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13. ABSTRACT *(Maximum 200* The deadly consequences of breast cancer are due to metastasis, a process in which tumor cells penetrate the blood vessels and enter other tissues to spread the cancer. This movement through vessels and tissues is attributed to a group of digestive enzymes (the matrix metalloproteinases or MMPs) that can destroy the matrix in advance of tumor cell movement. These MMPs are normally produced in small amount and are held in check by inhibitors in the tissues (tissue inhibitors of MMPs or TIMPs). We took 84 samples of breast tissues (benign tumors and various carcinomas) and measured the production of six different MMPs and 3 TIMPs in a unified multipronged approach. We used antibody methods to see which cells are producing these enzymes and inhibitors. The most prominent enzyme was MMP-9, also known as gelatinase B, which is able to break down the wall that forms around tumor cell clusters. While this, and several other MMPs were elevated in cancer, the TIMP inhibitors were produced at levels well below normal. This results in an imbalance in which the destructive proteases greatly outweigh the controlling inhibitors, facilitating the spread of the cancer. Biochemical quantification of MMPs by zymography showed an overall increase in all types of MMPs in cancer tissues. MMP-9 was the key MMP; it was present at levels 0.23 (*in situ* ductal) to 2.3 (infiltrating ductal) µg/g wet weight tissue in cancer tissues compared to unquantifiable amounts in normal (3/6) and benign neoplasm (10/11) tissues. Zymography also showed a fraction of MMP-9 and MMP-2 in their active forms in grade III breast cancers compared to normal and benign tissues Reverse zymography showed the presence of TIMPs -1, -2 and -3 in all breast tissues. Quantitation by TIMP-1 ELISA kit and reverse zymography clearly showed that total amounts of TIMPs were lower in cancer (1.6 µg/g wet weight tissue in adenocacinomas compared to higher amounts in normal (4.5 µg) and benign (3.5µg) tissues. The production of TIMPs in cancer tissues falls far short of the amount needed to counteract the excessive production of MMPs leading to an imbalance in enzyme and inhibitor expression. Semiquantitation of MMPand TIMP gene expresion (MMP-2, MMP-9, TIMP-1 and TIMP-2) by RT-PCR method. are in agreement with both zymography and immunohistochemical findings.

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Gerald Gregg Smith 9/29/97

PI - Signature Date

(4).Table of Contents

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

(5) INTRODUCTION

BACKGROUND

The overall objective of this research is to explore the role of matrix metalloproteinases (MMPs) and the tissue inhibitors of metalloproteinases (TIMPs) in the invasive and metastatic processes of human breast tumors [1-4]. The hypothesis to be tested is that an imbalance may arise through normal or elevated production of MMPs accompanied by a diminished production of inhibitory TIMPs. Metastasis of an initially 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 [6,7]. Recent evidence indicates that secreted matrix metalloproteases (MMPs) not only play a major role in the penetration of the basement membrane surrounding the tumor [8] but also play a role in the growth of primary and secondary tumors [9].

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

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

Since normal cells as well as non-invasive cells produce MMPs and also TIMPs, it seems likely that the extent of activation of MMPs and the levels of inhibitory TIMPs may be two key factors in the progression of normal to invasive cell type. Elevated levels of MMP-9 have been detected in the plasma of patients with breast cancer [21] although no such corelation was found for MMP-2. Not all of the MMPs and TIMPs need be related to the tumor cells. It is quite likely that tumor cells can stimulate neighboring stromal cells through cytokines to produce enzymes that degrade the matrix. So in certain breast tumors and other cancers of skin and colon, 92 kDa gelatinase was found at the tumor/stroma border but not in tumor cells [22].

In summary, there is a compelling evidence of a role for metalloproteases 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.

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

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

(6). BODY

6.A EXPERIMENTAL METHODS

6 A.1.Tissue Samples: The tissues (~ 1 cm^3, snap frozen, stored at -70° C) diagnosed as displaying breast carcinoma, normal or begnin 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.

In the fourth and final year the tissues will be chosen to fill gaps to generate meaningful statastical evaluations of different kinds of breast cancer. The Florida tumor registry can provide the history of patients showing the development of metastasis, surviva l rate of the patients up to five years and beyond. This service will help in collating the results in the fourth year of the grant. The snap frozen tissues kept for five years are suitable in some instances for Northerns (mRNA estimation) and most of them for RT - PCR as long as care is taken during the preparation of total tissue RNA

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

6.A.2. Explant cultures and cell lines: Tissues either fresh or frozen in 5% dimethyl sulfoxide are obtained according to State and Federal regulations from the Tissue 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 [23] 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°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 [23].

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. 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°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.3.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.

The polyclonal antibodies were found to be monospecific. They were further characterized for their reactivity to other MMPs using Western blot analysis [23].

6.A.4. Immunohistochemistry: These analyses are carried out in Principal Investigator's laboratory using Histostain™ SP Kit (Zymed Laboratories, San Francisco, CA) according to manufacturer's instructions with minor modifications. The stained slides are reviewed by Dr. Scott Y. Sittler, Department of Pathology, University of Miami Medical School. Paraffin sections (4 microns) are cleared of paraffin, blocked for endogenous peroxidase, washed in water, PBS, blocked with normal horse serum and then are treated with drops of specific primary antibodies in a humidity chamber (1h). The tissues are washed, and treated with a biotinylated secondary antibodies (1-3h), followed by avidin-biotin-peroxidase complex [25]. They are then washed and treated with the chromogen. The slides are counterstained with hematoxylin, washed, dehydrated and evaluated for the localization of various MMPs and TIMPs in specific cells in the tissue. The kit incorporates HorseRadish Peroxidase (HRP), streptavidin, and affinity-purified antibodies into the Labeled-[strept]Avidin-Biotin (LAB-SA) method. The chromogen/substrate system [aminoethyl carbazole (AEC) for a red signal or diaminobenzidine (DAB) for a brown signal] creates an intense color deposit around the antigen/antibody/enzyme complex in the tissue or cell sample.

6.A.5.Extraction and assays of metalloproteinases and inhibitors. Human breast tissues are weighed (100-200 mg), minced (finely) and homogenized (in the hood) in 7.5 volumes extraction buffer (**0.25% Triton-X 100 or 1 M GuHCL or 0.5 - 2% SDS in 50 mM Tris/HCl buffer, pH 7.5**) using Polytron homogenizer, centrifuged and the pellet reextracted with 2.5 volumes of appropriate extraction buffer, centrifuged and supernatants combined [26]. All steps are carried out at about 4°C. Extracts are dialyzed and stored in aliquots at -70°C.

Most tissues contain inhibitory activity which appears to be TIMP (tissue inhibitor of metalloproteinases). These can be destroyed, without affecting the metalloproteinases, by reduction (2 mM DTT) and alkylation [27]. This step also destroys any alpha-2-macroglobulin. Protein estimation of homogenates or concentrated media are by the use of BioRad protein estimation kit.

Tissue extracts (with or without reduction and alkylation) and column fractions (molecular seive or affinity chromatograophy) are assayed and quantitated for various MMPs using (1) ^{3}H-acetylated Type I gelatin to estimate the MMP-2 and MMP-9 gelatinases [26] (2) ^{3}H-acetylated Type I rat skin collagen for MMP-1 (interstitial collagenase [26] and ^{3}H-carboxymethylated transferrin [28] ^{3}H-acetylated proteoglycan monomer bead assay [22] 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 acheived by the inhibition of uterine MMP-7 and remaining activity assayed using Azocoll as substrate [27]. 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) [29] 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.6.Zymography and Immunoprecipitation. Gelatin zymography follows a modified procedure of Herron et al. [30] for detecting picograms of MMP-2 & -9 and nanograms of other MMPs and proteases. SDS-PAGE is performed in 7.5% or 10% polyacrylamide (31) containing 0.33 mg/ml gelatin (or other substates i.e. casein, soluble elastin or transferrin. The gels are then rinsed twice in 0.25% Triton X-100, and incubated (18 h, 37^{o} C in Tris-NaCl-$ZnCl_2$ -$ZnCl_2$,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 quantitated using densitometry analysis of gels (and also dried gels in membranes).The imager (Ultra Violet Products (UVP), Upland, CA) and GelBase/GelBlot Pro Software are used to scan and quantitate 250-500 gels generated per year.

Tissue extracts and filtered media from cell lines (diluted or concentrated) are immunoprecipitated with rabbit anti-MMP(s)or anti-TIMP(s) IgG(s) using protein A-agarose suspensions. Blanks are prepared with specific IgG alone, preimmune serum with enzyme fraction, and enzyme with protein A gels but no IgG. After the reacted agarose gels are washed, the immune complexes dissolved in sample buffer and analyzed by zymography for specific enzyme activity or for TIMPs by reverse zymography or for proteins by SDS-PAGE [32]. By this method, 90-95% of the antigen present is immunoprecipitated.

as GelBase/GelBlot Pro Software, CA). The PCR procedure ia repeated at least two or three times for each sample. Prime sequences for MMPs, TIMPs and GAPDH are recorded in **Table 1**. Sets of primers used are described previously [36,37].

Table 1. Primers for RT-PCR analysis

Genes		Sequence	Product (bp)
MMP-2	Antisense	5′-GCA GCC TAG GGA GTC GGA TTT GAT G-3′	480
	Sense	5′-CCA CGT GAC AAG CCC ATG GGG CCC C-3′	
MMP-9	Antisense	5′-GTC CTC AGG GCA CTG CAG GAT GTC ATA GGT-3′	640
	Sense	5′-GGT CCC CCC ACT GCT GGC CCT TCT ACG GCC-3′	
MMP-1	Antisense	5′-TTC CAG GTA TTT CTG GAC TAA GT-3′	185
	Sense	5′-ATT GGA GCA GCA AGA GGC TGG GA-3′	
MMP-3	Antisense	5′-TTC TAG ATA TTT CTG AAC AAG G-3′	155
	Sense	5′-GCA TAG AGA CAA CAT AGA GCT-3′	
GAPDH	Antisense	5′-TGA TTT TGG AGG GAT CTC GC-3′	230
	Sense	5′-ACG CAT TTG GTC GTA TTG GG-3′	
TIMP-1	Antisense	5′-GGC TAT CTG GGA CCG CAG GGA CTG CCA GGT-3′	551
	Sense	5′-TGC ACC TGT GTC CCA CCC CAC CCA CAG AGC-3′	
TIMP-2	Antisense	5′-GGA AGC TTT TAT GGG TCC TCG ATG TCG AG-3′	590
	Sense	5′-CCG AAT TCT GCA GCT GCT CCC CGG TGC ACC CG-3′	

Standard Northern blot technique was used [33]. Briefly, total RNA (3-10 μg) is electrophoresed through agarose-formaldehyde gels, transfered onto nylon membranes Nytran, Schleicher & Schuell) by capillary electrophoresis, followed by prehybridization of the membrane, and hybridization with the appropriate radiolabeled probe [38]. 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 Matriasin), stromelysin 2 (transin-2) in pUn121 by Matriasin), 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.

6.B.RESULTS:

The third year (September 1996-September 1997) of the four year granting period has generated fair amount of data that needs to be coordinated in the coming year. The results are presented in the **Tables 1-3** and **Figures 1 - 16**. We have analyzed approximately 102 samples of biopsied human breast tissues by substrate (gelatin,transferrin and elastin) zymography. Of **102, 82** breast tissue extracts were analyzed by zymography and measured and quantitated for the production of various MMPs and TIMPs(**Table 2 and Table 3**) Approximately **500 slides** (10 slides/breast tissue) were analyzed by immunohistochemical analysis and representing over 50 well characteized breast tissue samples. Each figure shows pictures of stained sections with different anti-MMPs and -TIMPs IgGs

The analysis of characterized breast tissues consisted of normal, benign tumors, lobular (in situ and infiltrating) carcinoma infiltrating ductal carcinomas (grade II and II) in situ ductal carcinoma, papillary carcinoma, medullary carcinoma and colloidal carcinoma.

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

The results in **Tables 2 and 3** categorically show that there is an **imbalance** in which the destructive proteases (**MMPs**) greatly outweigh the controlling inhibitors (**TIMPs**) facilitating the spread of cancer.

The tissues were extracted with 0.1%Triton X-100 followed by 0.5 and 1 M guanidine hydrochloride and 0.1% SDS solutions containing standard Tris buffer pH 7.4. The details of extraction, profiles and quantitation by imager have been reported in the 1995 and 1996 annual reports. The amount of MMPs and TIMPs present in the breast tissue extracts were

TABLE 2. Quantitation of Matrix Matalloproteinases--Gelatinases (A[@]+B[#]) and Collagenase (MMP-1, or -13)[^] in Breast Tissues.

