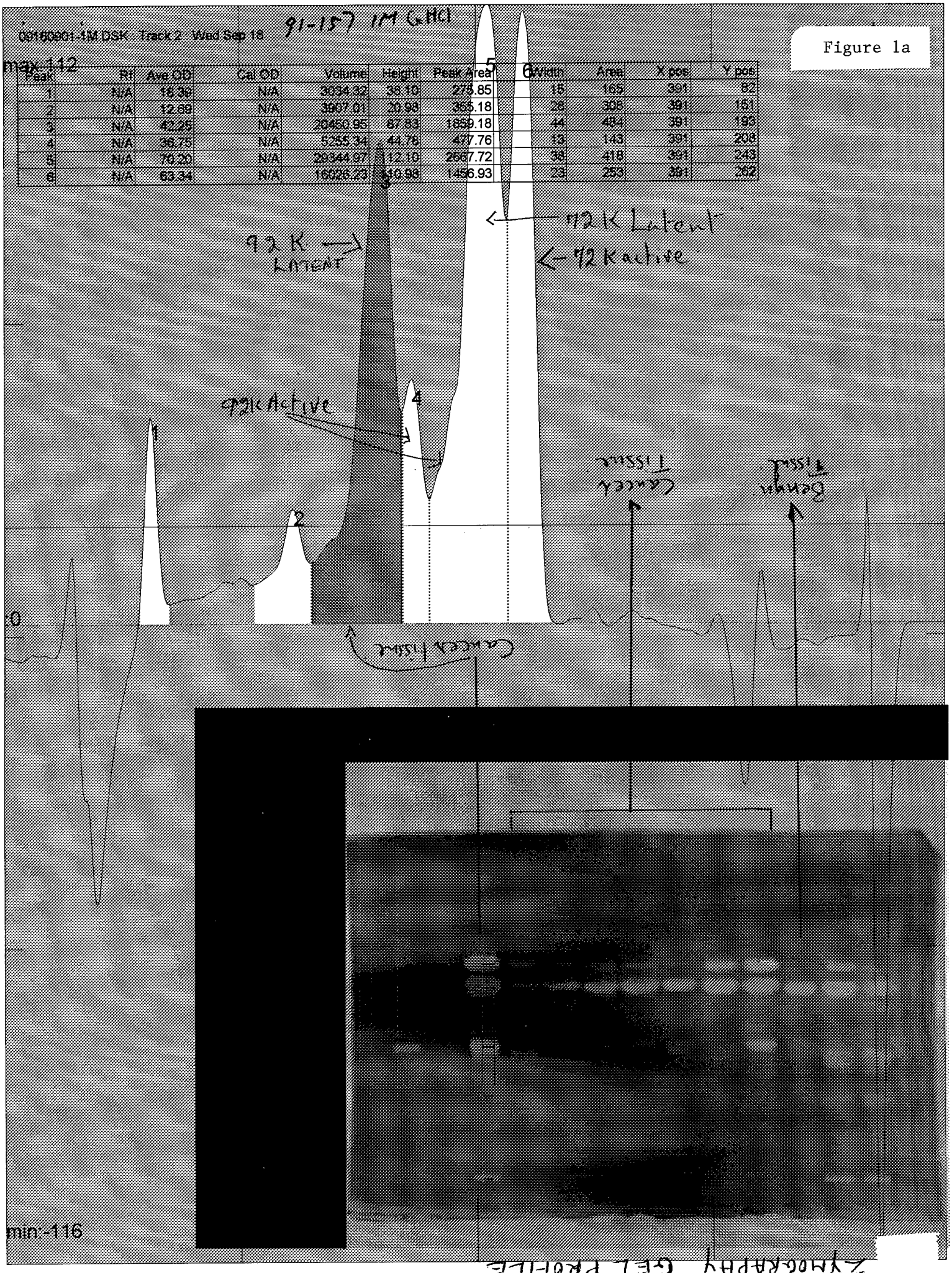
6.B.1. Quantitation and characterization of MMPs in breast tissue:

Breast tissues were homogenised using Polytron in 10 volumes of extraction buffer containing Triton-X 100, followed by 1 M Guanidine hydrochloride, **and further followed by 0.5% SDS** , dialyzed in standard Tris buffer, aliquoted and frozen at -70^{O} C. In latter experiments only **0.1% SDS-buffer solution** was used. SDS extracts are used to extreact TIMP-3 that binds to matrix and also extract any residual if any enzymes in tissues. The extracts were routinely analyzed for the presence of MMPs using substrates (gelatin, carboxy methylated transferrin, casein and sometimes soluble elastin) and 10%-7.5% acrylamide for SDS-PAGE electrophoresis. Gelatin gels gave a good profile of the MMPs present in the tissue extracts or media. The inclusion of 3 mM PMSF in substrate gel incubation buffer should mask serine proteases and reveal bands cleared mainly by MMPs on a coommassie blue stained gelatin gel. A typical profile of extracts analyzed by substrate zymography and quantitated by GELBASE software have been reported last year.

Figs. 1a, 1b; 2a, 2b show the zymography (gel) profile scanned by the UVP imager and analyzed on the computer by the software "GelBase/GelBase Pro" (UVP, Cambridge, England). The program tracks cleared bands in each track giving a profile of peaks. The peaks are calculated in computer numbers. Known amounts either in protein or the unit of activity of various MMPs (MMP-2, MMP-9) are analyzed with breast tissue extracts on the same gel. This allows the quantitation of MMPs in small amounts of biopsied breast tissue.

To date, 40 breast tissue samples have been analyzed using gelatin zymography **FIG. 1a** shows the imager-computer generated profile of gelatin zymography of **1 M Guanidine** extracts of breast tissues. This Figure shows two benign and nine breast cancer tissue extracts (**FIG. 1a.)** together with a prestained protein standards and MMP-2 and MMP-9 standards (known activity). The **FIG. 1a** also illustrates a peak profile of one breast cancer tissues showing several peaks of MMPs. They are mainly gelatinases MMP-2 and MMP-9 in active and latent forms. Each peak has values in number and these numbers are translated into units of enzyme for the values obtained for quantitated MMP-2 and MMP-9 standards. **FIG. 1a** also shows bands at Mrs higher than 92 kDa. and at Mrs lower than MMP-2. Higher than 92 kDa band can be attributed to either aggregates of active 72 kDa (~125 kDa) or lipocalin bound dimer 72 kDa. Band seen near the top of gel may be aggregate band of active

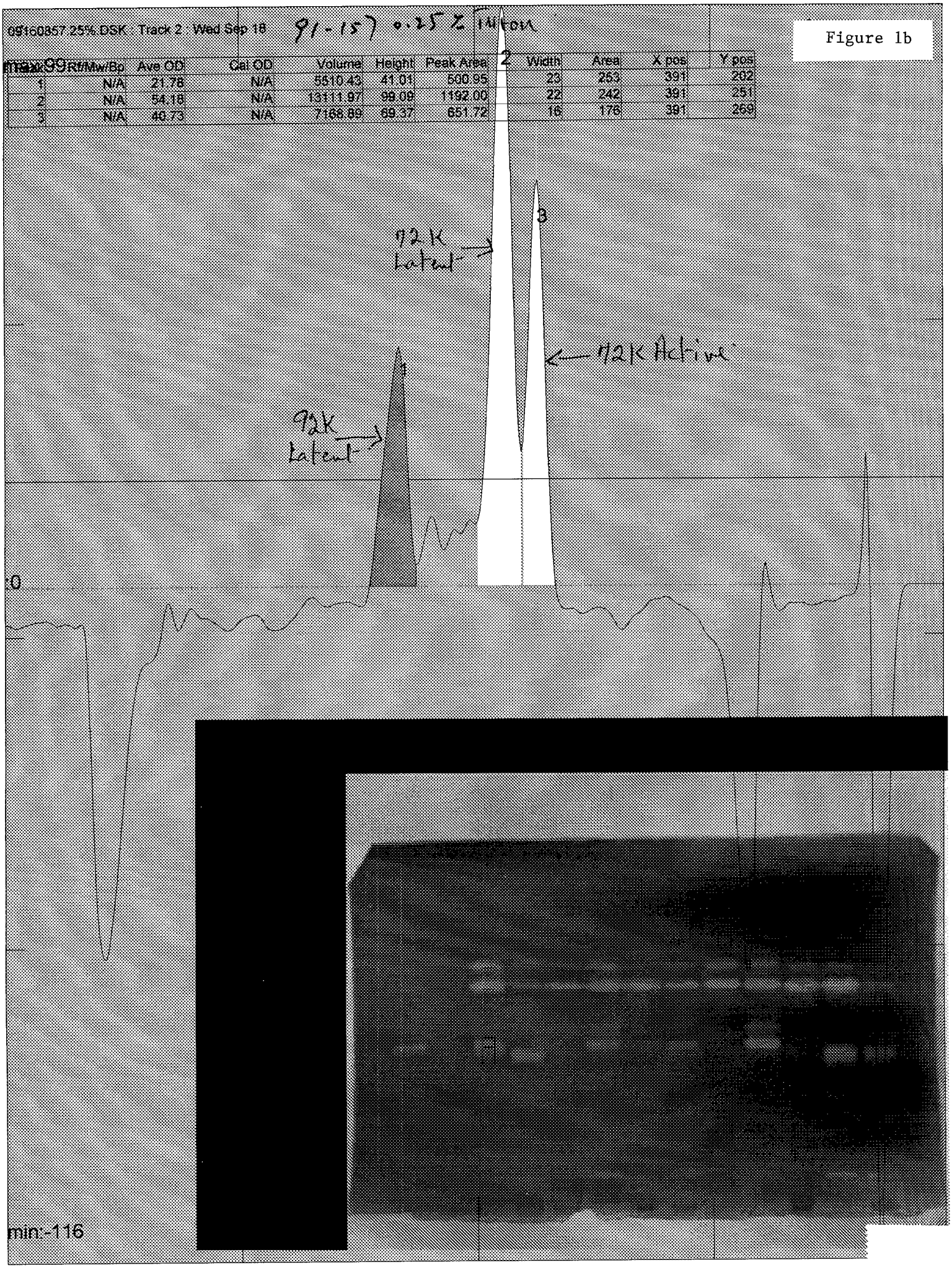
Figure 1a



Peak	Rf	Ave OD	Cal OD	Volume	Height	Peak Area	Width	Area	X pos	Y pos
1	N/A	18.39	N/A	3034.32	38.10	276.85	15	165	391	82
2	N/A	12.69	N/A	3907.01	20.98	365.18	28	305	391	151
3	N/A	42.25	N/A	20460.95	87.83	1859.18	44	484	391	193
4	N/A	36.75	N/A	5255.34	44.78	477.76	13	143	391	208
5	N/A	79.20	N/A	29344.97	112.10	2667.72	38	416	391	243
6	N/A	63.34	N/A	16026.23	140.98	1456.93	23	253	391	262

ZYMOGRAPHY GEL PROFILE

Figure 1b



	R/f/Mw/Bp	Ave OD	Cal OD	Volume	Height	Peak Area	Width	Area	X pos	Y pos
1	N/A	21.78	N/A	5510.43	41.01	500.95	23	253	391	202
2	N/A	54.18	N/A	13111.97	99.09	1192.00	22	242	391	251
3	N/A	40.73	N/A	7168.89	69.37	651.72	16	176	391	269

Figure 2a

05160901-1M DSK : Track 10 : Wed Sep 18 95-42M

Peak	Rf	Ave OD	Cal OD	Volume	Height	Peak Area	Width	Area	X pos	Y pos
1	N/A	37.22	N/A	17605.78	105.49	1600.53	43	473	127	239
2	N/A	17.27	N/A	3799.65	35.72	349.42	20	220	127	257

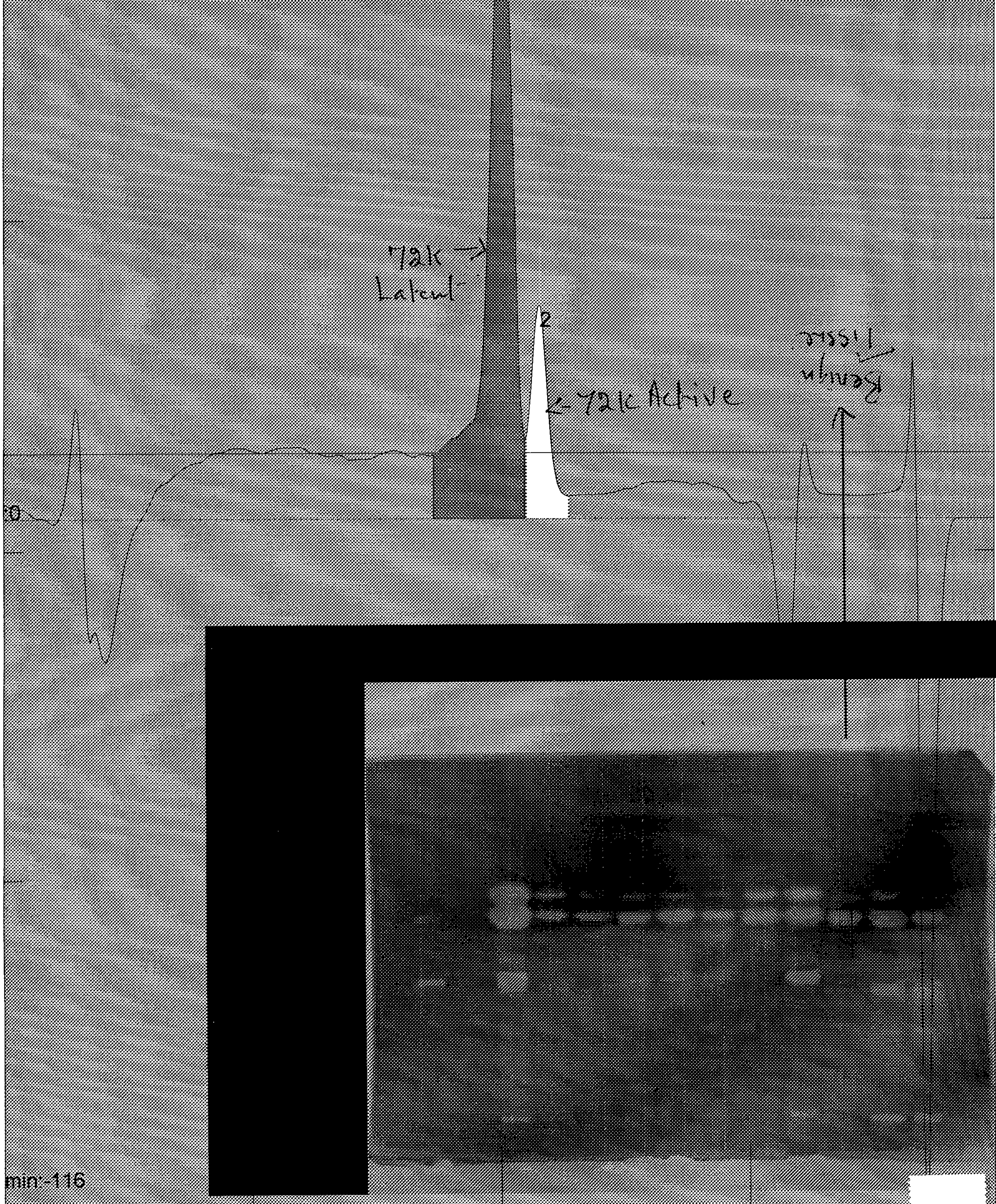


Figure 2b

09160857.25% DSK Track 10 Wed Sep 1 95-42 0.25 Z

Peak	Rf/Mw/Bp	Ave OD	Cal OD	Volume	Height	Peak Area	Width	Area	X pos	Y pos
1	N/A	30.09	N/A	12244.80	97.01	1113.16	37	407	127	251
2	N/A	8.82	N/A	1940.35	20.10	176.40	20	220	127	267

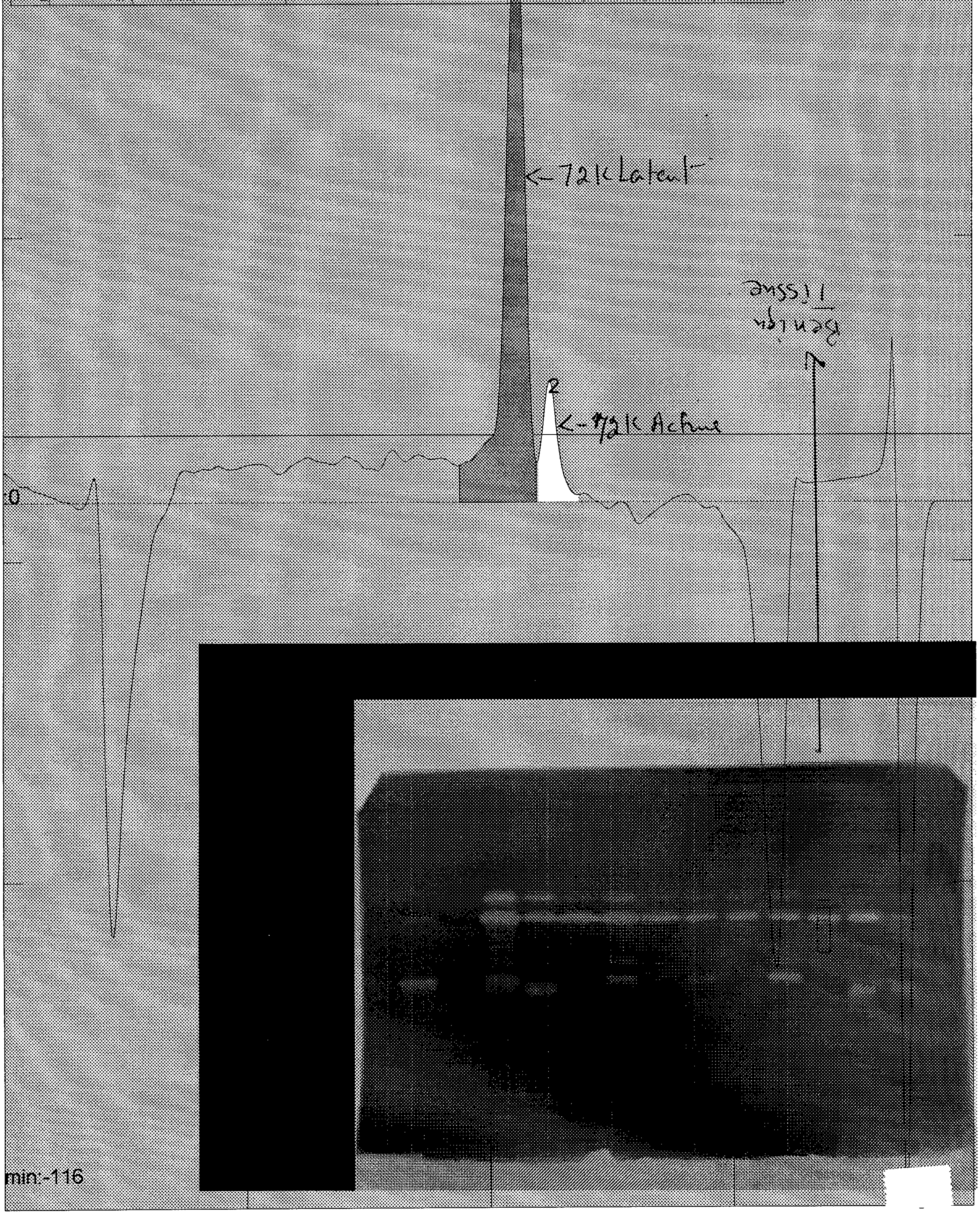


Figure 3a

Gelatin Zymography of Breast Tissue Extracts

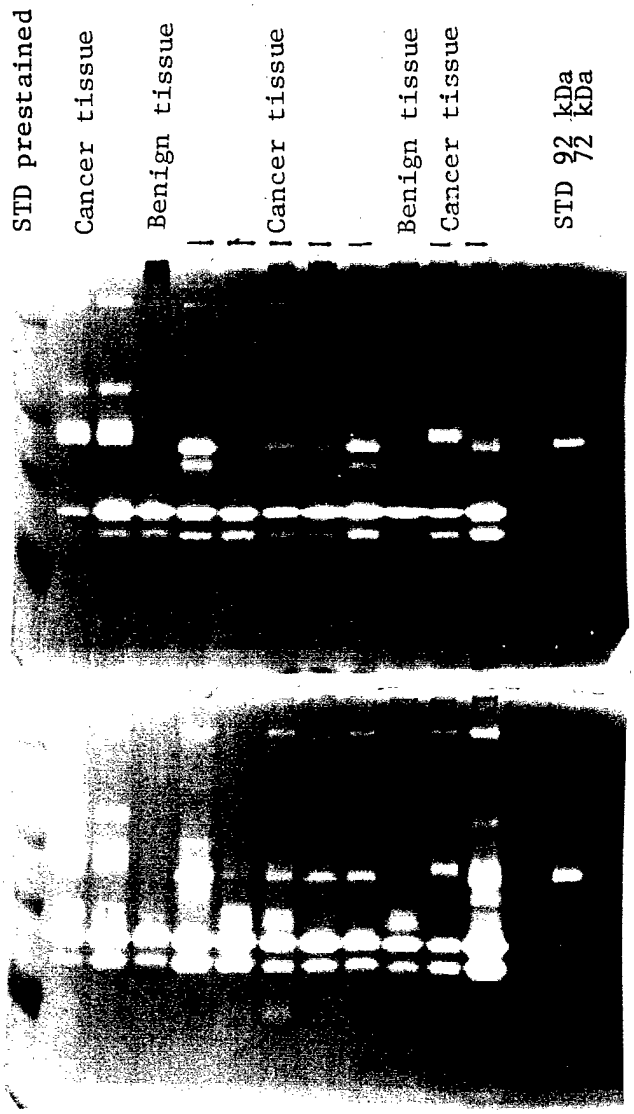
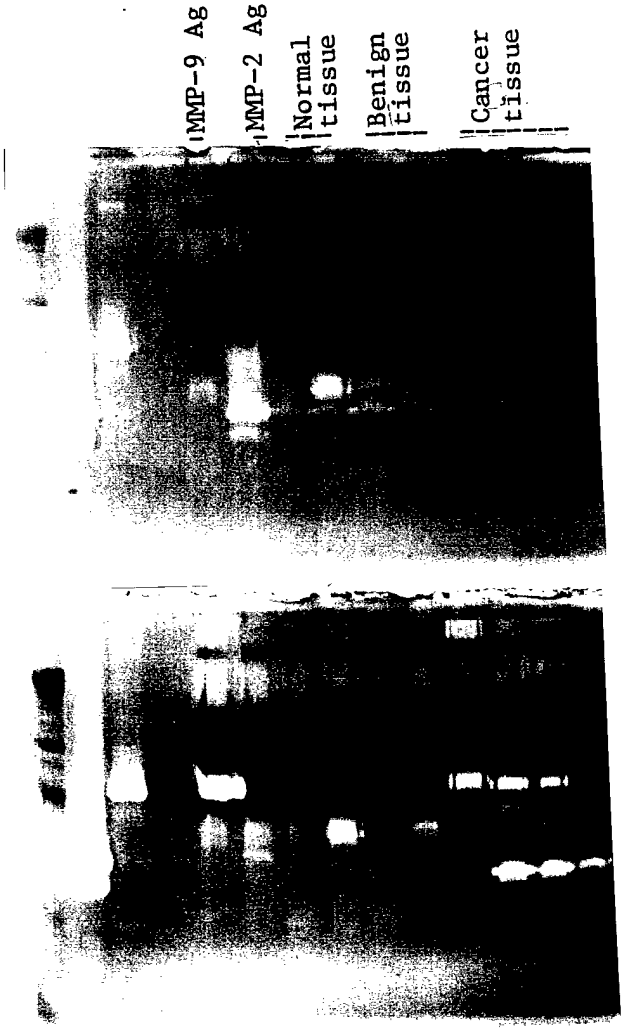


Figure 3b

Gelatin Zymography of Immunoprecipitated Breast Tissue Extracts
Extracts and antigens MMP-2 and MMP-9 were immunoprecipitated with anti-MMP-2 and anti-MMP-9 IgGs.



92 kDa or lipocalin bound dimer of 92 kDa. MMP-1 and MMP-3 digest the gelatin substrate 1000 times lower than the two gelatinases.

FIG. 1b shows gelatin zymographical analyses of 0.25% Triton breast tissue extracts of the same breast tissue samples shown in **FIG. 1a** . These profiles show latent and active MMP-2 and MMP-9 is only in latent form. These profiles show that extractions of enzymes needs different extraction procedures. Use of Triton X-100 is mainly to remove membrane and all soluble proteins and enzymes. 1 M Guanidine-buffer solution breaks hydrophobic bonds and extracts bound proteins (releasing more MMPs and TIMPs) and SDS extracts matrix bound components (TIMP-3). These three extractions are necessary - Triton removes soluble proteins away from the TIMPs that are extracted (90%) mainly by 1 M Guanidine buffer solution.

In contrast benign breast tissue extracts, **Fig 2a** for 1 M guanidine extracts and **FIG. 2b** , 0.25% Triton extracts reveal clear bands at molecular weight corresponding to MMP-2 (72 kDa, gelatinase A) only. The latent enzyme band is at Mr 68 kDa and active enzyme band at 62 kDa. Benign tissue shows exclusively the bands for latent and active MMP-2 (72 kDa). Zymography of normal breast tissue extracts reveal mainly latent form of MMP-2.