Tissue	No.	MMP-2[@] + MMP-9[#] μg[*]	MMP-9 L/A	MMP-2 L/A	MMP-9 μg[*]	MMP-1[^] μg[*]	Total Enzyme μg
Normal	6	0.62 ± 0.02	N/A	7.8	N/A	0.05 ± 0.01	1.0
Benign Tumor	11	1.58 ± 0.01	N/A	3.58	N/A	0.68	1.63
Benign Tumor Tubular	3	3.41 ± 0.5	N/A	1.6	N/A	0.24 ±0.02	3.55
Adeno-carcinoma GRIII	32	5.1 ±0.06	1.9	1.5	2.3 ±0.04	0.66 ±0.01	5.76
GRII	20	2.1 ±0.09	2.01	2.55	0.53 ± 0.02	0.62 ±0.11	2.72
Lobular carcinoma	6	2.2 ± 0.04	0.71	2.05 -	0.66 ±0.01	0.7 ±0.6	2.95
Colloidal carcinoma	3	1.5 ±0.03	1.1	1.9	--	---	--
In Situ ductal carcinoma	3	0.97 ±0.03	0.84	4.18	0.23 ±0.01	0.04 ±0.01	1.01

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 μg/g wet weight breast tissue. -- units of enzyme activity converted to μg.
Values are given as mean ± SEM(standard error of the mean).
L/A = Latent enzyme/Active enzyme ratio
[%] Amount of MMP-9 enzyme from total gelatinase
Adenocarcinoma = Infiltrating Ductal Carcinoma - Nuclear Grade III or Nuclear Grade II
^ Interstitial collagenase - MMP-1 or MMP-13.

calculated as total units or μg of enzyme/g wet weight tissue for MMPs or μg inhibitor/g wet weight tissue for TIMPs.

The results are presented in **Table 2.** showing the quantitation of gelatinases (MMP-9 and MMP-2) by zymography and enzyme substrate (tritiated gelatin) assays. MMP-1 (interstitial collagenase) or MMP-13 (collagenase -3) were quantitated using tritiated rat soluble collagen. MMP-1, MMP-13 and MMP-8 (neutrophil collgenase) digest the collagen in the assay system used [26]. MMP-1 in many cases cannot be detected by gelatin zymography due to poor digestion of gelatin by MMP-1. The ratios of latent to active MMP-9 and MMP-2 were obtained from the zymographical analysis Stromelysin-1 or MMP-7 (matrilysin, PUMP-1) were not detected in any of the breast tissue extracts. No bands were obtained in transferrin zymography. We have not included the zymographical profiles of the breast tissues analyzed in the last year. Most profiles for each category of breast tissues were similar to those presented in the previous reports. No attempt was made to quantitate stromelysin-3 last year. Now that we have successfully mastered the RT-PCR technique, we plan to develop specific primers and quantitate stromelysin-3 and MT1-MMP.

Biochemical quantification of MMPs showed an overall increase in all types of MMPs in cancer tissues (**Table 2**). MMP-9 was the key MMP, it was present at levels 11.5.units (0.23 μg)/g wet weight tissue in *in situ* iductal to 2.3 μg (27 units)/g wet weight tissue in infiltrating ductal carcinoma (grade III). Zymography also shows a fraction of MMP-9 and MMP-2 in their active forms. There is a corelation of constitutively expressed MMP-1 (0.04 - 0.66 μg/g wet weight tissue) in cancer tissues. Constitutively expressed MMP-2 also increases.

We encountered 2-3 tissues characterized as benign having MMP-9 in quantifiable amounts however, on close examination of tissues by histology (H&E stain) showed questionable infiltration of other cell types. It was difficult to find in literature, the analysis of benign breast tissues by zymography. The levels of MMP-9 are highest in infiltrating and poorly differentiated ductal carcinoma (Grade III) breast tissue extracts than any of the other carcinomas . Lobular carcinoma also showed high levels of MMP-9. Latent to active enzyme ratio was below one (**0.71**) in lobular and in situ ductal carcinoma breast tissue extracts. Results in **Table 2** clearly show that MMP-9, 92 kDa type IV collagenase or gelatinase B is found in varying amounts in cancer tissues when compared with normal and benign breast tissues.

It is possible, we may need to process more tissues in the fourth year to fill in the gaps for statistical evaluations of certain type of breast cancer. Considerable portion of the third year was spent in establishing molecular biology and immunohistostaining techniques in my laboratory.

MT1-MMP (membrane type metalloproteinase) was not quantifiable by zymography. Confocal analysis using anti-MT1-MMP) IgGs (kindly provided

Table 3. Amounts of Tissue Inhibitors of Metalloproteinases (TIMPs -1, -2) in dialyzed Triton X 100 & 1M GuHCl extracts of Breast Tissues.

Tissue	No.	TIMP-1	TIMP-2
Normal	5	2.6μg	1..9μg
Benign	6	1.7μg	1.8μg
Adeno-carcinoma	28	0.1μg	1.55μg

The values in μg for TIMP-1 and TIMP-2 are obtained from breast tissue extracts analyzed by TIMP-1 (Cat # QIA31) & TIMP-2 (CAT#QIA40) kits (BIOTRAK) purchased from Calbiochem (Oncogene Research Products)

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

These values reported may not be accurate. They differ from values calculated from reverse zymography.

by Dr. G. Goldberg, Washington university, St. Louis), showed that MT1-MMP was seen only in breast cancer tissues. This evaluation was reported last year (Progress Report 1996).

The breast tissue extracts were scanned for **TIMPs** by reverse zymography. **TIMP-1, -2 & -3** are detected in ng quantities. The tissue extracts are fractionated by SDS-PAGE (12,5% acrylamide, gelatin and enzyme media) 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 incubation. Conditioned cell media can be successfully quantitated for TIMPs. To refine the method of reverse zymography is an ongoing priority in the next few months. We still beleive that refining the conditions of reduction and alkylation that destroys the TIMPs may help in evaluating the absolute amounts of TIMPs present in the breast tissues.

We next proceeded to quantitate the dialyzed breast tissue extracts by ELISA systems for TIMP-1 and TIMP-2 purchased from Oncogene Research Products (Calbiochem, Cambridge, MA). This procedure was not satisfactory to quantitate absolute amounts of TIMPs present in the tissue. Solutions containing TIMPs standards showed a good corelation upon dilution of the solutions. However, this was not the case with tissue extracts. Dilutions of breast extract solutions did not match with undiluted dialyzed breast tissue extracts. So we analyzed some of the tissue extracts (undiluted) by ELISA and results are reported in **Table 3**. This Table shows (although may be inaccurate in absolute amounts) however, a trend of lower amounts of TIMP-1 and TIMP-2 (µg/g wet weight tissue) found in 28 cancer tissues compared to the higher amount found in normal (5 samples) and benign (6 samples) tissue.

The reults shown in **Tables 2 and 3** clearly indicate that the amounts of MMPs present in cancer breast tissues (6µg, Grade III cancer) has available only 2.5 -3.5µg of TIMPs for arresting the destruction of tissue by MMPs. There are other MMPs and TIMPs present in the tissue and their quantitation has not been accounted in this study..

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

Immunohistochemical analyses were established in my laboratory. The main reason being that Dr. Nadji's laboratory could not titrate the anti-MMP and anti-TIMP IgGs to obtain best staining of tissues. The time constraint and a workload of a diagnostic laboratory prevented the tissue section staining for research purposes in his laboratory. Over 500 slides comprising and representing over 50 human breast tissues were carried out in my laboratory. We titrated the IgGs for maximum staining

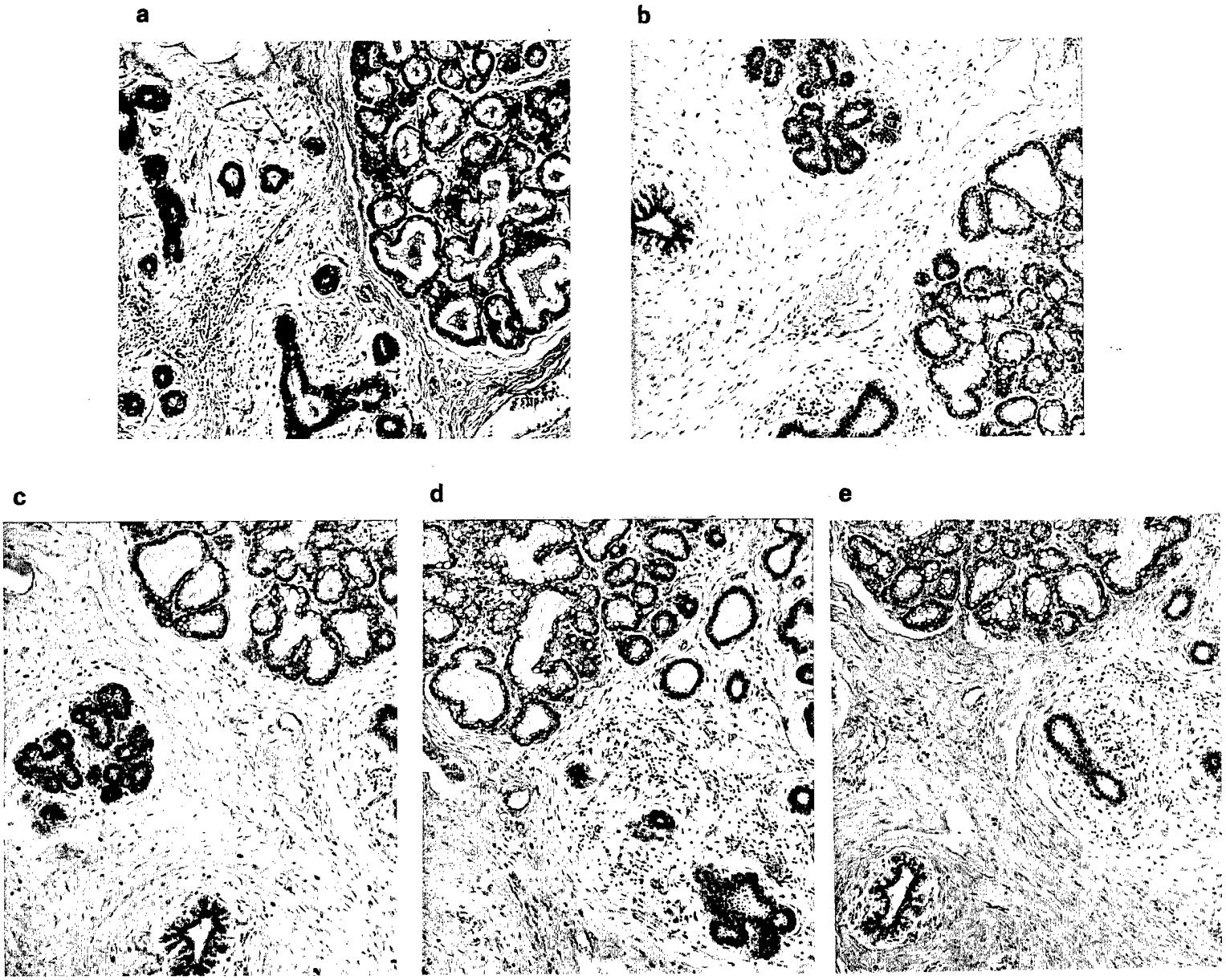


Figure. 1. Immunohistochemical staining [x 10] of human breast tissue (**normal breast tissue**) using immunoperoxidase reagents revealing location of the antigen as red deposit with AEC substrate-chromogen. **a: H & E stain. b: no primary IgGs. c: anti-MMP-9** showing no staining. **d: Anti-MMP-2** showing 2^+ staining of epithelium and stroma. **e: anti- MMP-1** showing 2^+ staining of both epithelium and stroma.