There is a clear difference in the secretion pattern in of MMPs in normal, begnin and cancer breast tissue. The results therefore indicated that 92 kDa MMP is a predominant enzyme in cancer tissue. Several reports in literature have implicated the role of MMP-9 (92 kDa, gelatinase A) in breast and several other cancer tissues [44, 45].

FIG. 3a shows the imager generated profile of zymography of breast tissues extracts -- 0.25% Triton and 1 M Guanidine. This figure shows clearly that only cancer breast tissue extracts reveal MMP-9 bands (active and latent).

Quantitation of MMPs by zymography with the use of the GelBase software has facilitated the analysis of many breast tissue samples. This method also separates out the TIMP proteins away from the bands of enzymes. The activities estimated with the above procedure shows 60% of the activity quantitated by substrate assays. Quantitation by substrate assays require lengthy procedures of molecular seive column chromatography for the separation of enzymes and their inhibitors.

Presence of tissue inhibitors in tissue extracts prevent the enzyme inhibitor analysis directly from the tissue extracts as the activated enzymes bind directly to TIMPs and hence render them inactive. TIMPs can be destroyed by reduction and alkylation of tissue extracts without excessive destruction of the MMPs [29]. However, in the case of breast tissue extracts, TIMPs destruction was not complete as detected by reverse zymography.(results shown below) of reduced and alkylated extracts. Substrate assays require lengthy separation procedures and larger amounts of extracts.

SDS-PAGE substrate (zymography) analysis of extracts separates the proteins by the molecular weights and proteins and enzymes can be quantitated in µl quantities.

The identity of each MMP secreted in breast tissue was further characterized using ACA54 molecular seive column. The fractions so obtained were scanned for enzyme bands by gelatin substrate zymography. The aliquots of fractions for each peak observed were then combined, reanalyzed by zymography. Peak sample from each gelatinases (92 kDa) and (72 kDa) were treated with APMA and the activated products analyzed by zymography. Results are presented in **Fig. 10** .This study shows that the enzymes found in breast tissues are gelatinases (metalloproteinases). Mrs of activated enzyme products were 84 kDa, 64 kDa for proenzyme 92 kDa and Mrs 62 kDa and faint bands at 45 kDa for proenzyme 72 kDa. The enzyme bands were inhibited by EDTA and 1,10 phenophthrailein (results not shown) by zymographic and substrate assay analysis.

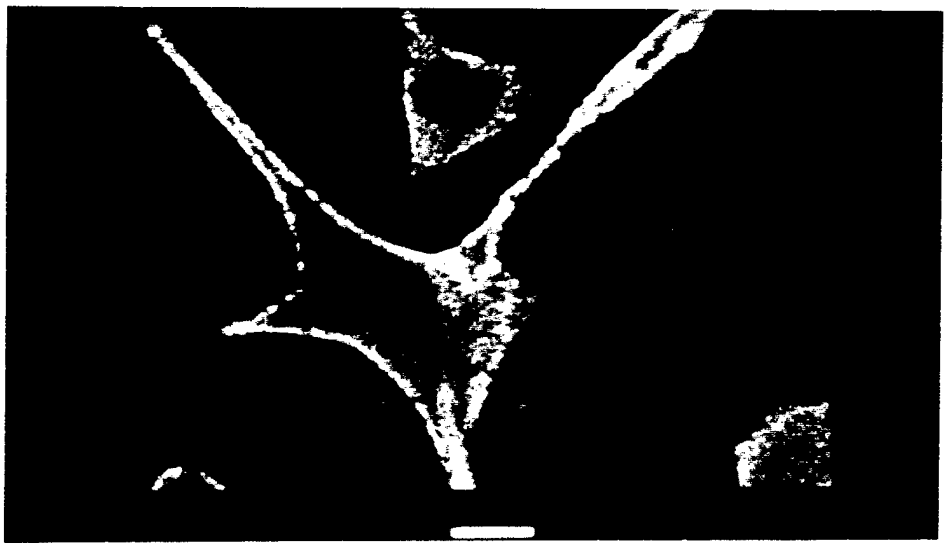
The breast tissues have been characterized from the reports supplied by Jackson Memorial Hospital/Pathology Department, University of Miami Medical School. So far, we have analyzed **five normals**, **five** termed as **benign**, **two** characterized as **tubular benign** (**rich in epithelium**), **fifteen** characterized as **infiltrating ductal carcinoma where nine were characterized as Nuclear Grade III and six were characterized as Nuclear Grade II.** There were **three** breast tissue samples characterized as **lobular carcinoma** and **two** as **in situ ductal carcinoma.** The tissue termed as fibrocystic disease reported in Table 1 of 1996 report was erroneous characterisation and we have discarded those tissues from our study. As explained in the covering letter, the system of storing tissues was not well documented at our institution. We now process tissues only after the pathology reports are made available. there were only 6 tissues that in error and therefore, we consider ourselves lucky that 6 /55 we processed not acceptable.

The breast extracts were analyzed for gelatinase activities (MMP-2 and MMP-9) using the substrate (tritiated gelatin) and for collagenase (MMP-1 or MMP-13, interstitial collagenase,) using the substrate (tritiated collgen). The substrate method used for collagenase will also quantitate collagenase-3 or MMP-13 and this enzyme has been reported in human normal and breast carcinoma tissues[46]. The values obtained by activity using tritiated gelatin were compared with the activity values obtained from gelatin zymography using predetermined (activity) of gelatinase samples for MMP-2 and MMP-9. The zymographical evaluated activities were 85% of the substrate activity results. The results are presented in **Table 1**. The **Table 1** shows total gelatinase activity (MMP-2+MMP-9), total collagenase (MMP-1 or MMP-13), Ratios of latent to active enzyme for both gelatinases (MMP-2 and MMP-9) and lastly the percentage of MMP-9 of the total gelatinase activity found in each characterized breast tissue.

The results in **Table 1** clearly show the absence of MMP-9 in the normal and benign breast tissues. MMP-9 is found in otherwise

a:

b:



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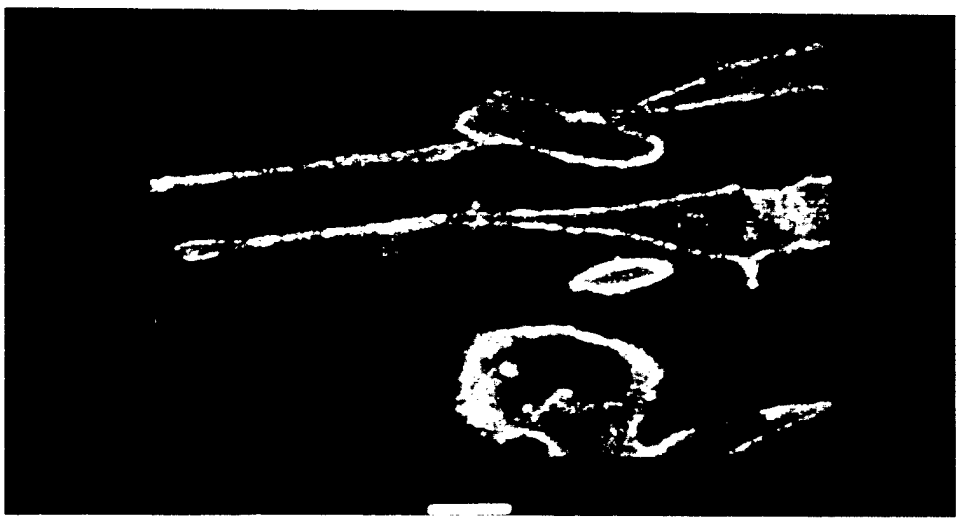


FIG. 10. Immunofluorescence staining of MET-1 Cells using confocal microscope. Rat anti-CD44 IgG , rabbit anti-MT-MMP-1, rabbit anti-MMP-9 & rabbit anti-MMP-2 were used followed by fluorescein-labeled (green color) for MMPs and rhodamine-conjugated (red color) for CD44. For colocation of two antigens, YELLOW COLOR is observed. MT-MMP-1, MMP-9 & MMP- 2 show colocation with CD44 on transmembrane.

TABLE 1. Quantitation of Matrix Matalloproteinases--Gelatinases (A[@]+B[#]) and Collagenase (MMP-1, or -13)[^] in Breast Tissues.

Tissue	No.	MMP-2[@] + MMP-9[#] Total Units[*]	MMP-9 L/A	MMP-2 L/A	MMP-9 %	MMP-1[^] Total Units[*]
Normal	5	4.6 ± 0.86	N/A	7.8	N/A	0.27 ± 0.1
Benign Tumor	5	17.1 ± 0.54	N/A	3.58 ± 0.96	N/A	0.68 ±0.19
Benign Tumor Tubular[@]	2	50.1	N/A	1.6	N/A	1.2
Adeno-carcinoma GRIII	9	65[*] ± 7.3	1.9 ± 0.6	1.5 ± 0.29	27 ±8.0	2.9 ±1.5
GRII	6	20.1 ± 3.2	2.01 ±0.45	2.55 ± 0.4	28 ± 6.3	0.62 ± 0.11
Lobular carcinoma	3	22.2 ± 8.1	0.71 ±0.15	2.05 -	35	2.4
In Situ ductal carcinoma	2	11.5	0.84	4.18	30	0.13

Gelatinase A + B =MMP-2[@] + MMP-9[#], Total Units = latent + active enzyme from breast extracts. 0.25% Triton extracts + 1M GuHCl extracts.
One enzyme unit = 1μg of substate digested /min at 37° C for gelatinases & at 30° C for collagenase.
[*] Values expressed as enzyme units/g wet weight breast tissue.
Values are given as mean ± SEM(standard error of the mean).
L/A = Latent enzyme/Active enzyme ratio
% = Amount of MMP-9 enzyme from total gelatinase
Adenocarcinoma = Infiltrating Ductal Carcinoma - Nuclear Grade III or Nuclear Grade II
^ Interstitial collagenase - MMP-1 or MMP-13.

characterized adenocarcinoma breast tissues in varying amounts. The highest activity of MMP-9 is observed in poorly differentiated (Nuclear Grade III). infiltrating ductal carcinoma (**18 units, 1μg**). Grade II carcinoma breast tissue shows **5.7 units** and lobular carcinoma has **7.7 units** of MMP-9. The ratios of latent to active (**L/A**) enzyme for both enzymes, MMP-2 and MMP-9 are also shown in the **Table 1**. There are no clear cut differences observed when samples in each category are combined. Some individual samples showed all active enzyme for MMP-9 and MMP-2 (results not shown). Values for interstitial collagenase, MMP-1 or MMP-13 show a nice corelation between Nuclear Grade III (**2.9 units, 0.6 μg**) carcinoma and Nuclear Grade II carcinoma (**0.62 units, 0.13μg**). Again another MMP, collagenase is observed in higher amounts in adenocarcima tissues. This is enough to digest the collagen and together with high gelatinases, these enzymes can disrupt the ECM and allow the tumor cells to escape In situ ductal carcinoma tissue shows relatively low MMP-1 (**0.13 units**) activity , **3.45 units** of of relatively larger proportion of active MMP-9 and higher portion of latent MMP-2 enzyme (**7 units**).

The **L/A** ratio for **MMP-2** (**Table 1**) shows the presence of more latent MMP-2 enzyme for normal tissues (**7.8**). The L/A ratio (**3.6**) in benign tumor and L/A ratio (**1.6**)in benign tumor containing more epithelium (tubular) shows the presence of more active MMP-2. This is a nice corelation for MMP-2 between normal and benign tissues and shows a clear cut difference in the presence of one particular gelatinase (MMP-2) in normal and benign tissues. Interstitial collagenases is elevated in benign tissues (**0.68, 1.2 units**) compared to normals (**0.27 units**).

The results presented in **Table 1** for quantitation of some matrix metalloproteinases (MMP-1 or MMP-13, MMP-2 and MMP-9) in extracts of characterized breast tissues show a corelation between presence and absence of certain MMPs. We show that MMP-2 is found in normal and benign breast tissues and MMP-9 an enzyme associated with inflammatory response [47] is found mainly in different forms of adenocarcinomas of breast tissues. More tissues of characterized tissues in the category of lobular, in situ carcinomas need to be identified and analyzed. This will be attempted in the third and fourth year of the granting period. Definitely, numbers are needed for other types of breast tissues.

No stromelysin-1 was found in any extracts. Stromelysin-3 cannot be estimated by protein substrate assay and was not quantitated. No metallo-elastase or PUMP-1 (matrilysin) was detected in breast tissue. Several bands of serine proteases were observed in carboxymethylated transferrin zymography (result not shown). No attempt was made to characterize or quantitate them. This line of research may be pursued in later years.

FIG. 3b shows gelatin zymography of immunoprecipitated breast tissue extracts. The beads were coated (see Methods section) with anti-MMP-2 and anti-MMP-9 and after washing are reacted with breast extracts. Immunoprecipitated extracts are then analyzed by gelatin zymography. MMP-9 immunoprecipitated in all breast cancer tissues and in three with active form of MMP-9. This further confirms the presence of MMP-9 in breast cancer tissues.

6.B.2. Analysis of TIMPs in breast tissue:

The breast tissue extracts were scanned for TIMPs by reverse zymography. TIMP-1, -2 & -3 are detected in ng quantities. The tissue extracts are fractionated by SDS-PAGE (12,5% acrylamide, gelatin and enzyme media) electophoresis The uncleared blue bands are due to the TIMPs present in the extracts or conditioned media. All 3 TIMPs are present in different amounts in the breast tissue extracts Of the three different extracts, only 1M GuHCl and SDS extracts can be scanned for TIMPs. The SDS extraction step is necessary to remove TIMP-3 entrapped in the matrix of the tissue. The Triton extracts contain other soluble proteins and it becomes difficult to distinguish the TIMP bands from the regular same molecular weight protein bands that did not diffuse out of gel during (buffer and gel) incubation. Conditioned cell media can be successfully quantitated for TIMPs. Also, the background color varies in intensity from one gel to another using the reverse zymography kit. The media in the kit has other proteases (serine) of different molecular weights digesting the gelatin more.

The breast tissue extracts (0.25% Triton, 1M Guanidine and 0.5% SDS) were scanned by reverse zymography and the results are presented **in Table 2 & FIG. 4. Table 2** shows the amounts of TIMP-1 and TIMP-2 present in the I M Guanidine extracts of breast tissue. The present **Table 2** replaces the **Table 2** sent with the report for 1996. The breast tissue extracts were scanned by reverse zymography before and after reduction and alkylation of the extracts. It was evident that protein bands existed in the gel **(FIG 4).** There are still protein bands found in the areas where three TIMPs migrate in the gel. It is known in our laboratory that for unknown reasons all TIMPs (TIMP-2 and TIMP-3 specifically) are difficult to remove by reduction and alkylation step. It would be ideal to establish a more suitable reverse zymography procedure. It would be helpful in identifying individual TIMP and then quantitate each TIMP by the GELBASE program. We have tried to quantitate TIMP-1 and TIMP-2 by the technique mentioned above. TIMP-3 caliberated standard was not available.

The highest amount of TIMP-2 (**0.7 µg**) was found in benign tissues, adenocarcinoma tissues on the other hand contain only **0.07µg** of TIMP-2. The highest amount of TIMPs are observed in benign extracts (**0.93µg**). These results are very preliminary and once the reliable method isobtained, the extracts will be analyzed again. We speculate that there is an imbalance of MMP-TIMP ratio in the breast adenocarcinoma [48].

We are still in the middle of developing a reliable quantitation method (reverse zymography) for the TIMPs. TIMPs are also difficult to quantitate by enzyme binding assays [33] . The enzymic method using small uterine metalloproteinase (see method section) was not suitable for SDS extraction fraction (TIMP-3). SDS hinders the analysis and is difficult to remove by dialysis. Also, uterine MMP (MMP-7) has varied sensitivity (binding capacity) for vaious TIMPs to obtain reproducible

Figure 4

Rever Zymography of Breast Tissue Extracts
With and without Reduction and Alkylation

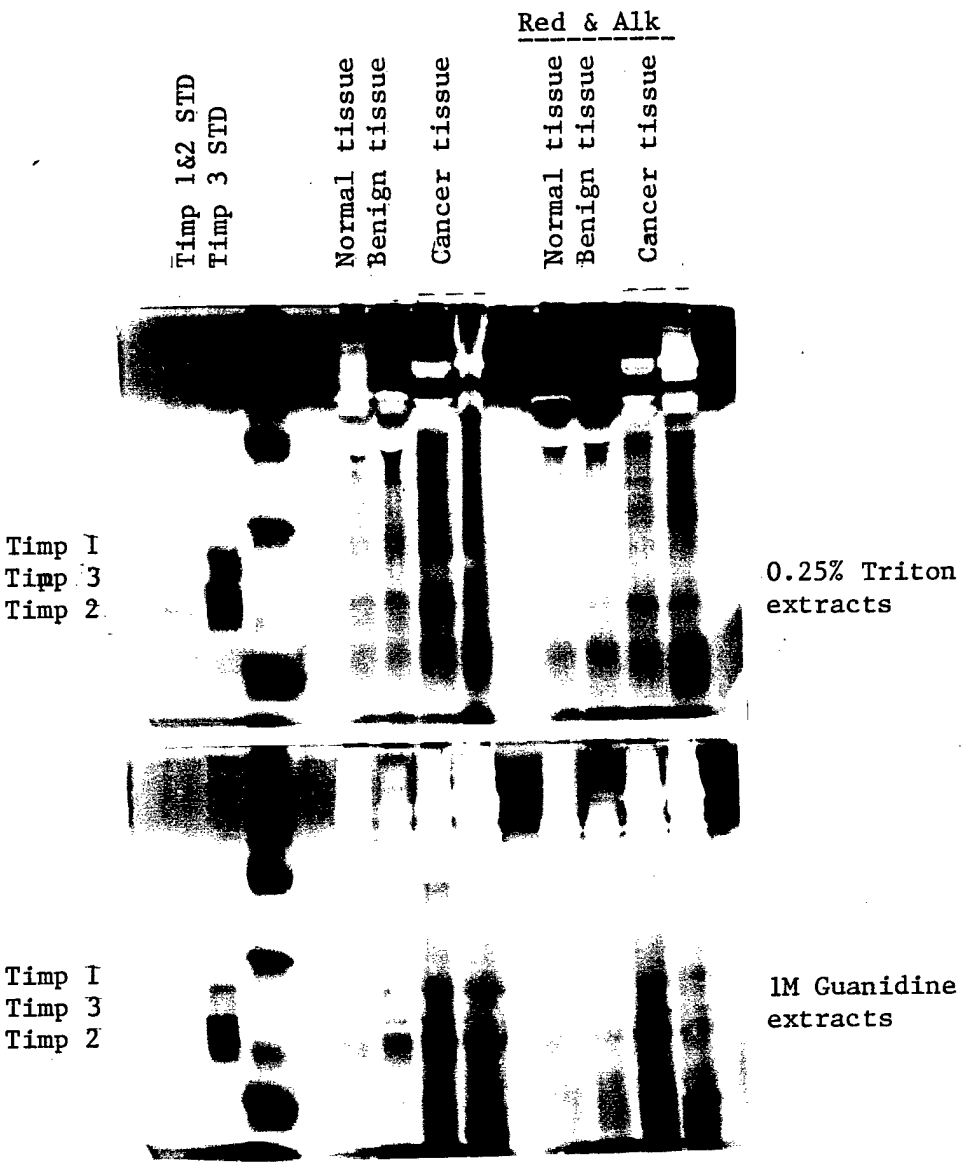


Table 2. Amounts of Tissue Inhibitors of Metalloproteinases (TIMPs 1, 2 and 3) in 1M GuHCl extracts of Breast Tissues.

Tissue	No.	TIMP-1	TIMP-2	TIMP-3
Normal	4	0.10μg	0.5μg	N/A
Benign	3	0.23μg	0.7μg	N/A
Adeno-carcinoma	11	0.12μg	0.07μg	N/A

The values in μg for TIMP-1 and TIMP-2 are obtained from breast tissue extracts analyzed by reverse zymograms before and after reduction and alkylation.

Caliberated TIMP standards of known values (ng) are used for calculation by Imager/Computer program.

These values reported may not be accurate.

Caliberated TIMP-3 standard was not available.

results. Improving the existing methods that are suitable for quantitation are **time consumig** but necessary to obtain reliable results.

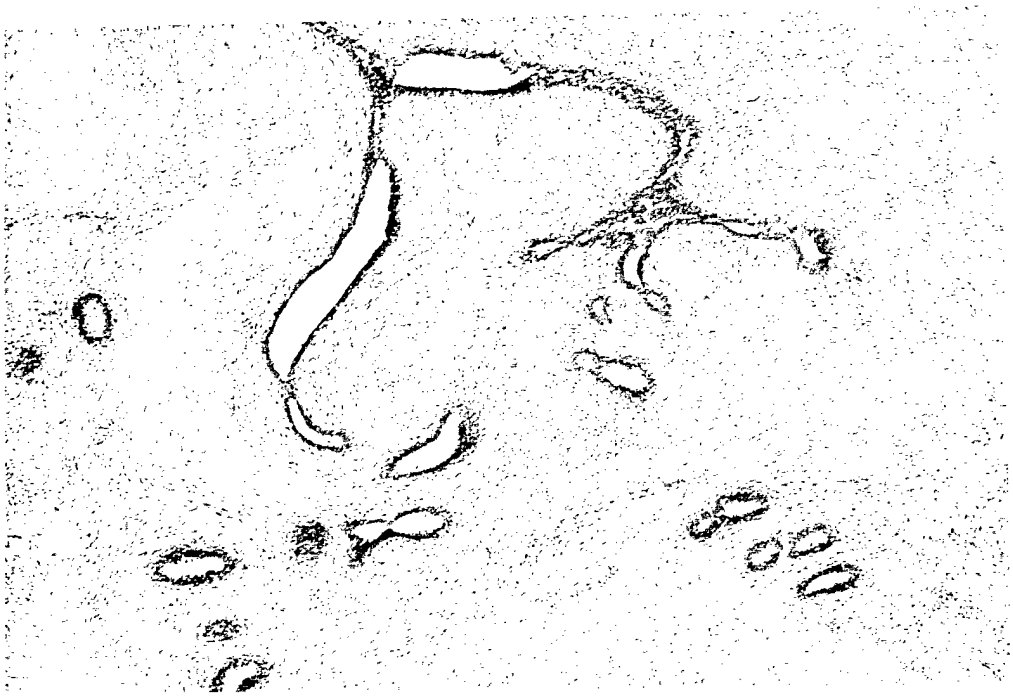
We plan to trimline the reverse zymography method for the quantitation of the TIMPs. We hope to reduce crosslinking of Bis-achrylamide complex to facilitate the diffusion of comigrating extraneous protein bands. Also rummaging through our records of home-made reverse zymography, we found that the quantitation of TIMPS (TIMP-1 and -2) was linear. Also, the background was uniform. This is not the case when the TIMPs are quantitated using the kit. One diffence is that we used partially purified 72 kDa gelatinase from the skin fibroblast to externally digest the non bound gelatin. The kit supplies a media containing many proteases (also serine proteases) clearing the areas unevenly. It is difficult to stockpile the enzyme for a length of time (loss of activity on storage) to use this enzyme routinely. We hope to develop this procedure using partially purified gelatinase for incorporation into the acrylamide gel. Preliminary results show that indeed we can successfully quantitate the TIMPs by the use of partially purified skin fibroblast gelatinase. We will scan the breast tissue extracts for TIMPs using the kit and final quantitation of TIMPs using the purified gelatinase..TIMP ELISA kits are unsuitable for our extracts.

6.B.3. Immunohistochemical evaluations of breast tissue:

The results are presented in **FIG. 5-7.** . The parrafin sections are stained with anti- MMP-9, anti-MMP-2, anti-MMP-1, anti-TIMP-1 and anti-TIMP-2 IgGs; followed by IgG-peroxidase as reported in Methods section. Immunoperoxidase staining reveals brown or red granules at the site of antigen-antibody binding in this study. The breast tissues were stained with five different antibodies -Anti-TIMP-1 (whole molecule), -MMP-9 (MBR, metal binding region peptides) and -MMP-2 (MBR, metal binding region peptide) anti-MMP-1 (fibroblast collagenase) and anti-TIMP-2.. **Fig. 5a** shows the routine H&E (hematoxylin and eosin, histopathology stain) staining of benign breast tissue and exhibiting pink colored cytoplasm and purple nuclei. Epithelial to stroma ratio (E:S) was 1:10. Results of staining benign tissue with anti-MMP-9 or anti-MMP-2 anti-TIMP-1 or or anti-MMP-1 or anti-TIMP-2 are presented in in **Fig. 5b, 5c, 5d 5e and 5f** respectively. Weak staining of epithelium only; stroma (connective tissue component of benign and malignant tumors) is negative. There was negative staining of stroma and epithelial but weak staining of macrophages with anti-MMP-9 IgGs (**Fig. 5b**). On the other hand, no staining of epithelial but staining of stroma was found with anti-MMP-2 (**Fig. 5c**). Anti-TIMP-1 staining shows weak staining of epithelium in **FIG. 5d.** Negative staining of stroma or epithelium is seen with anti-MMP-1 or anti TIMP-2 IgGs (**FIG. 5e & 5f**). Staining with anti-MT-MMP-1 IgG also showed negative staining.

Fig. 6 show staining of breast carcinoma tissues (IDC, infiltrating ductal carcinoma). **Fig. 6a** shows routine H & E staining for stroma and tumor cells. Stroma was negative for the IDC tissue. The important

a



b

Figure 5a Immunohistochemical staining of breast tissue (fibroadenoma, benign tumor). a: H & E staining. b: Immunoperoxidase staining with anti-MMP-9 IgG showing negative of epithelium and stroma.