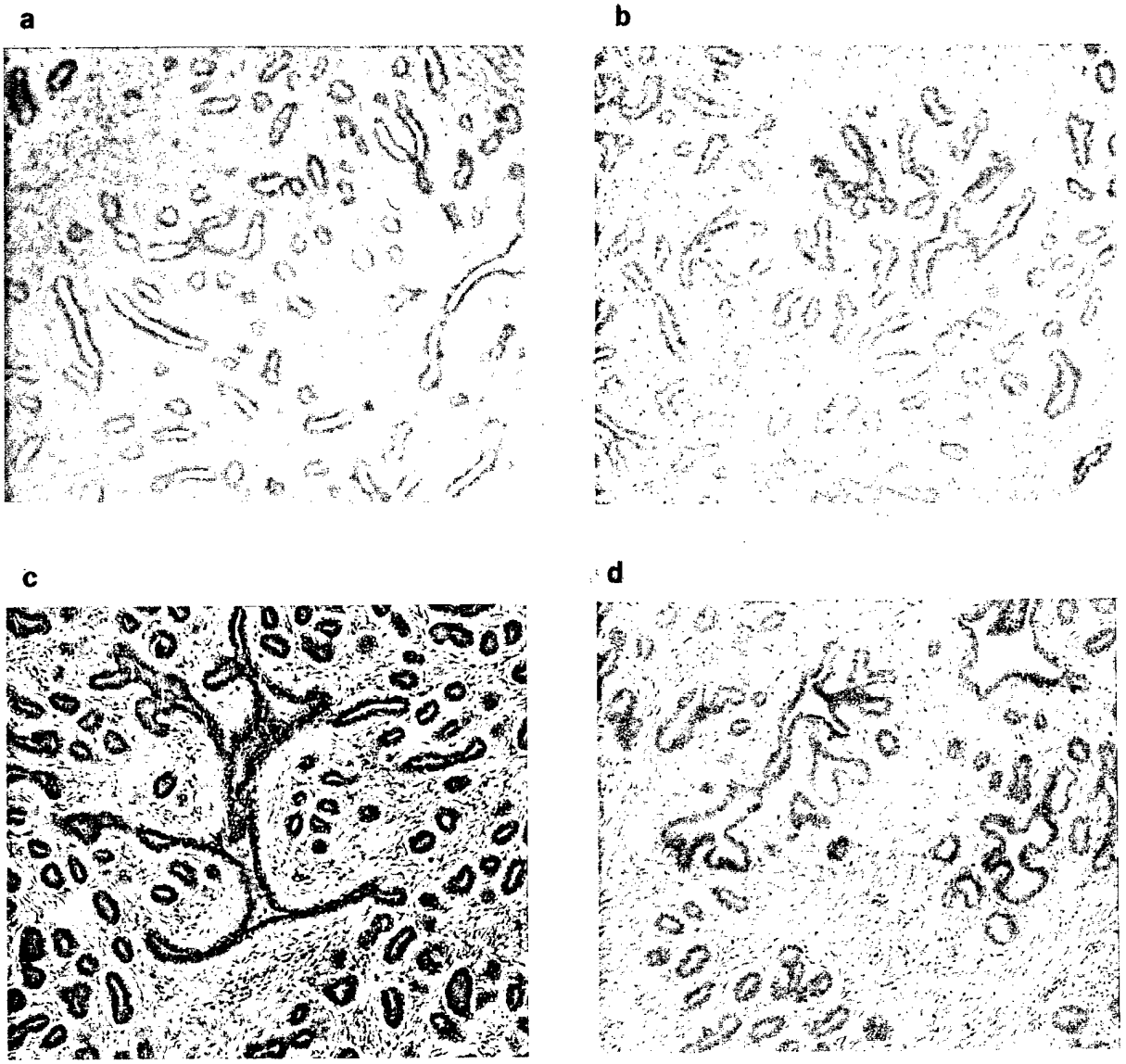


Figure. 2. Immunohistochemical staining [x 10] of human breast tissue (**benign tumor, fibroadenoma**) using immunoperoxidase reagents revealing location of the antigen as red deposit with AEC substrate-chromogen. **.a: H & E** staining. **b: no primary** IgGs **c: anti-MMP-9** showing very weak staining of epithelium and stroma. **d: anti-MMP-1** showing 1^{+} staining of stroma and weak staining of epithelium.

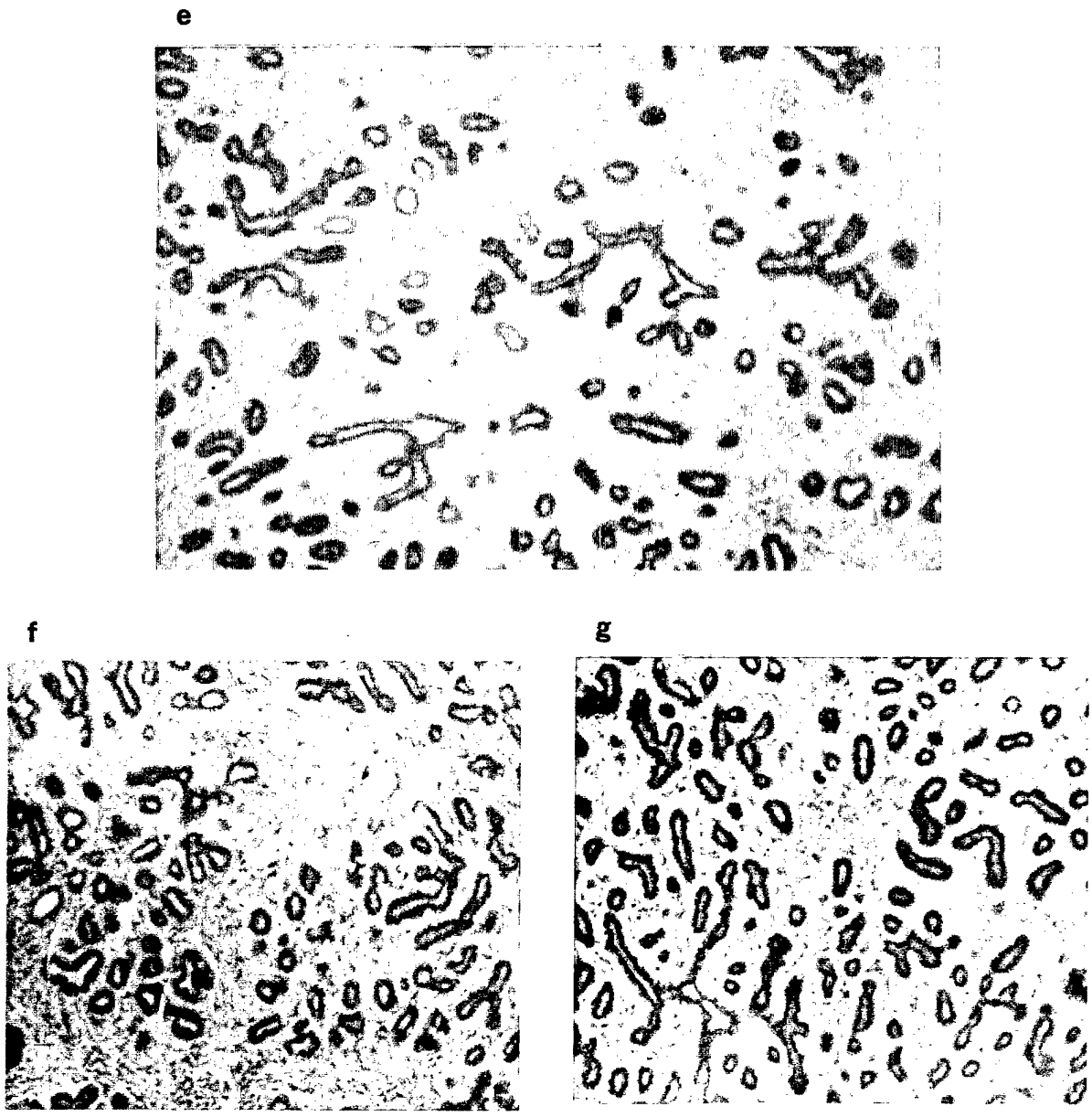


Figure. 3. Immunohistochemical staining [x 10] of human breast tissue (**benign tumor, fibroadenoma**) using immunoperoxidase reagents revealing location of the antigen as red deposit with AEC substrate-chromogen. **e: anti-MMP-2** showing 3^+ staining of epithelium and weak staining of stroma. **f: anti-TIMP-1** showing 1^+ staining of stroma only. **g: anti-TIMP-2** showing 1^+ staining of both epithelium and stroma.

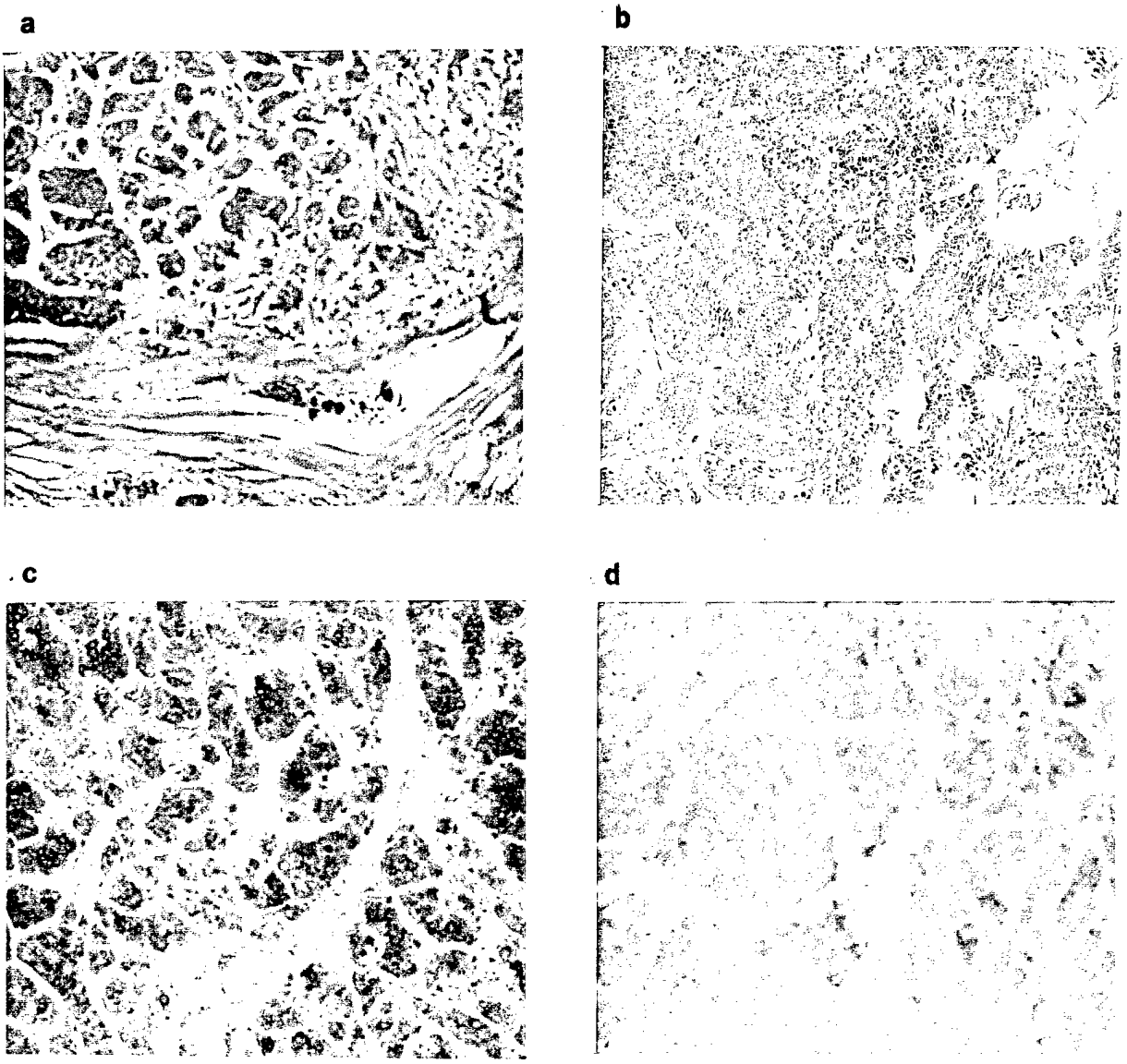
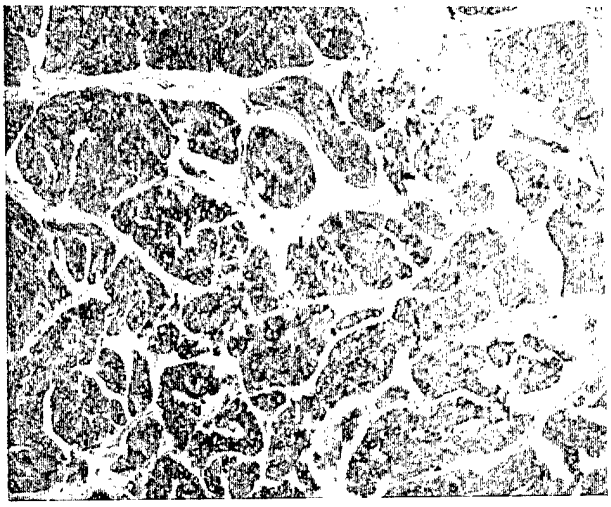


Figure. 4. Immunohistochemical staining [x 10] of human breast tissue (**infiltrating and poorly differentiated ductal [IDC] carcinoma**) using immunoperoxidase reagents revealing location of the antigen as red deposit with AEC substrate-chromogen. **a: H & E stain. b: no primary IgGs. c: anti-MMP-1** 3^+ staining of tumor cells only. **d: anti-TIMP-1** showing 1^+ staining of stroma and some staining of tumor cells.

e



f

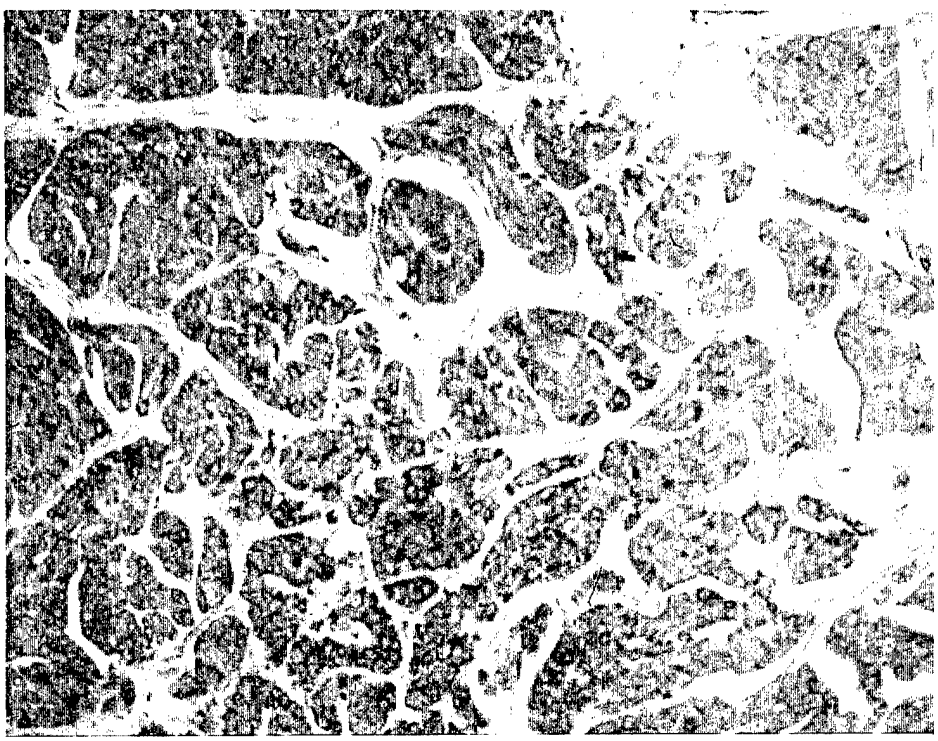


Figure. 5. Immunohistochemical staining of human breast tissue (**infiltrating and poorly differentiated ductal [IDC] carcinoma**) using immunoperoxidase reagents revealing location of the antigen as red deposit with AEC substrate-chromogen. **e: anti MMP-9 [x 10]. f: anti-MMP-9 [x 40]** showing 3^+ staining of tumor cells only.

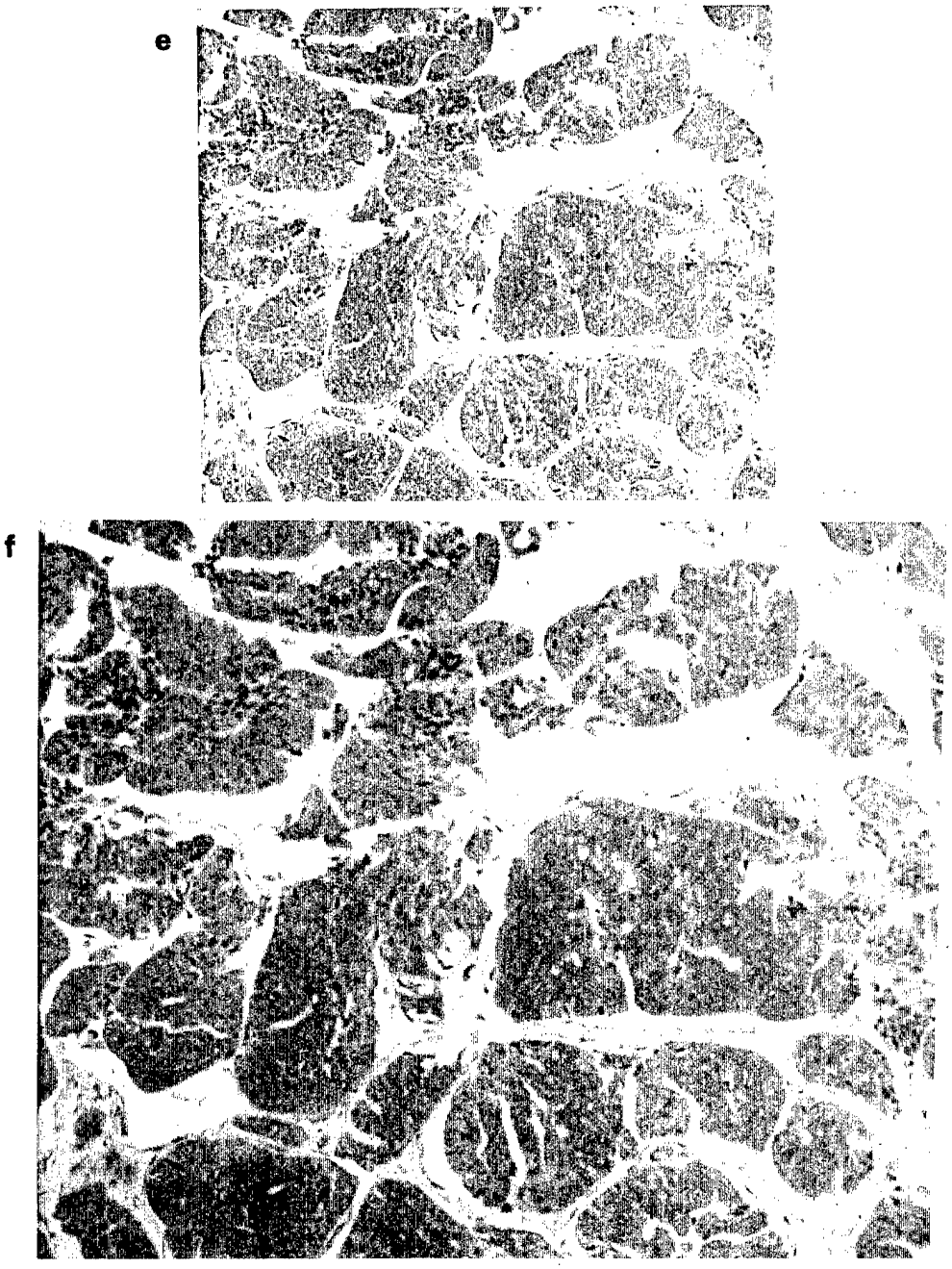


Figure. 6. Immunohistochemical staining of human breast tissue (**infiltrating and poorly differentiated ductal [IDC] carcinoma**) using immunoperoxidase reagents revealing location of the antigen as red deposit with AEC substrate-chromogen. **e: anti MMP-2 [x 10]. f: anti-MMP-2 [x 40]** showing 3^+ staining ofarea around tumor cells only.

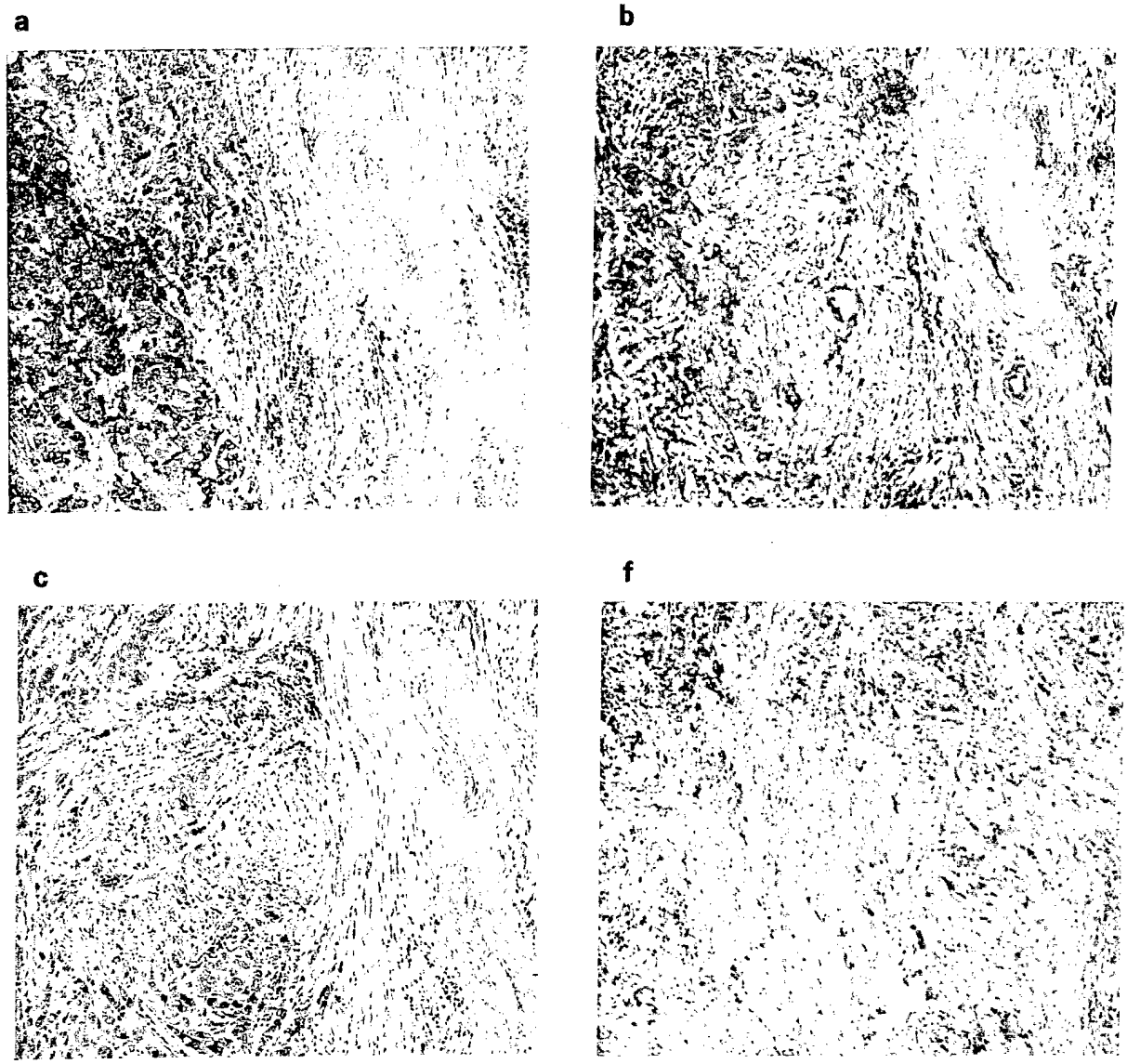


Figure. 7. Immunohistochemical staining [x 10] of human breast tissue (**infiltrating ductal carcinoma, Grade II**) using immunoperoxidase reagents revealing location of the antigen as red deposit with AEC substrate-chromogen. **a: anti-MMP-9** showing 3^+ staining of tumor cells only. **b: anti-MMP-1** showing 3^+ staining of stroma only. **c: anti MMP-2** showing 1^+ staining of tumor cells and surrounding stroma. **f: anti-TIMP-1** showing weak staining of stroma.