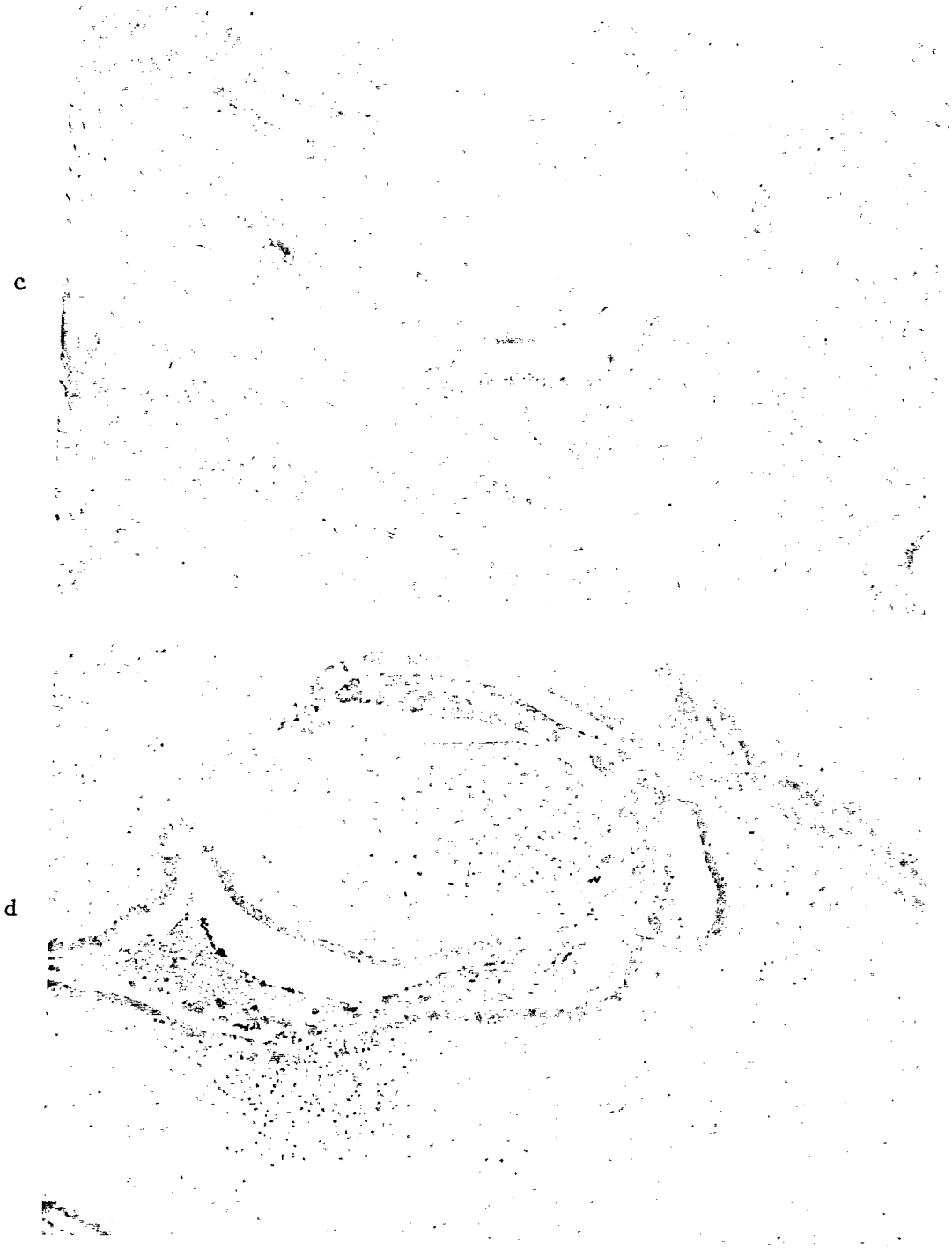


Figure 5b Immunohistochemical staining breast tissue. (fibroadenoma, benign tumor). **c**: Immunoperoxidase staining with anti-MMP-2 IgG showing negative staining of epithelium; positive stainin of stroma. **d**: anti-TIMP-1 IgG showingweak staining of epithelium.

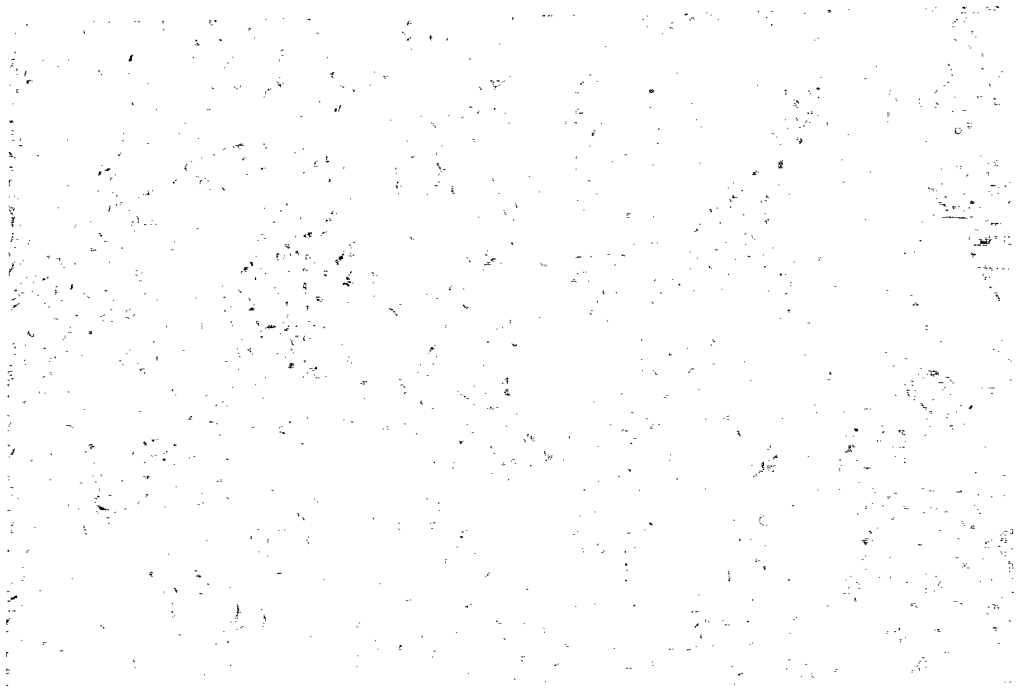
e

f

Figure 5c

Immunohistochemical staining of breast tissue (fibroadenoma, benign tumor). e: Immunoperoxidase staining with anti-MMP-1 IgG showing negative staining in epithelium and stroma (arrow). f: anti-TIMP-2 IgG, negative staining of epithelium and stroma.

a



b

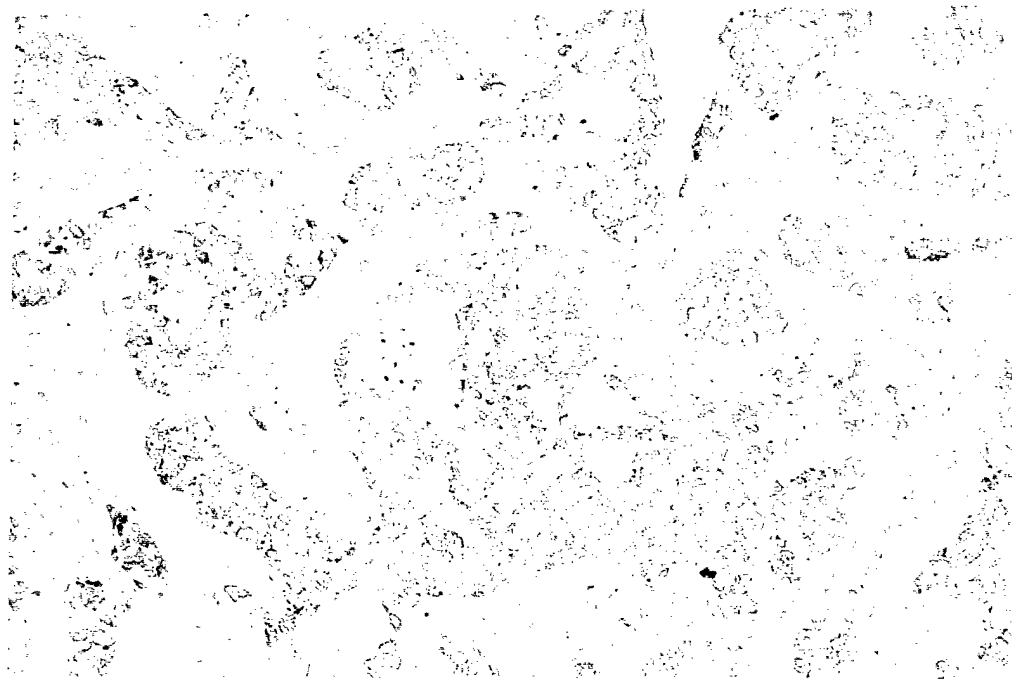
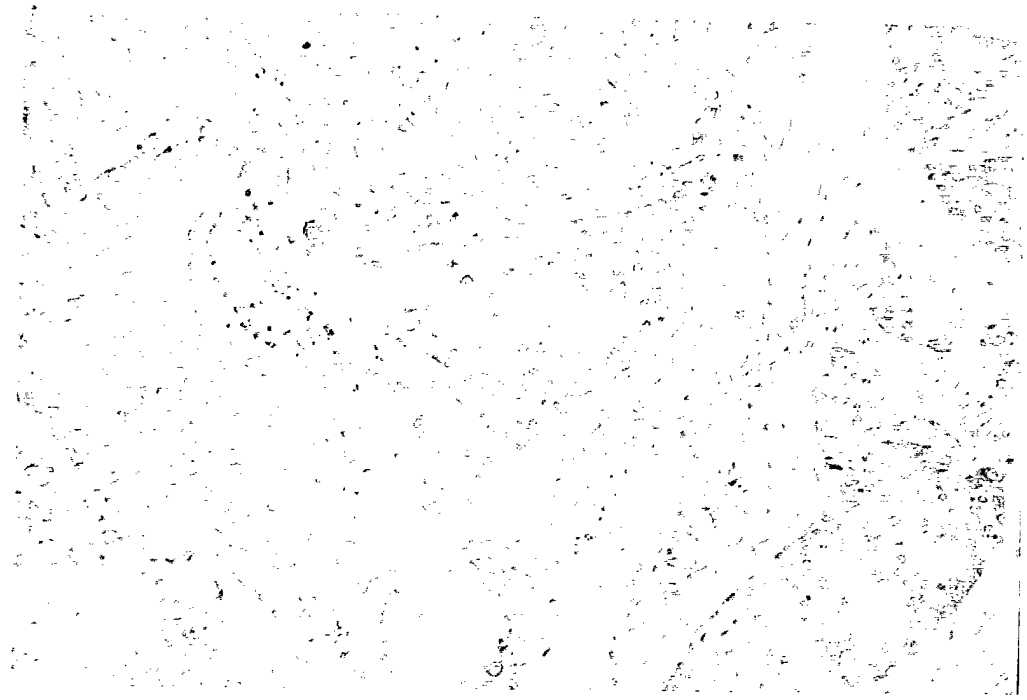


Figure 6a Immunohistochemical staining of breast tissue (Infiltrating ductal carcinoma, IDC). a: H & E staining. b: Immunoperoxidase staining with anti-MMP-9 IgG showing positive staining of tumor cells (epithelium) but negative staining of stroma.

c



d

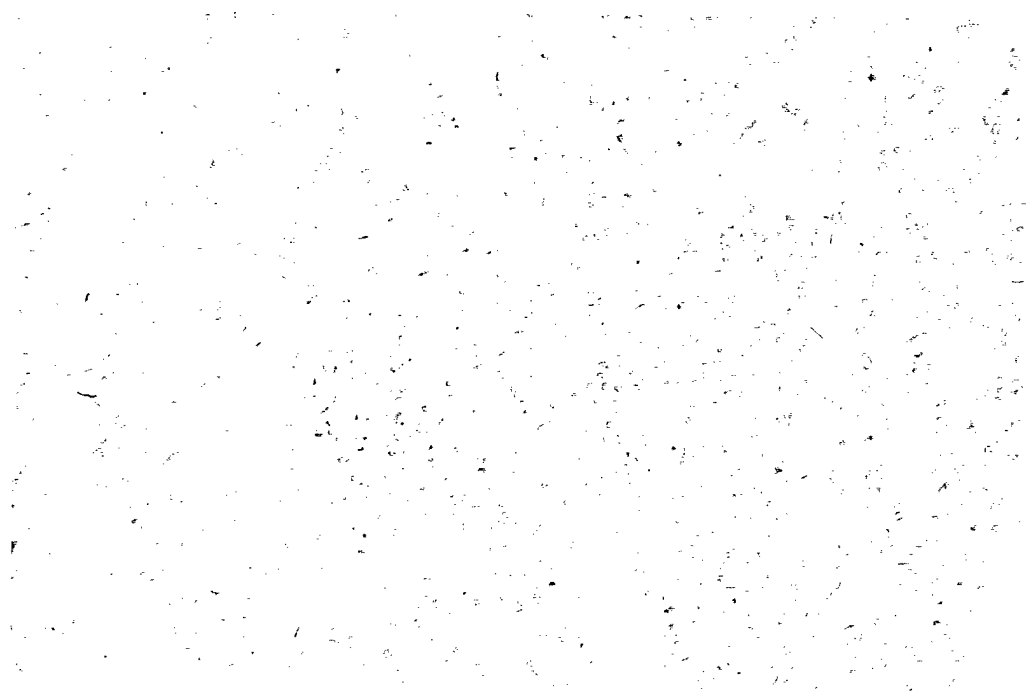


Figure 6b Immunohistochemical staining of breast tissue

c: Immunoperoxidase ataining with anti-MMP-2 IgG showing weak staining of tumor cells and stroma. d: anti-TIMP-1 IgG showing weak staining of tumor cells, no staining of stroma.