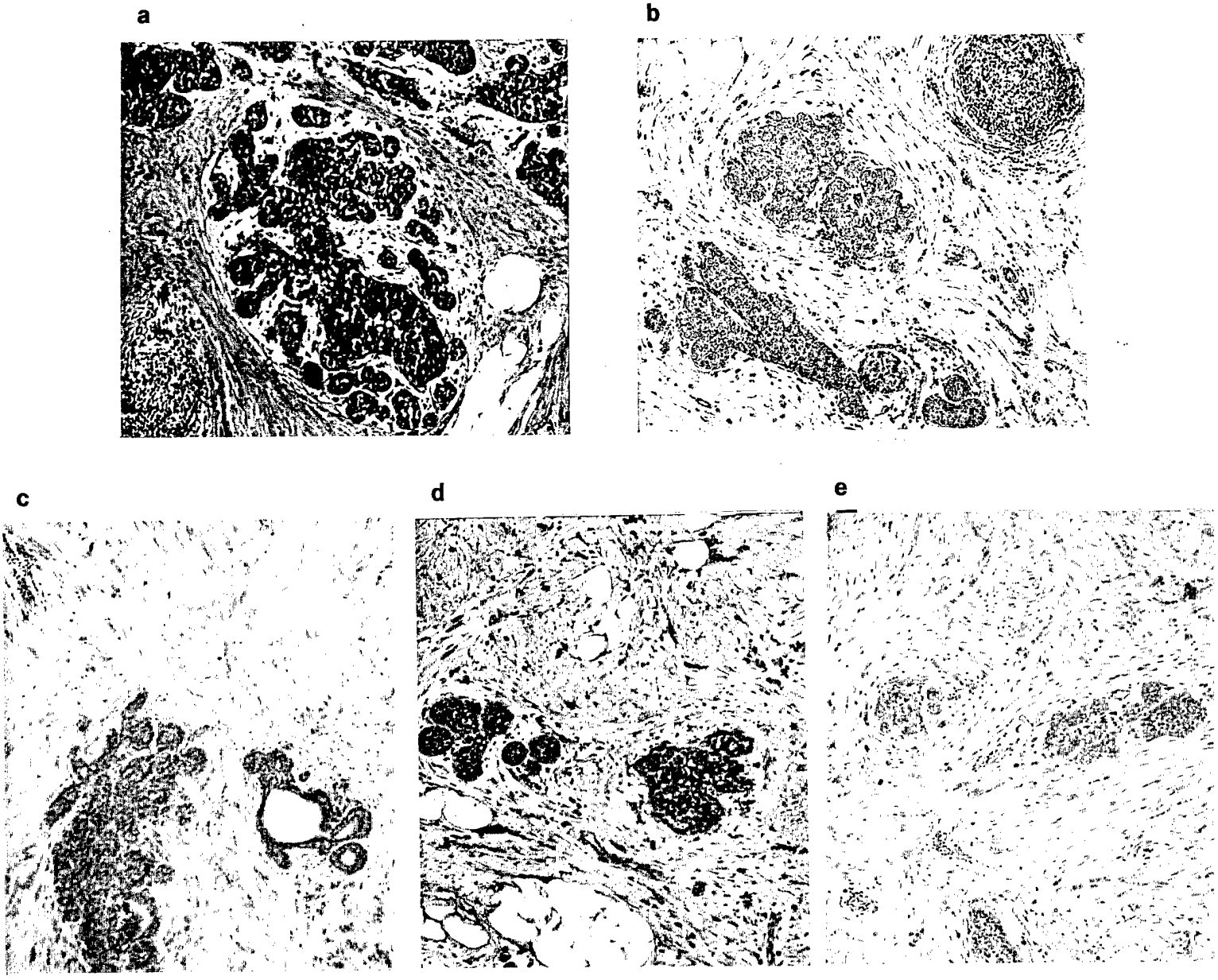


Figure. 8. Immunohistochemical staining [x 10] of human breast tissue (**lobular [in situ] carcinoma**) using immunoperoxidase reagents revealing location of the antigen as red deposit with AEC substrate-chromogen. **a: H & E stain. b: anti-MMP-9** showing 3^+ staining equally of epithelium and stroma. **c: anti MMP-2** showing 3^+ staining of tumor cells and surrounding stroma. **d: anti-MMP-1** showing 4^+ staining equally of epithelium and stroma. **e: Anti-TIMP-1** showing 1^+ staining of epithelium and stroma.

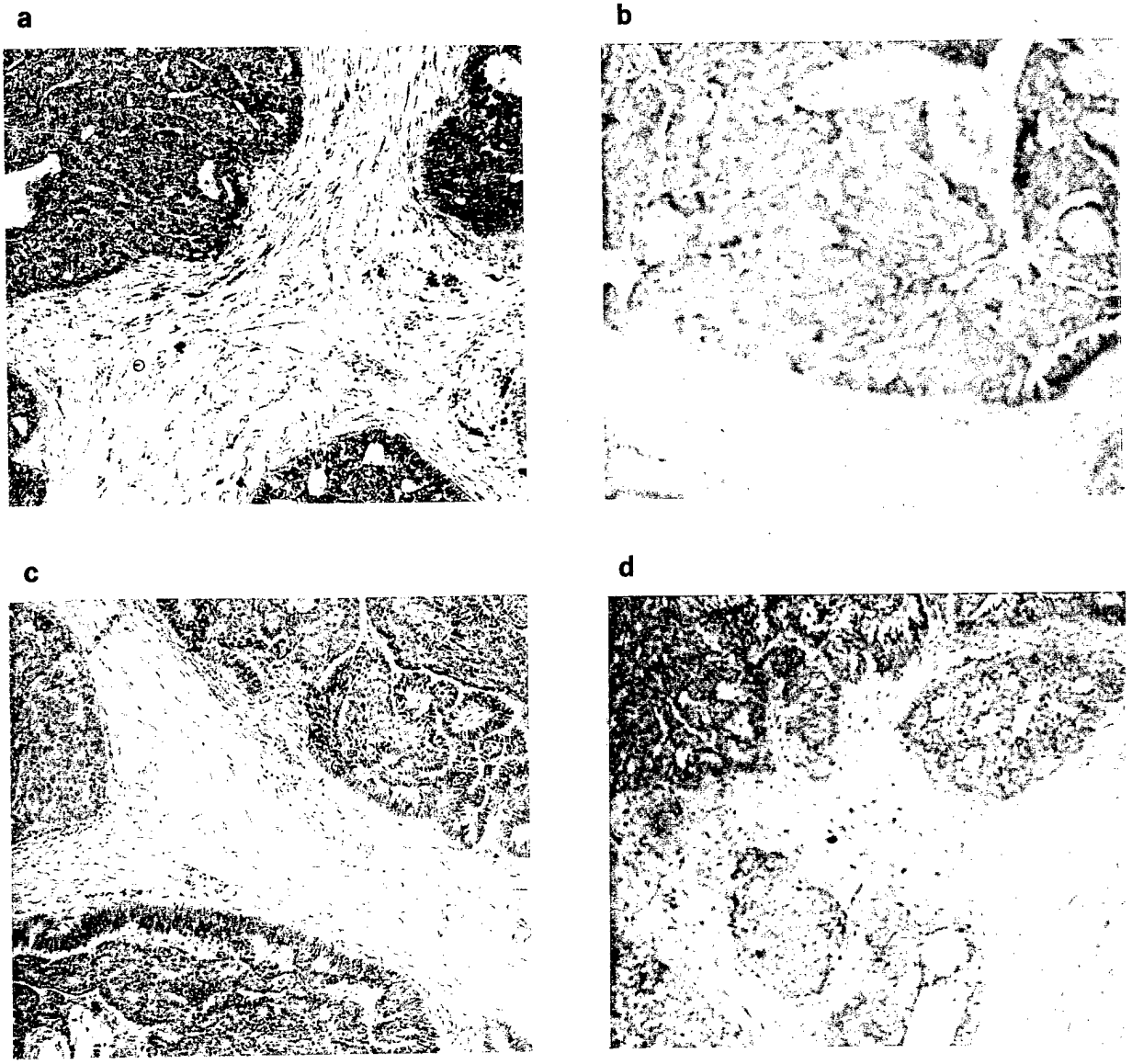


Figure. 9. Immunohistochemical staining [x 10] of human breast tissue (**papillary carcinoma**) using immunoperoxidase reagents revealing location of the antigen as red deposit with AEC substrate-chromogen. **a: anti-MMP-1** showing 4^+ staining equally of epithelium and stroma. **b: anti MMP-9** showing weak staining of tumor cells. **c: anti-MMP-2** showing 1^+ staining equally of epithelium and stroma. **d: Anti-TIMP-1** showing no staining.

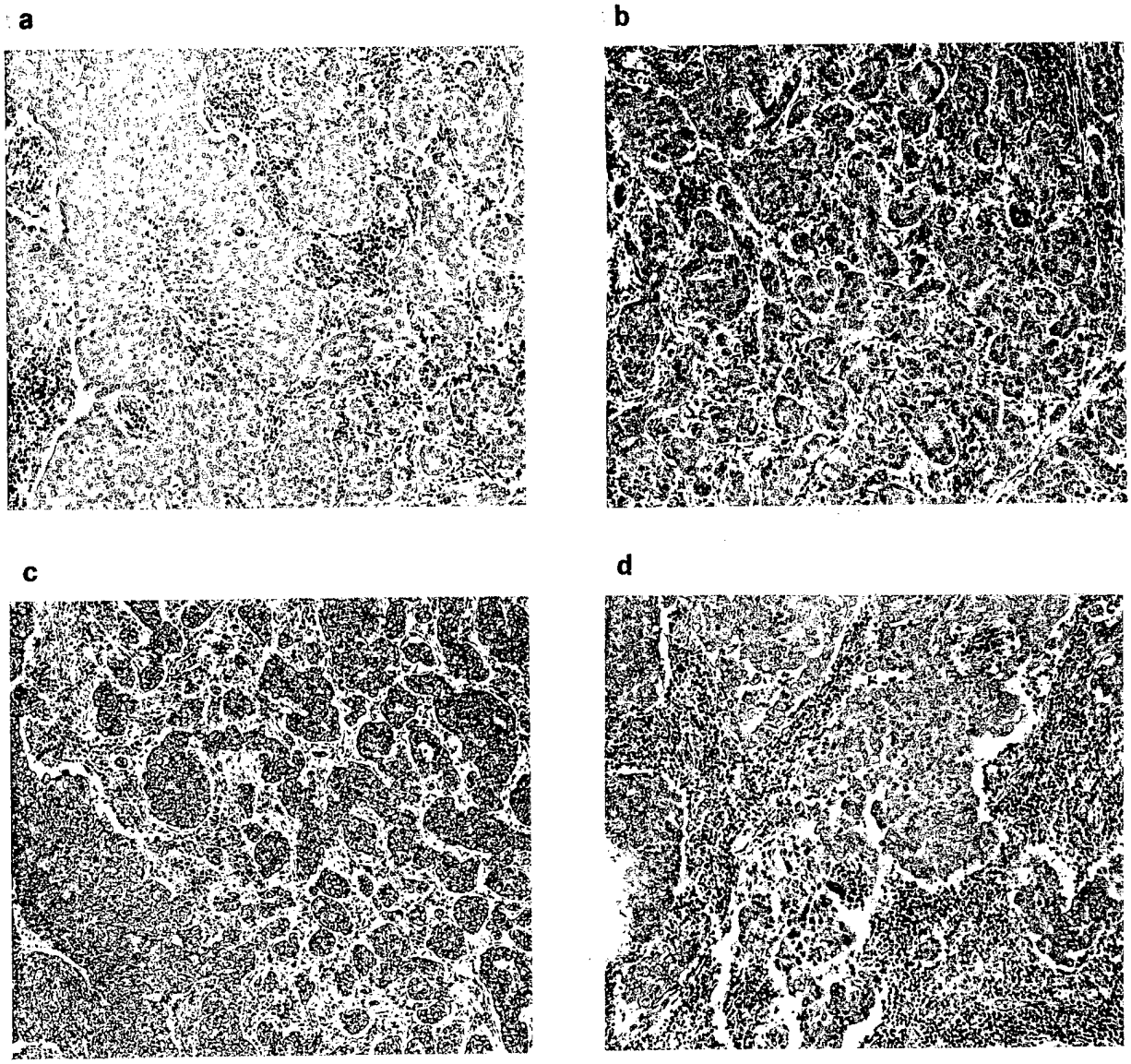


Figure. 10. Immunohistochemical staining [x 10] of human breast tissue (**medullary carcinoma**) using immunoperoxidase reagents revealing location of the antigen as red deposit with AEC substrate-chromogen. **a: anti-TIMP-1** showing weak staining equally of tumor cells.and stroma **b: anti MMP-2** showing 2^+ staining of tumor cells only. **c: anti-MMP-9** showing 4^+ staining of tumor cells only. **d: Anti-MMP-1** showing 3^+ staining in tumor cells and less staining in stroma.