(Infiltrating ductal carcinoma, IDC)

Figure 6c Immunohistochemical staining of breast tissue (Infiltrating ductal carcinoma, IDC). e: Immunoperoxidase staining with anti-MMP-1 IgG showing positive staining of tumor cells and stroma. f: anti-TIMP-2 IgG showing negative of stroma and tumor cells.

a

b

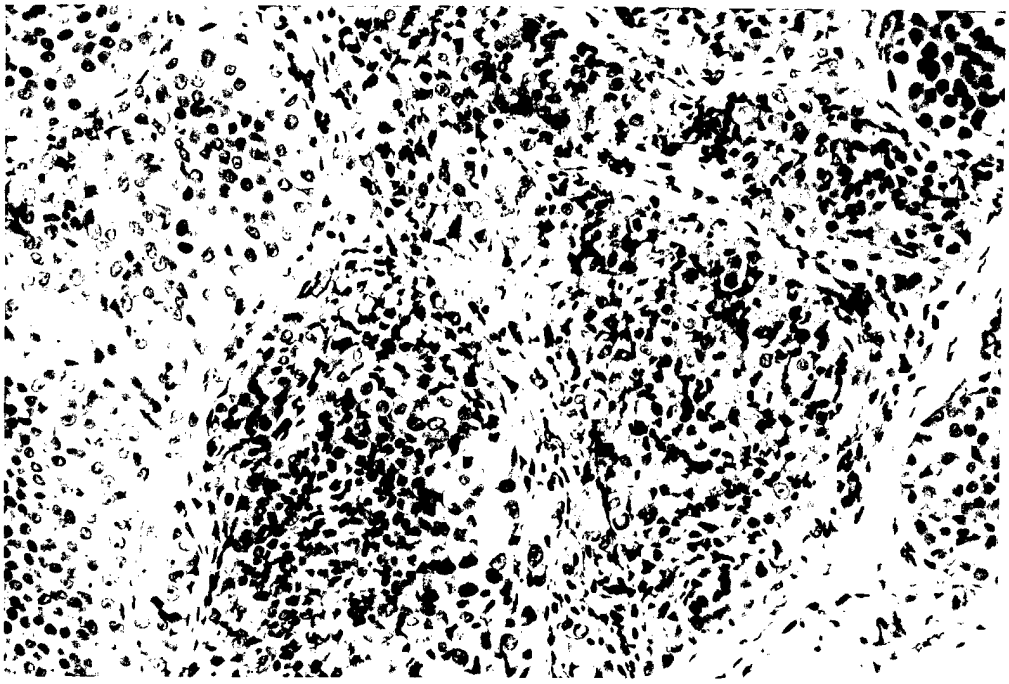
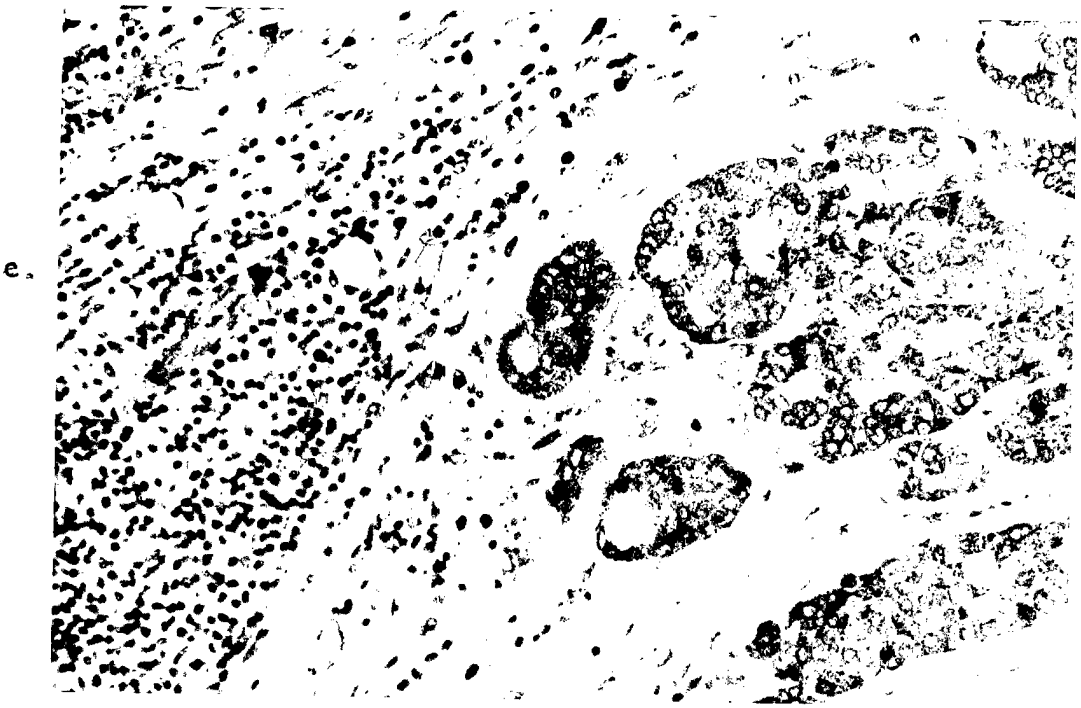
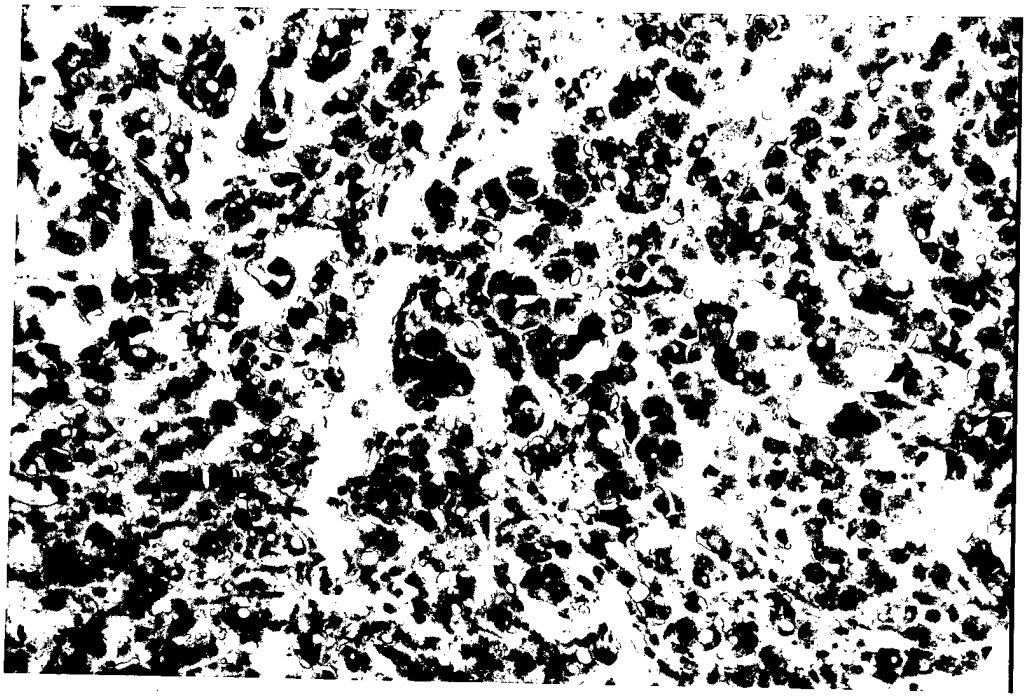


Figure 7a Immunohistochemical staining of breast tissue

a: Fibroadenoma, Benign; immunoperoxidase staining (AEC-red stain) with anti-MMP-2 IgG showing staining of epithelium, around capillary and some stroma. b: Lobular in-situ carcinoma; staining with anti-MMP-9 and showing no staining.



c



d

Figure 7b Immunohistochemical staining of breast tissue

c: Invasive lobular carcinoma, ILC; immunoperoxidase staining (AEC-redstain) with anti-MMP-9 IgG showing positive staining of tumor cells. d: Invasive ductal carcinoma, IDC; staining with anti-MMP-9 showing staining of tumor cells, negative staining of stroma.

finding is presented in **Figs. 6b** confirming the presence of MMP-9 in the IDC tissues as seen by zymography of tissue extracts. There is an intense staining of tumor cells with anti-MMP-9 IgG. There is weak staining of tumor cells and stroma with anti-MMP-2 IgG (**FIG. 6c**).Anti-TIMP-1 stained weakly positive (**Fig. 6d**) for tumor cells in IDC tissue. Anti-TIMP-1 IgG shows positive staining of tumor cells and stroma while anti-TIMP-2 shows negative stroma and tumor cells (**FIG. 6f**).

FIG. 7 shows some interesting results - again confirming the presence of MMP-9 in carcinoma. **FIG. 7a** shows staining of epithelium (red color) with anti-MMP-2 IgG of benign (fibroadenoma) tissue. There is no staining of cells with anti-MMP-9 IgG in breast tissue of **in situ lobular carcinoma FIG. 7b**. Staining of however, invasive lobular carcinoma (**FIG. 7c**) tissue with anti-MMP-9 IgG showed positive staining of tumor cells only in the invasive part and not in the in situ part of the tissue. Infiltrating ductal carcinoma tissue also stained red in tumor cells (**FIG. 7d**). **We have more stained tissue of lobular in situ and invasive lobular carcinoma with other IgGs and repeatedly it shows that MMP-9 is definitely a marker for breast cancer.**

6.B.4. Analysis of secreted MMPs and TIMPs in various tumor cell lines:

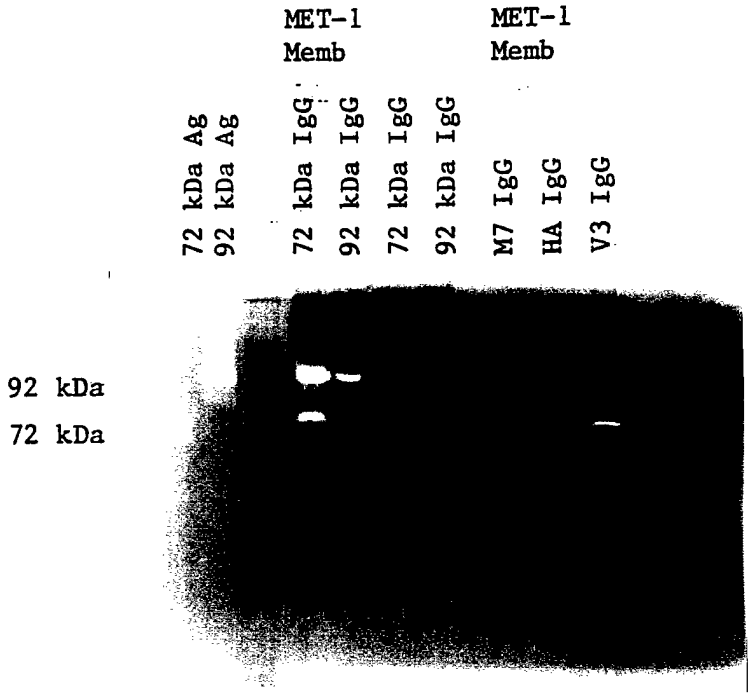
Several cell lines (MCF-7, DA-3, 101A-HMT, PPC-1, PC-3 and MDA-MB) were scanned for secreted MMPs and TIMPs and the results were reported earlier. Now we report the secreted MMPs from cell lines MET-1 and PM-1 derived from tumors of different metastic potential from a transgenic mouse system expressing polyomavirus middle T oncogene (provided by Dr. Robert , Cardiff University of California, Davis, CA to Dr. Lily Bourguignon). The cell lines were of high metastic potential.and unlike non-metastic cells, Met-1 cells have been shown to form membrane spikes or "invadopodia" structures (**FIG. 10, a, b, c**) , see confocal studies below.