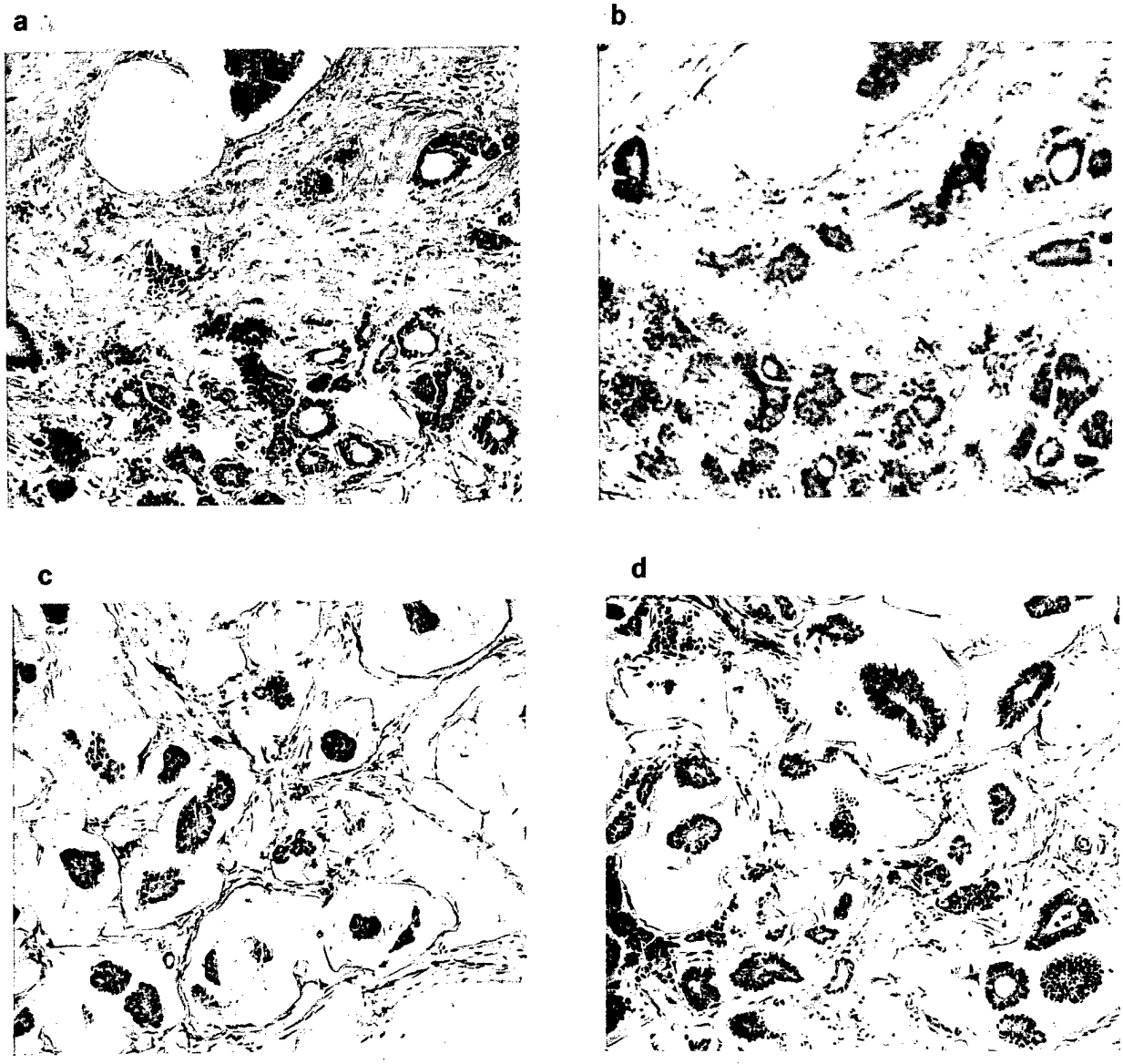


Figure. 11. Immunohistochemical staining [x 10] of human breast tissue (**colloidal carcinoma**) using immunoperoxidase reagents revealing location of the antigen as red deposit with AEC substrate-chromogen. **a: anti-MMP-1** showing 3^+ staining equally of tumor cells and stroma. **b: anti MMP-9** showing 3^+ staining equally of tumor cells and stroma. **c: anti-MMP-2** showing 1^+ staining of tumor cells only. **d: Anti-MMP-1** showing negative staining of tissue section.

for almost all the MMPs and TIMPs. The results are presented in **Figures 1 - 11.**

The tissue sections (4μ) were prepared from the paraffin blocks adjacent to the tissue block used for biochemical and molecular biological analyses. Most of the tissue sections were stained with purified IgGs; 1) Hematoxylin-eosin stain, 2) anti-MMP-2, 3) anti-MMP-9, 4)anti-MMP-1, 5) anti-MMP-7, 6) anti-stromelysin, 7) anti-TIMP-1, 8) anti-TIMP-2, 9) anti-TIMP-3, 10) ani-MT1-MMP, 11) no primary IgGs. The kit Histostain™ SP (CAT. # 95-9743) using AEC (aminoethyl carbazole, red signal instead of DAB, brown signal) was purchased from Zymed Laboratories Inc. San Francisco, CA. The kit uses HRP (horse radish peroxidase) labeled with Streptavidin-Biotin Amplification method. The protocol was followed according to the Manufacturer's instructions and reviewed under the microscope. The red signal is notable specifically around the epithelial cells or tumor cells or fibroblasts and the stroma.

Each tissue section is photographed for a slide using the microscope available in the Pathology Department, University of Miami. The slides are carefully evaluated with Dr. Scott Sittler, Assistant Professor, Department of Pathology, University of Miami Fl and recorded using grading system of 1-4 with + sign against each number as 1^+, 2^+ to 4. The terminology of negative or positive to IgGs are also used besides the grading system. Immunohistochemical staining of tissue sections was time consuming but a worth while effort to evaluate the tissue samples. The results are reported in **Figures. 1 - 11.** As reported in Progress Report 1995-1996, all IgGs used in this study were monospecific and evaluated priodically by western blots.

Eleven types of breast tissues have been stained. All figures are self explanatory. **Figure 1** shows the staining of normal breast tissue. There is no staining with anti-MMP-9 and shows staining only with MMP-2 and MMP-1. **Figures 2 and 3** show staining of benign, fibroadenoma breast tissue sections. There is virtually no staining with anti-MMP-9 IgGs, but stains heavily with anti-MMP-2. **Figure 4, 5 and 6** show infiltrating and poorly differentiated ductal carcinoma, Grade III (IDC) that stains heavily with anti-MMP-2,-MMP-9, -MMP-1 and yet show only weak staining with anti-TIMP-1. This suggests an imbalance of MMPs and TIMPs in IDC. Medullary, in situ lobular stain highly for enzymes overall and has weak staining for TIMP-1.Papillary carcinoma sections did not stain for anti-MMP-9. These results show that our anti-MMP and -TIMP IgGs are monospecific.

6.B.3. Reverse transcription polymerase chain reaction (RT-PCR) analysis. Detection of low-abundance mRNAs by RT-PCR has now become a standard technique to determine gene expression by tissues. To determine relative or absolute copy numbers of specific mRNAs is difficult without internal standards as control for sample to sample variation. In these set of experiments, we determined by RT-PCR analysis to determine the expression of MMPs and TIMPs in breast tissue samples. Northern blot

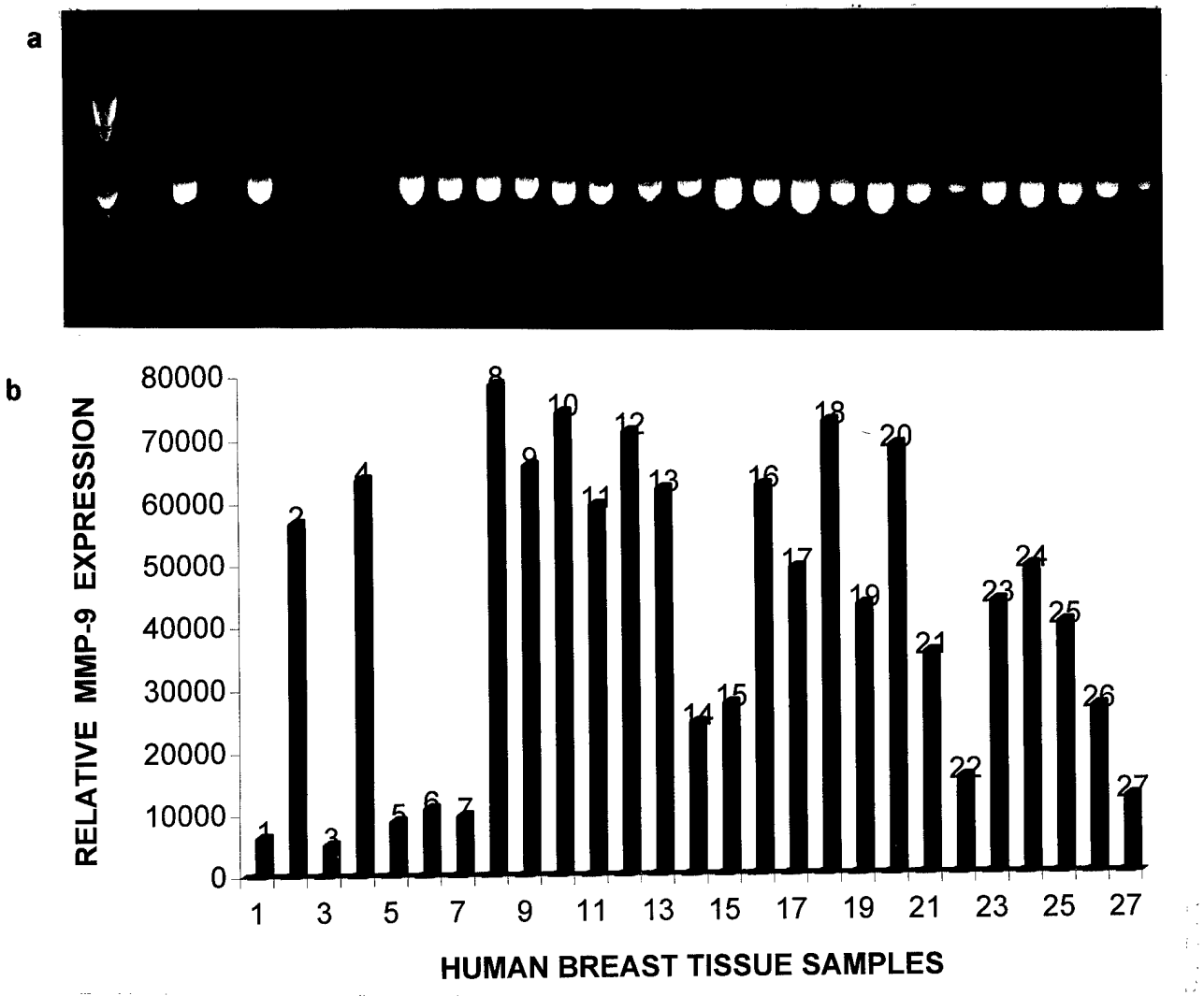


Figure. 12. (**a**) Expression of MMP-9 (640 bp fragment), a househeeping gene (internal standard) as determined by PCR. CDNA was prepared from breast tissues and RT-PCR was performed using the specific primer pairs. The amplified products were separated on 1.5% agarose gels and stained with ethidium bromide. DNA markers; **lane 1**, normal breast tissue; **lanes 3, 5 , 6, 7**, benign breast tissues; **lanes 14, 20, 21, 23, 27**, infiltrating ductal carcinoma, Grade II; **lanes 2, 4, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18**, infiltrating and poorly differentiated ductal carcinoma, Grade III; **lanes, 19, 22, 24, 25, 26**, lobular carcinoma. (**b**) Semi-quantitative analysis of MMP-9 in breast tissues. The MMP-9 bands were scanned by an imager and analyzed by GELBASE/GELBLOT PRO software (UVP Products, Upland CA). Units of peak area are arbitary.

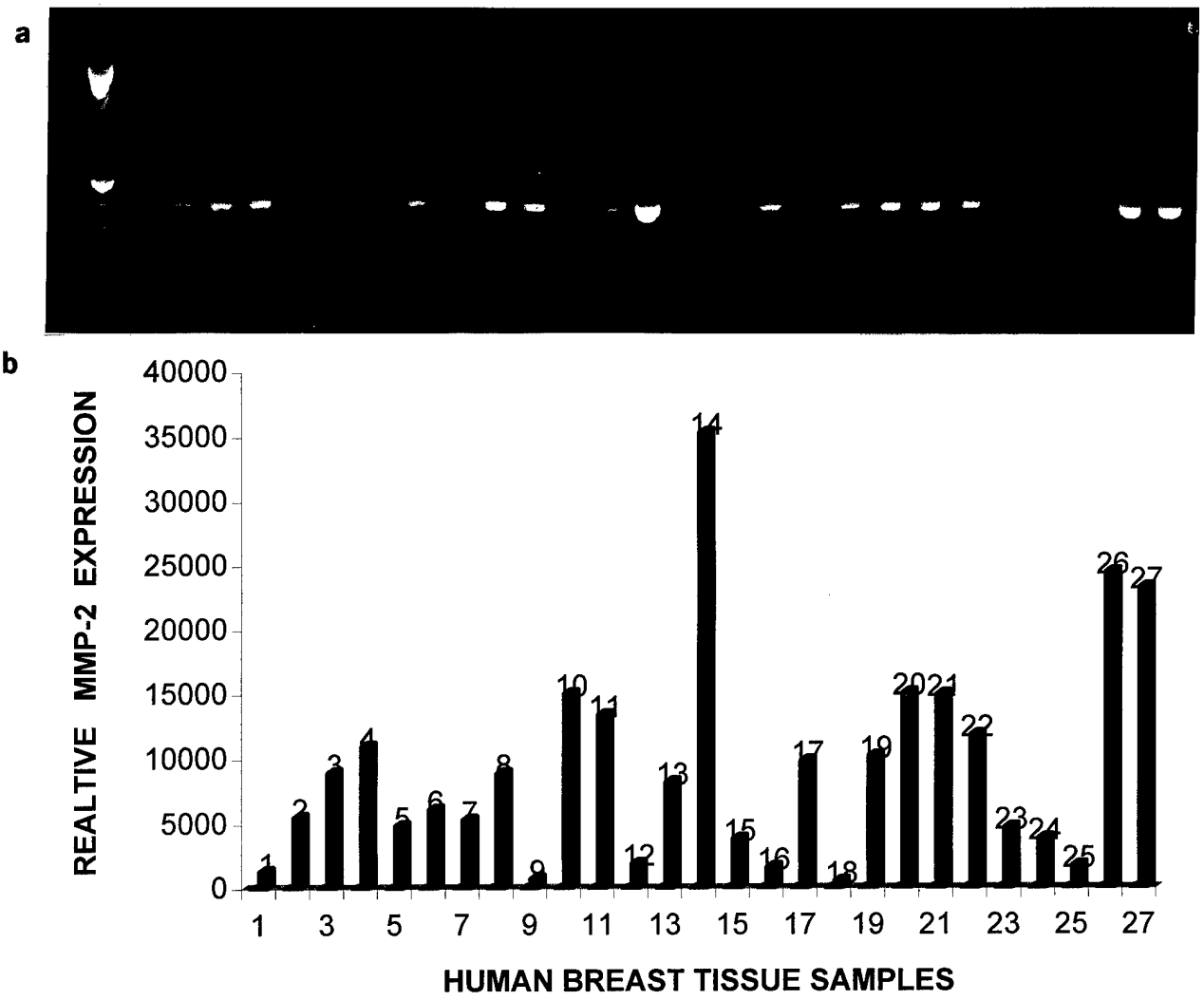


Figure. 13. (**a**) Expression of MMP-2 (480 bp fragment), a househeeping gene (internal standard) as determined by PCR. CDNA was prepared from breast tissues and RT-PCR was performed using the specific primer pairs. The amplified products were separated on 1.5% agarose gels and stained with ethidium bromide. DNA markers; **lane 1**, normal breast tissue; **lanes 3, 5 , 6, 7**, benign breast tissues; **lanes 14, 20, 21, 23, 27**, infiltrating ductal carcinoma, Grade II; **lanes 2, 4, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18**, infiltrating and poorly differentiated ductal carcinoma, Grade III; **lanes, 19, 22, 24, 25, 26**, lobular carcinoma. (**b**) Semi-quantitative analysis of MMP-9 in breast tissues. The MMP-9 bands were scanned by an imager and analyzed by GELBASE/GELBLOT PRO software (UVP Products, Upland CA). Units of peak area are arbitary.

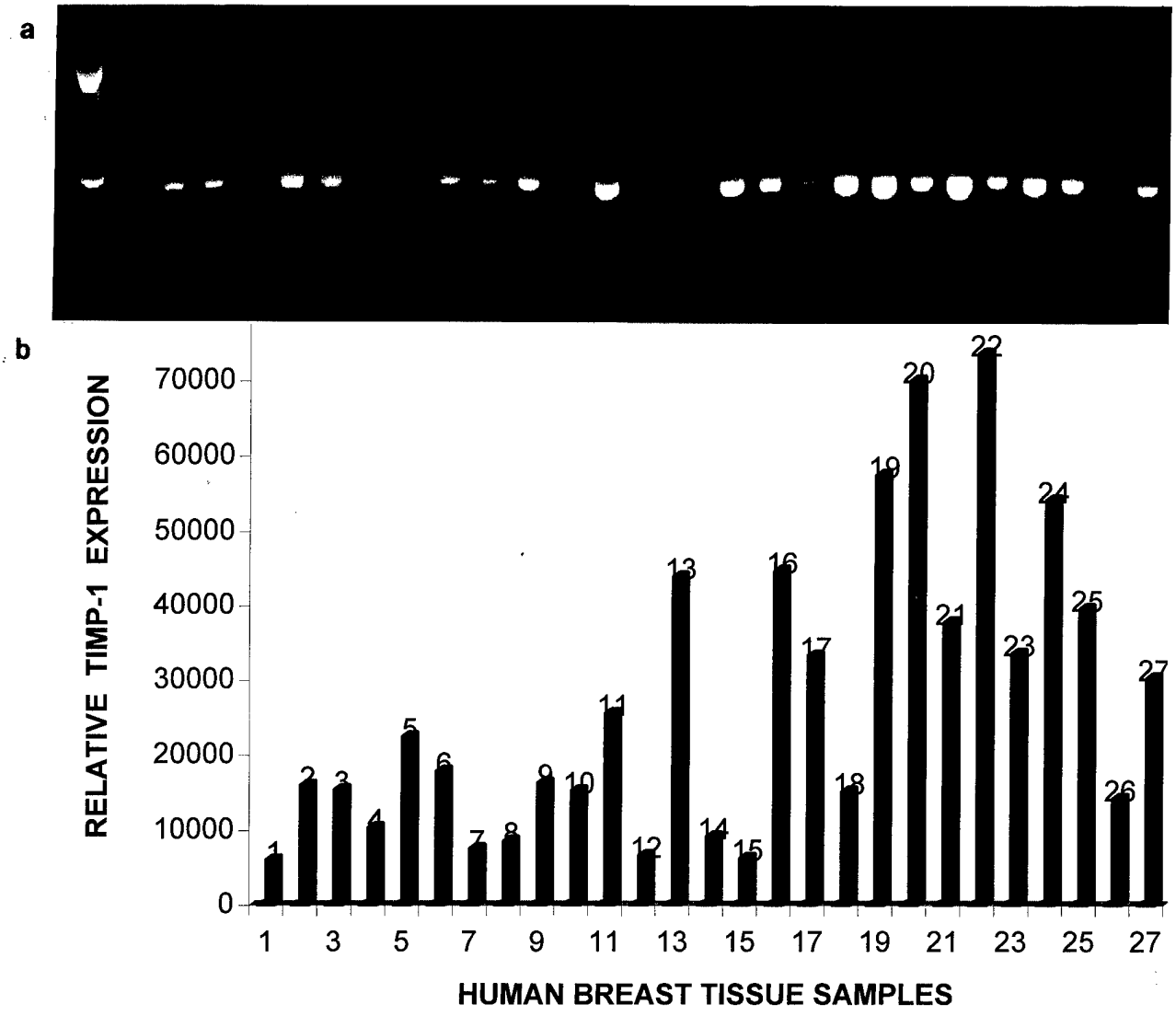


Figure. 14. (**a**) Expression of TIMP-1 (551 bp fragment), a househeeping gene (internal standard) as determined by PCR. CDNA was prepared from breast tissues and RT-PCR was performed using the specific primer pairs. The amplified products were separated on 1.5% agarose gels and stained with ethidium bromide. DNA markers; **lane 1**, normal breast tissue; **lanes 3, 5 , 6, 7**, benign breast tissues; **lanes 14, 20, 21, 23, 27**, infiltrating ductal carcinoma, Grade II; **lanes 2, 4, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18**, infiltrating and poorly differentiated ductal carcinoma, Grade III; **lanes, 19, 22, 24, 25, 26**, lobular carcinoma. (**b**) Semi-quantitative analysis of MMP-9 in breast tissues. The MMP-9 bands were scanned by an imager and analyzed by GELBASE/GELBLOT PRO software (UVP Products, Upland CA). Units of peak area are arbitary.

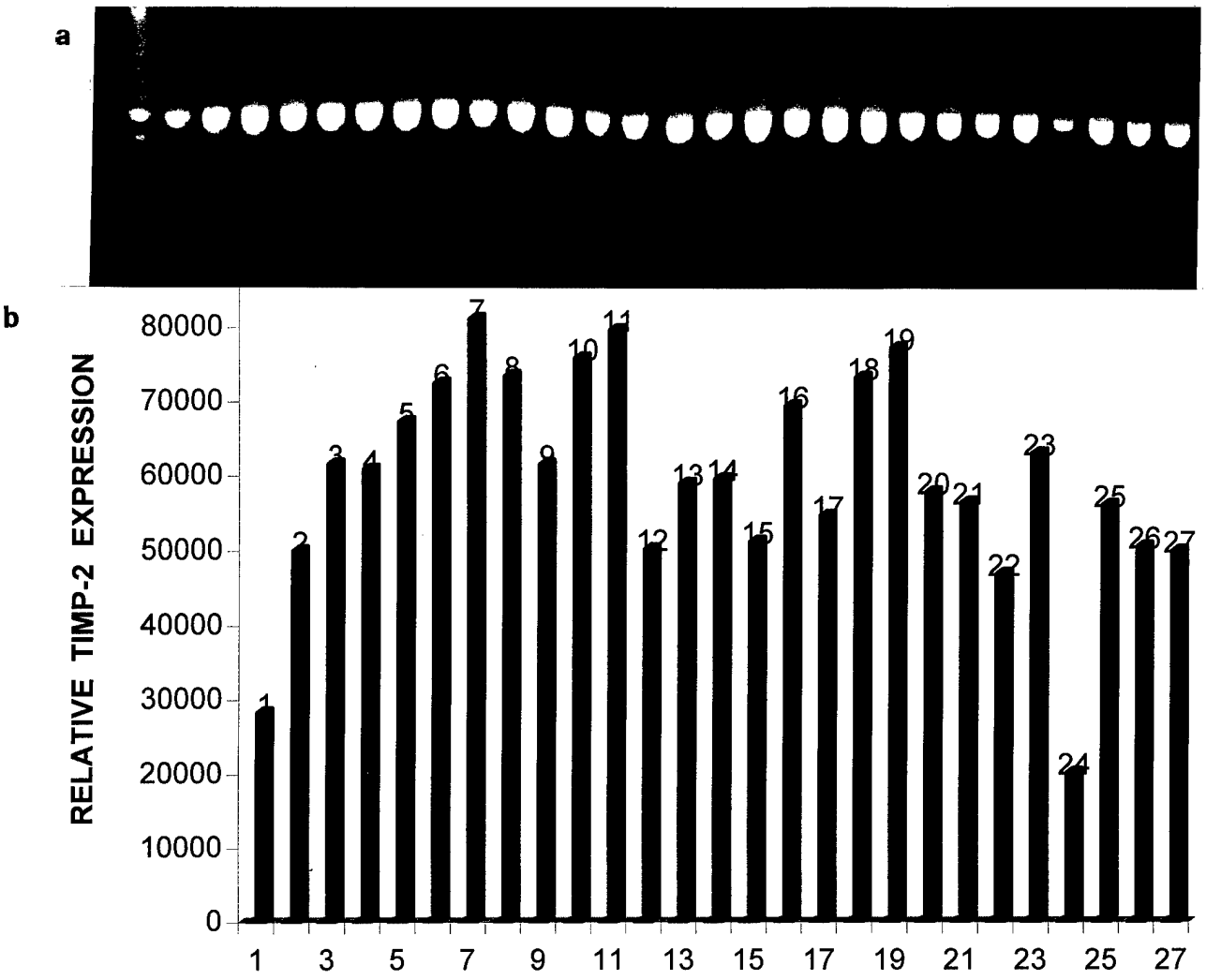


Figure. 15. (**a**) Expression of TIMP-2 (590 bp fragment), a housekeeping gene (internal standard) as determined by PCR. CDNA was prepared from breast tissues and RT-PCR was performed using the specific primer pairs. The amplified products were separated on 1.5% agarose gels and stained with ethidium bromide. DNA markers; **lane 1**, normal breast tissue; **lanes 3, 5 , 6, 7**, benign breast tissues; **lanes 14, 20, 21, 23, 27**, infiltrating ductal carcinoma, Grade II; **lanes 2, 4, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18**, infiltrating and poorly differentiated ductal carcinoma, Grade III; **lanes, 19, 22, 24, 25, 26**, lobular carcinoma. (**b**) Semi-quantitative analysis of MMP-9 in breast tissues. The MMP-9 bands were scanned by an imager and analyzed by GELBASE/GELBLOT PRO software (UVP Products, Upland CA). Units of peak area are arbitary.