Both cell lines secreted MMP-9 only . This finding is similar to that observed for MCF-7 (reported earlier). **FIG. 8a** shows the gelatin zymography of immunoprecipitated membrane fractions of cell lines MET-1 and PM-1. Anti-MMP-9, anti-CD44v-III, -CD44v-VII, anti- HA binding region and anti-MMP-2 IgGs were used. Anti-MMP-9 IgG immunoprecipitated CD44v-III (result not shown). Anti-CD44v-III IgG immunoprecipitated with it MMP-9 only and not MMP-2. This is extremely exciting as CD44 variants are known to be associated with several cancers including breast cancer [42]. These findings are first of a report of association or colocation of MMPs and CD44 variants and manuscript is in preparation (Journal of Biological Chemistry).

Similar results were obtained with membrane fractions of PMA-stimulated MCF-7 cell line (FIG. 8b). One interesting observation was that in all three cell lines, anti-MMP-2 IgG immunoprecipitated a fraction containing MMP-2 and MMP-9. This suggests that MMP-9 is localized as a complex with CD44 or with MT-MMP-1 through of TIMP-2 cascade.

Figure 8a

Gelatin Zymography showing Immunoprecipitation of membrane fractions of cells - MET-1 and PM-1 with anti-CD44; anti-72 kDa and anti-92 kDa gelatinases



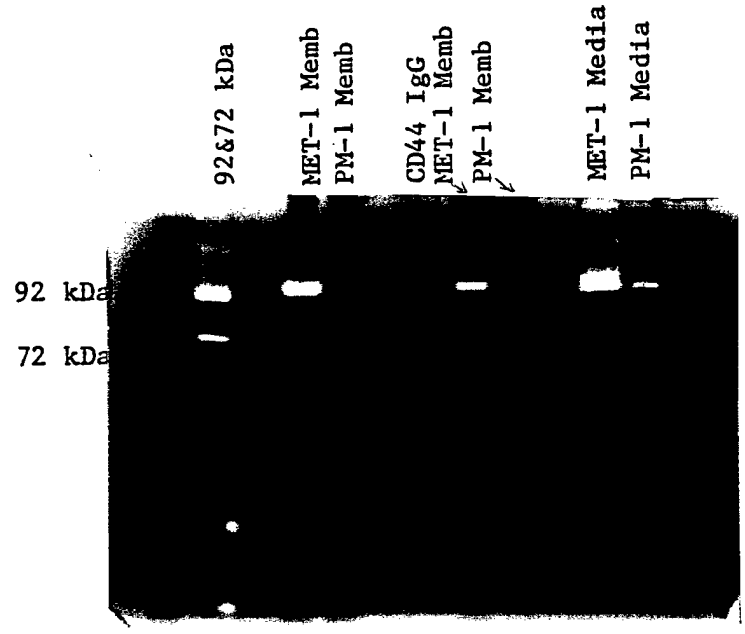
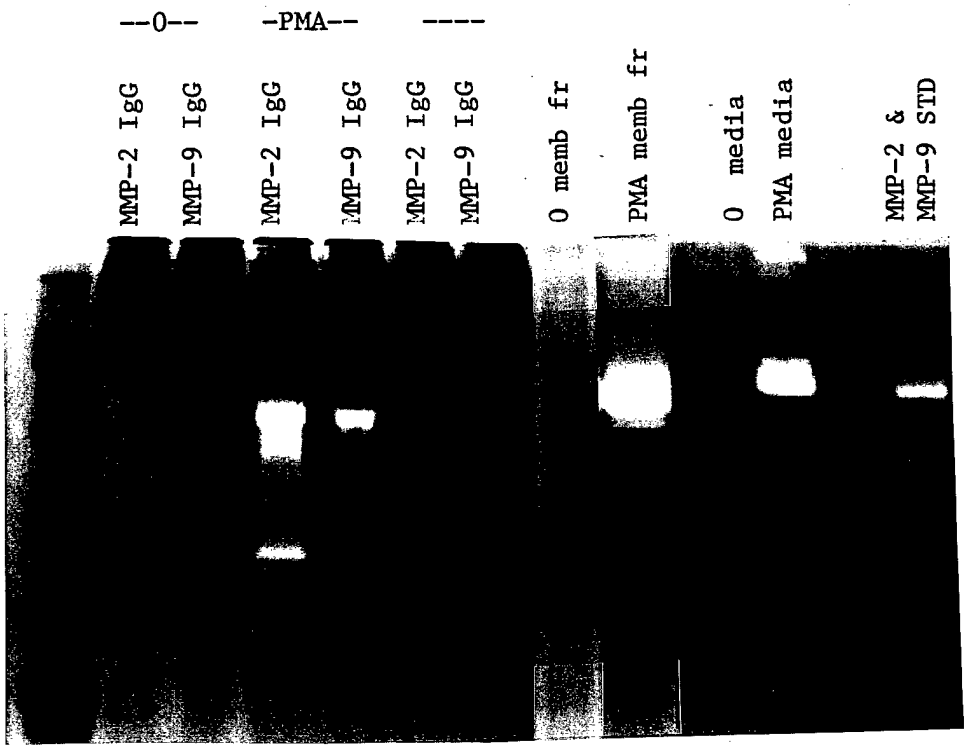


Figure 8b

Gelatin Zymography Showing Immunoprecipitation of Membrane Fractions.

Cells used were MCF-7 without - 0 and with PMA, phorbol ester stimulation. Anti-MMP-2 and anti-MMP-9 IgGs were used.



a:

b:

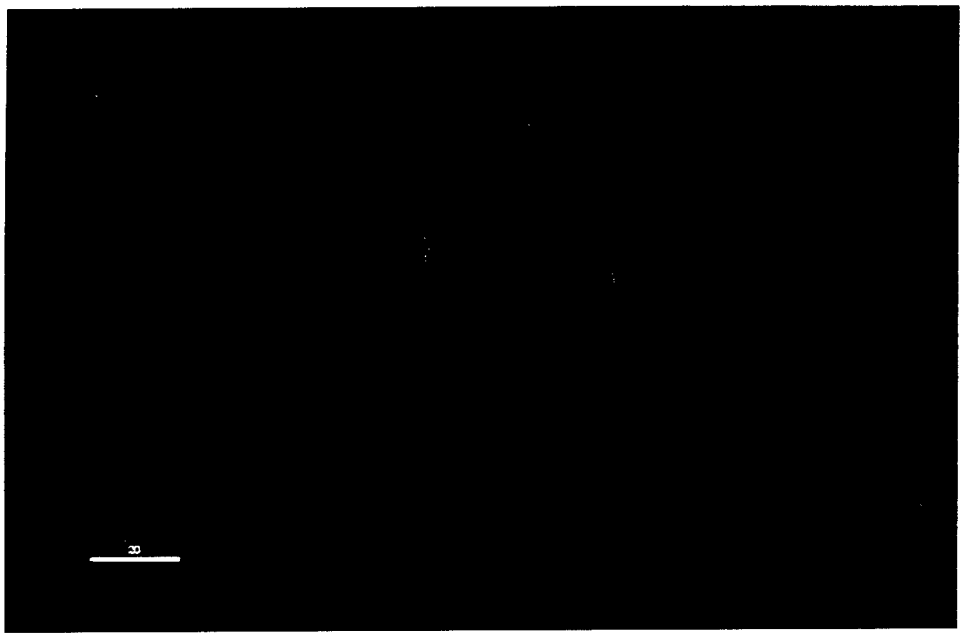


FIG. 9. Immunofluorescence staining of MT-MMP-1 in Breast Tissue using confocal microscope. Rabbit anti-MT-MMP-1 was used followed by fluorescein-labeled (green color) goat anti-rabbit IgG.

a: Benign tissue b: Infiltrating ductal carcinoma,IDC tissue

6.B.5. Immunofluorescense staining and confocal microscope analyses:

Breast cells lines e.g. MCF-7 (human) and MET (murine) were incubated with rhodamine-labeled rat anti-CD44 IgG and fluorescine-labeled rabbit anti-MTMMP-1 (membrane type MMP) IgG, or rabbit anti-MMP-9 IgG or rabbit anti-MMP-2 IgG; cells were washed and labeled samples excited with a krypton-argon ion mixed-gas laser and examined with a laser scanning Confocal microscope (Multiprobe 2001 Invert CLSM System, Molecular Dynamics) using a 63 X-oil immersion and an imaging processing device. Cells were photographed with Kodak Tri-X-film. The results of the study with MCF-7 were presented in last year's report.In this report, we present the results from similar study using MET-1 cell line.

The results are presented in **FIG 9 and 10.** Staining of tissue sections with anti-MT-MMP-1 were not sharp enough to show the stained membranes. So we proceeded to label the tissue sections with anti-rabbit IgG fluorescein- conjugate to locate the MT-MMP-1 stain using confocal microscope . The results are presented in **Fig.9a and 9b. a**: represents benign tissue section and **b**: represents breast cancer tissue section. **The staining for MT-MMP-1 is intense only in the cancer tissue.** MT-MMP-1 gene has been implicated to be expressed by resident matrix fibroblast cells of tumor stroma in human breast carcinoma rather than tumor cells[49]. We plan to scan more cancer tissues with anti-MT-MMP-1 IgGs and also find the gene signal using Northern Blots.

The MET-1 cells show membrane spikes or "invadopodia" structures as shown in **FIGs. 10a,b,c** using confocal microscope. **FIG. 10a**, shows staining of cells with anti-CD44 (red color) and anti-MTMMP-1 (green color) The yellow color (green and red = yellow color) shows the colocation of MTMMP-1 and CD44 in the transmembrane location of the MET-1 cells. Similarly, **FIG. 10b** shows the colocation of MMP-9 (92 kDa gelatinase) and CD44. There are very distinct and broad areas of colocation of two proteins. Colocation of MMP-2 (72 kDa gelatinase) and CD44 are shown in **FIG. 10c**. Colocation is distinct but not as broad as those found for MMP-9 and CD44. These results are very exciting and the first report of co-anchoring of MMP-9 (or of other MMPs) with CD44 in the transmembrane portion of a metastatic cell line.

6.B.6. RNA Extraction and Northern Blot Analysis

Molecular biology techniques were - RNA extraction from cells and tissues; ; purification and analysis of DNA; transfer to membrane, fix RNA onto membrane, prehybridize (blocking), hybridize labeled probes, wash excess probe and detection of the hybridized probe using non-radioactive or radioactive methods. Good available kits were purchased whenever necessary.

MMP-2 (collagenase type IV 72K, PBS-GEL); MMP-9 (collagenase type IV 92K, PBS-92 174) and MTMMP-1 (PBS-MT-MMP-1) cDNA clones were kindly provided by Dr. G. Goldberg, Washington University School of Medicine, St. Louis, MO. cDNA Clone for GAPDH was provided by Dr. P. Mehta, U. of Miami Medical School, Miami, FL. Human fibroblast collagenase from PMA-treated cells clone was purchased from ATCC as purified plasmid DNA

(57685). TIMP-1 and TIMP-3 cDNAs are provided by Dr. Keith Brew, Dept. of Mol. Biol. & Biochemistry, U. of Miami Med. School.

The cDNA inserts transferred to appropriate vetors were removed by appropriate enzymes and used for northern blots.

We first started with RNA extraction and Northern blotting procedure using fresh MCF-7 cells for the detection of **mRNA for MMP-9** and human neonatal fibroblast cell line (NFC, LHZ) for the detection of **mRNA for MMP-2**. Cells were chosen to establish the non-radioactive detection technique We started with non-radioactive detection method due to health reasons. We were successful in obtaining a strong signal for both MMPs (**FIG. 12**) in cells but no signal for both MMPs could be detected from breast cancer tissue.

The temperature was also critical for hybridization procedure. There was non-specific binding at 42°C and this disappeared when hybridization temperature was raised to 68°C (**FIG. 12**). The probe was MMP-2 provided by Dr. G. Goldberg. The benign and cancer tissues also showed non-specific binding at 42°C (hybridization temperature). We tried to cut the vector only by enzyme for cDNA for MMP-2, to minimize the non-specific binding. it helped a little, however, the signals are too low in the breast tissues. ^{32}P-labeled probe for MMP-2 was also used for NFC cells and breast tissues (**FIG. 12**), there was again non-specific binding at temperature 42°C (hybridization).

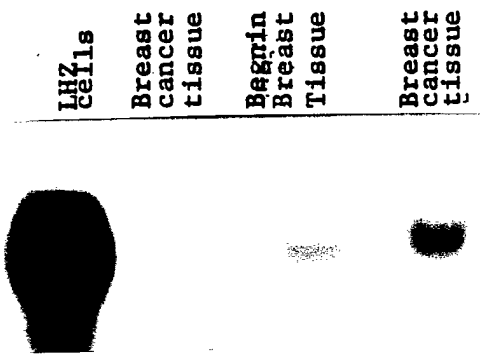
We turned our attention to generation of highly sensitive probes labeled with DIG-dUTP in the polymerase chain reaction (PCR) The kit was purchased from Boehringer Mannheim,(Cat. No. 1636 090, Boehringer Manheim, Indianapolis, IN) and dig-labeled PCR probes were prepared for MMP-2 and MMP-9 using the kit according to Manufacturer's instructions. This procedure generates highly sensitive hybridization probes suitable for detection of low (single) copy target sequences. The nucleotide concentration in the PCR Dig probe synthesis mix ensures the identification of single copy genes in genomic blots after hybridization to DIG-labeled in 10 µg of genomic DNA.

The results using PCR probes for MMP-9 and MMP-2 are presented in **FIG. 11**. The background was low and the signal for MMP-9 was observed for breast cancer tissues. Dig-labeled probes for fibroblast collagenase (MMP-1) and GAPDH showed mRNA signals successfully (**FIG. 11**) using fresh MCF-7 cells. Future experiments will be carried out with PCR DIG-labeled probes. The sequences for most MMPs and TIMPs are known and as the signal in breast tissue is low, PCR Dig-labeled probes are needed. We have probes for most MMPs and TIMPs and the third year will be devoted to molecular biology experiments.

For Northern Blot procedure, total RNA was obtained from the autopsied breast tissues that have been frozen at -70°C for 2-5 years. Several tissue samples were not enough for all analysis. Again, several samples (in number) had to be processed for RNA extraction. Most samples were found to have degraded RNA. **Five to seven samples from twenty five sammples revealed good functional RNA to proceed further for Northern blotting procedure. This is time consuming and shows that samples having**

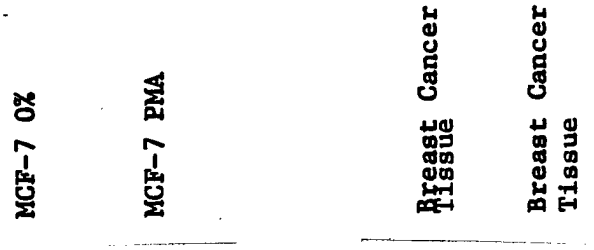
Figure 11

NORTHERN BLOTS : Non-radioactive DIG LAB Method (also PCR Dig Lab)



72 kDa Probe PCR-Dig-Labeled

GAPDH Dig-Labeled



92 kDa PCR Dig-Labeled

Fibroblast collagenase
MMP-1 Dig-Labeled

GAPDH Dig-labeled

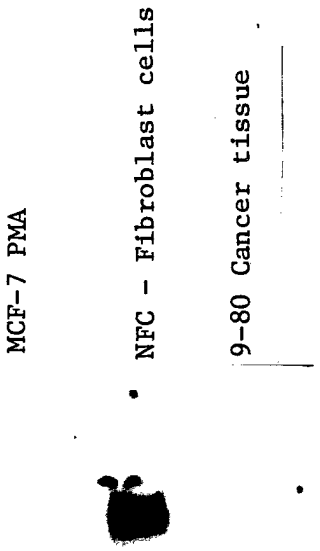
Figure 12

Northern Blots of Breast Cancer Tissues with

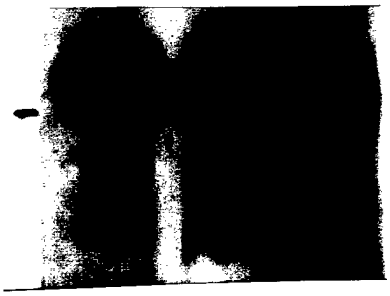
DIG-labeled blots at 68°C and at 42°C

^{32}P-labeled at 42°C

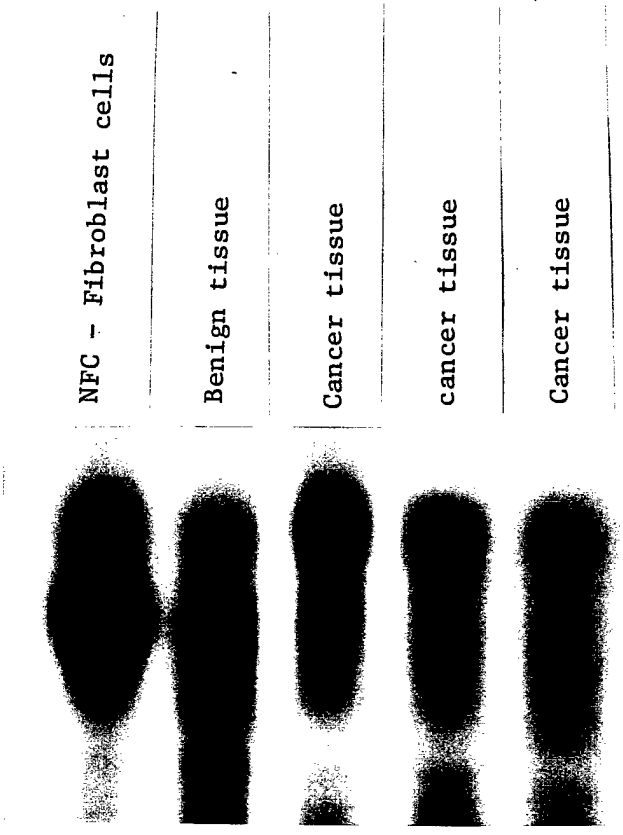
Probes were MMP-2, with cells and breast tissue



DIG-labeled 68°C Hyb
MMP-2 probe



DIG-labeled 42°C Hyb
MMP-2 probe



^{32}-P-labeled 42°C Hyb
MMP-2 probe

degraded RNA and already used for zymographical evaluations of breast tissues wil not have companion Northern Blots In future, we hope to evaluate the RNA extraction of breast tissues prior to the zymographical and immunohistochemical evaluations for MMPs and TIMPs. This tactic may reduce the number of samples that can be evaluated for all parameters.

7. CONCLUSIONS:

MMPs and TIMPs represent a class of metalloproteases and their tissue inhibitors secreted by various types of cells, including epithelial, fibroblasts, and macrophages MMPs have been implicated in degradation of basement membrane during cancer invasion and metastasis Their activity is controlled, in part, by natural inhibitors (TIMPs). The imbalance created in the secretion of their tissue inhibitors (TIMPs) has been implicated by us for prostate cancer [26] and by others in breast carcinoma [48].The present study in its first two years clearly demonstrates that 1) extraction of enzymes from the tissues and 2) immunohistochemical analysis, showsthe presence of different levels of latent and active forms of MMPs in human normal, begnin and cancer breast tissues. The extraction of these tissues also show the presence of diferent types and levels of TIMPs by reverse zymography of tissue extracts. These findings strongly suggest that the basement membrane underlying breast epithelium probably undergoes rapid turnover due to matrix degrading enzymes secreted by various resident cells in the breast tissue.

A novel finding of this study is that gelatinase B (MMP-9) was found only in breast tumor tissue as demonstrated by gelatin zymography (**Table 1**) and immunohistochemical analysis of the same tissues. Other classes of MMPs such as MMP-1 0r MMP-13 (interstitial collagenase-1 and -3) and gelatinase A (MMP-2) were found in all classes of breast tissue. Normal and benign tissues showed the presence of only MMP-2 and MMP-1 was found in lower amount than that found in breast carcinoma tissues. Presence of stromelysin-1 or stromelysin-3 could not be demonstrated by zymography or substrate assays. Presence of stromelysin-3 can only be demonstrated by measuring mRNA levels [19], as the proteolytic activity of this enzyme appears to be very weak[20].

Immunohistochemical analysis have been established in the laboratory. This allows us to quickly evaluate the integrity of the tissue used for various biochemical analyses. Immunohistochemical analysis of breast tissues will be an ongoing part of the study for next two years. Samples from other institutions will also be obtained to fill the void of particular type of samples. Samples of in situ lobular and infiltrating lobular carcinoma are rare in University of Miami archives.

Reverse zymography will be stream-lined to quantitate the TIMPs present in breast tissue extracts. Presence of other proteins in extracts do not diffuse out sufficiently from the gel. This was demonstrated by examining the extracts before and after reduction and

alkylation procedure. This procedure should destroy the TIMPs. We have found that TIMP-2 and TIMP-3 are difficult to be knocked out and degraded than TIMP-1. This will be taken into consideration. Besides, we found that use of partially purified MMP-2 (skin fibroblasts) gave an even background compared to the use of media supplied in the kit. Also, the lowering of BIS in percentage may allow the extraneous proteins in TIMP area to diffuse out.

Meaningful statistical analysis of 50-100 samples are now feasible by the analysis of gelatinase MMPs by zymography and quantitation of MMP bands by use of GelBase/GelBlot Pro software. Storage of fresh frozen samples over five years and the availibility of information from Florida Tumor Registry of follow-ups of patients will allow us to fine tune the collected information. These findings may lead to the development of markers for early diagnosis in breast cancer.

A manuscript is in preparation to present the zymographical and immunohistochemical evaluations of breast cancer tissues.

The measurement of mRNA levels of MMPs and TIMPs have begun and preliminary results show that MMP-9 mRNA signal coud be detected in breast cancer tissues. **We had to process several breast tissue samples to obtain intact (not degraded) RNA to carry out successful Northern blots.** Stistically **only 2-3 samples from ten showed undegraded RN**A that could be processed. We also found that unless the signal was strong as found with fresh cells, regular no-radioactive or radioactive DIG-labeled procedure showed high backgroung or no potential signal for a particular cDNA probe. In some instances, the temperature requirements for hybridization had to be changed from 42°C-68°C for lower background. The MCF-7 and skin fibroblast cell lines have been useful in estabilishing all the molecular biology techniques.

We finally decided to turn to PCR DIG Probe Synthesis kit for detection of *low* (single) copy genes in genomic blots after hybridization to DIG-labeled PCR products. PCR primers containing 210-220 base pairs of primers from MMP-2 and MMP-9 sequences and subsequently dig-labeled were used. It is possible that procurrement of fresh breast tissues may be necessary to evaluate breast tissue by enzymic and molecular biology techniques. We hope to establish relationship with an Oncology surgeon to obtain at least some fresh breast tissue samples.

Immunofluorescence staining and confocal microscope analyses of tumor cell lines (MET-1 and MCF-7) has led to some important finding that either MMP-2 or MMP-9 are associated with CD44 (a transmembrane glycoprotein) isoforms in tumor cells. Preliminary results indicated that anti-CD44v-III isoform found in breast tumors activated the secreted MMP-9. MCF-7 cells did not secrete MMP-2 into the media as shown by zymography earlier. Immunofluorescence staining with anti-MMP-2 IgG showed staining in the cell membrane suggesting the presence of MMP-2 bound to the membrane (report 1995).

Experiments with membrane fraction of MET-1 cell line shows that MMP-9 and CD44v-III isoform are colocalized in the transmembrane. The

membrane fractions were immunoprecipitated with anti-CD44v-III IgG or anti-MMP-9 IgG bound to Protein-A-Sepharose beads independently or individually and the bound product examined by zymography for MMP and SDS-PAGE-western blot (chemiluminescent method) for CD44 and its variants. MMP-9 antibody immunoprecipitated only CD44 variant v-III and MMP-9. No MMP-2 was observed by zymography. Immunoprecipitation with MMP-2 IgG did not co-precipitate CD44v-III variant. Anti MMP-2 did immunoprecipitate MMP-9 and a band coresponding to MMP-2 or MT-MMP-1 but no variant CD44.

Experiments with membrane fraction of MCF-7 immunoprecipitated MMP-9 with anti-MMP-9 or anti-CD44v-VI variant. Anti-MMP-2 immuno-precipitated only MMP-9 and its active forms. A manuscript is in preparation to report these findings with MCF-7 and MET-1 cell line.

Recently, an MMP has been identified as an integral part of plasma membrane protein [50, 51,52] and is termed as membrane-type protein (MT-MMP). This MT-MMP can activate MMP-2, which in turn can activate MMP-9 and other MMPs. MT-MMP has a dual role in assissting cell migration. It is membrane associated and can localize ECM digestion in the vicinity of the cell surface and also amplify the destruction of matrix by activating other MMPs. MCF-7 tumor cell line was shown to have high capacity to bind exogenous MMP-2 by use of radioreceptor -binding assay [49]. Our findings by confocal studies of MCF-7 cells for MMP-2 localization in transmembrane may sggest that MMP-2 made by the stimulated cells bind to TIMP-2, which in turn binds to MT-MMP [51].

There are several exciting findings in our present study that need to be expanded in the third year of the granting period. The study on analysing the breast tissues for MMPs and TIMPs will be an ongoing project for the third year and then correlate these findings obtained to be a part of the fourth year.

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