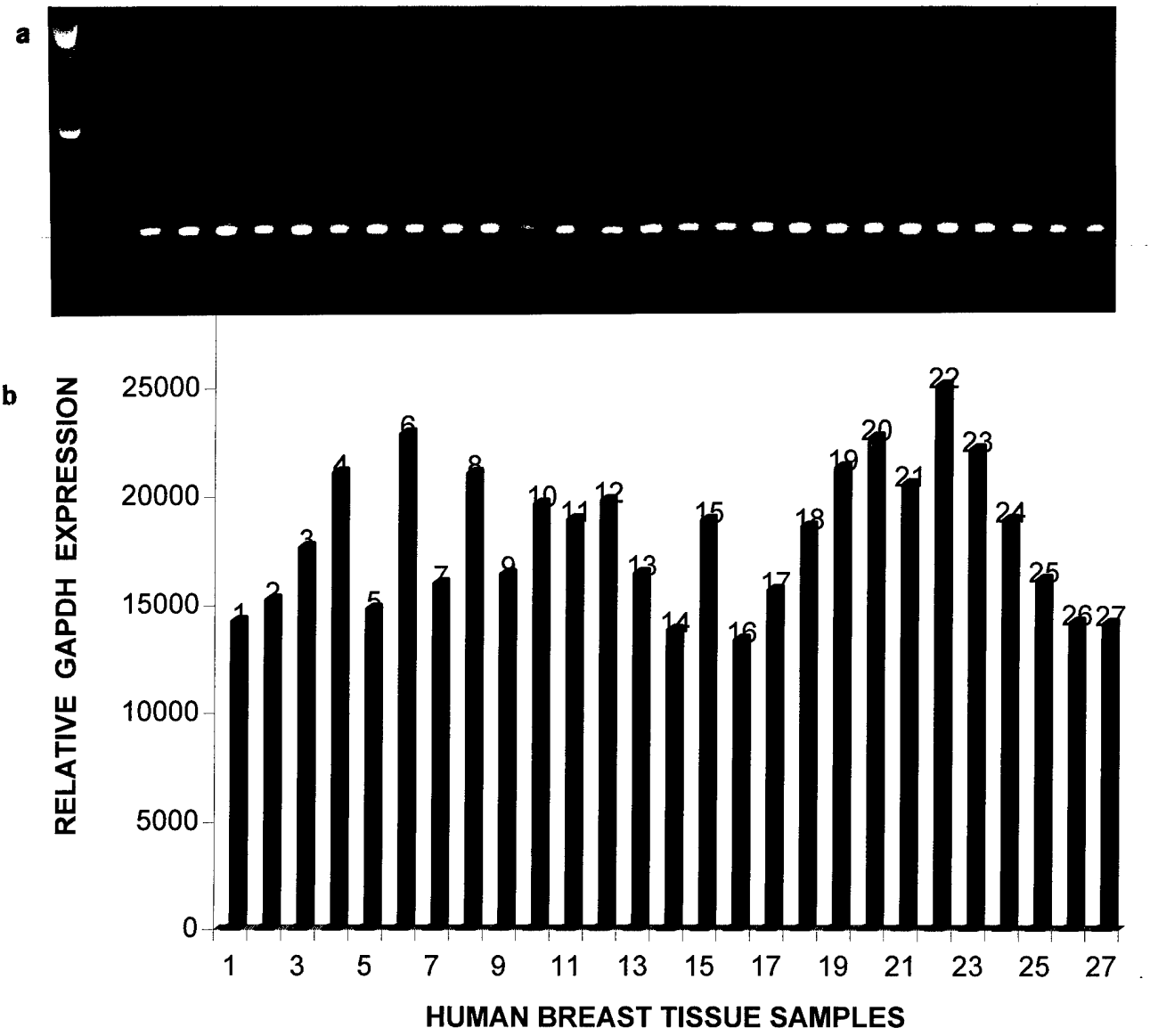


Figure. 16. (**a**) Expression of GAPDH (230 bp fragment), a househeeping gene (internal standard) as determined by PCR. CDNA was prepared from breast tissues and RT-PCR was performed using the specific primer pairs. The amplified products were separated on 1.5% agarose gels and stained with ethidium bromide. DNA markers; **lane 1**, normal breast tissue; **lanes 3, 5 , 6, 7**, benign breast tissues; **lanes 14, 20, 21, 23, 27**, infiltrating ductal carcinoma, Grade II; **lanes 2, 4, 8, 9, 10, 11, 12, 13, 15, 16, 17, 18**, infiltrating and poorly differentiated ductal carcinoma, Grade III; **lanes, 19, 22, 24, 25, 26**, lobular carcinoma. (**b**) Semi-quantitative analysis of MMP-9 in breast tissues. The MMP-9 bands were scanned by an imager and analyzed by GELBASE/GELBLOT PRO software (UVP Products, Upland CA). Units of peak area are arbitary.

analysis were not successful using RNA isolated from various breast tissues. Freshly harvested breast tissue and cells from different cell lines showed the expression of MMPs and TIMPs by northern blots. The feasibility of acquiring 100 plus fresh samples for this multipronged study was impossible. We therefore traded my antibodies for MMPs and TIMPs with primer pairs from Dr. V.H. Rao, Nebraska Medical center, Omaha, NE. The primers selected for amplification (see **Table 1**) have been shown to produce a single productof the appropriate size for MMP-9, MMP-2 [36], TIMP-2, TIMP-1 and GAPDH, a house keeping gene [37]. Oligonucleotide primers used in this study amplify 680 bp for MMP-9 cDNA; 480 bp for MMP-2 cDNA; 551 bp for TIMP-1 cDNA; 590 bp for TIMP-2 cDNA and 230 bp for GAPDH cDNA. We were successful in processing 27 breast tissue samples. The results are reported in **Figures 12-16**. So far, we have analyzed one normal; four, benign tumors; five, infiltrating ductal carcinoma (IDC), Grade II; eleven, IDC, Grade III and five, lobular carcinoma tissues.

The results obtained by this RT-PCR method are semi quantitative. However, **Figure 12** shows low expression of MMP-9 in benign tissue compared to higher levels observed in all other cancer tissues. These results are in agreement with both zymographical quantitation and immunohistochemical findings. **Figure 13** evaluates MMP-2 expression in breast tissues. MMP-2 seems to be expressed in low amounts in all except one tissue (grade III, IDC) **Figure 14** shows the expression of TIMP-1 in lower amounts compared to expression of TIMP-2 (**Figure 15**). Expression of house keeping gene, GAPDH seems fairly constant (**Figure 16**). It is possible that the variation in expression of GAPDH could be improved, when we repeat these experiments. We have recognized that there may be an error in the amount of RNA aliquot used for ceratain tissues. RT-PCR method proved to be useful and at least we can utilize the extracted RNA's from One to five year frozen breast tissue samples that we have processed for zymography and immunohistochemical analysis. Exact number of expressed copies can be acheived by competitive RNA templates for detection of MMPs and TIMPs by RT-PCR procedure described by Tarnuzzer et al. [39]. This method will eliminate sample to sample variation and several MMPs and TIMPs can be expressed from as little as 4 μg of total RNA.

The abstract titled "Expression of gelatinase B (MMP-9) in MET-1 (invasive, mouse) and MCF-7 (human, non-invasive) breast cancer cell lines" was presented at The International Congress of Biochemistry and Molecular Biogy, FASEB at San Francisco, CA. The abstract has appeared in the Faseb Journal, vol. 11, Abst # 2336, A1256, 1997.and is included below:

EXPRESSION OF GELATINASE B (MMP-9) IN MET-1 (INVASIVE, MOUSE) AND MCF-7 (HUMAN, NON-INVASIVE) BREAST CANCER CELL LINES,

Z. Gunja-Smith, Y. Liu and L. Bourguignon, Univ. of Miami Medical School, Miami, Fl 33101, USA.

Gelatinase B (MMP-9, both latent and active forms) is constitutively secreted by mouse tumor cells (MET-1, high metastatic potential) in serum-free media. MMP-9 is also constitutively secreted (mainly in latent form) by the noninvasive human cell line MCF-7; these cells must be treated with phorbol ester (PMA) to secrete quantities of MMP-9 comparable to basal levels found in MET-1 cells. By use of monospecific antibodies (IgGs), confocal laser microscopy showed colocalization of MMP-9 and CD44 (a cell surface adhesion molecule);in particular, interaction with CD44 was prominent in the invadopodia of MET-1 cells. Immuno-precipitation of a membrane fraction with either monospecific antibody followed by gelatin zymography (to assay MMP-9) and immunoblot (CD44) confirms the close association of active MMP-9 and CD44 isoforms. However, latent MMP-9 was found to be closely associated with multiple CD44 isoforms in the resting and PMA-treated MCF-7 cells. These findings suggest that association of proteolytically active form of MMP-9 with CD44 isoforms of a surface adhesion molecule play a key role in rendering a cell line tumorigenic and ultimately of metastatic potential.Supported by DOD grants DAMD 17-94-J-4295/4121 (ZGS/LB).

The profiles and figures can be found in Reports 1995 and 1996.

We obtained some fresh breast tissues consisting of one benign, and three IDC tissues to evaluate the explant cultures. We were successful in obtaining and sustaining the growth of epithelial cell line with the prostate tissue explants [23] Unfortunately, the electricity shut off for half a day ruined the cultures and we have not yet repeated the breast explant cultures. This will be attempted in the coming year.

(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 [23]. We have in the last three years demonstrated that biopsied breast tissues is suitable to carry out a multi pronged study to obtain data using biochemical, immunohistochemical and molecular biological evalutions of MMPs and TIMPs. This multipronged study points to the role of MMP-9 as the key enzyme that is elevated in cancer tissues. This finding is in agreement with RT-PCR, Zymography and immunohistochemical findings. Role

of MMP-9 in cancer has been implicated by several workers [9, 20, 41] Immunohistochemical analysis showed that in many cancer tissues, we observed the red signal of antigen (MMP-9) mainly around the tumor cell clusters. Higher magnification X 100 clearly showed the red signal of MMP-9 in the tumor cells and not in the stroma surrounding tumor cells.

Besides elevated levels of MMP-9, several other MMPs were also elevated in breast cancer tissues. Levels of MMP-1 although low in amounts showed an increase from 0.04 - 0.66 µg/g tissue in cancer tissues. The activity range of MMP-1 (0.66 µg) can disrupt the extracellular matrix by digesting the collagen molecules present. The tumor cells come in contact with growth factors present in the matrix and support the growth of tumor cells [9]. MMP-1, MMP-8 and MMP-13 all digest the collagen fibres and our assay method for collagenase quantitates all three types of collagenase. We hope to use RT-PCR to quantitate the three collagenases in the coming year. We have not been able to corelate the MMP-1 activity profile with several of the immunohistochemical evaluations of tissues using anti-MMP-1 IgGs. We suspect that MMP-8 and MMP-13 collagenase-3 may be present in higher amounts than MMP-1 in the cancer tissues. Collagenase-3 (MMP-13) has been implicated in breast cancer tissues [18].

The breast tissue extracts showed the presence of diferent types and levels of TIMPs by reverse zymography. We were able to detect TIMP-1, TIMP-2 and TIMP-3. The reverse zymographical profiles were included in Preogress Reports 1995 and 1996. We have quantitated the TIMP-1 and TIMP-2 by ELISAs purchased from Oncogene Research Products. The results by this method should be considered preliminary as we found difficulty in evaluationg the dialyzed breast extracts. The breast tissue sections stained with anti-TIMP-1, anti-TIMP-2 and anti-TIMP-3 IgGs showed weak staining of stromas. Some cancer tissues showed staining around the tumor cell clusters. The RT-PCR also exhibited comparatively low expression of TIMP-1 and combining these findings from multi pronged study shows that there is an imbalance in which the destructive proteases greatly outweigh the controlling inhibitors, facilitating the spread of cancer. These findings strongly suggest that the basement membrane underlying breast epithelium probably undergoes rapid turnover due to matrix degrading enzymes secreted by various resident cells in the breast tissue.

The measurement of mRNA levels of TIMPs and MMPs by Northern blots has been frustrating. However, RT-PCR method has been established in my laboratory and scanning of 27 breast mRNAs showed that MMPs and TIMPs signal could be detected in biopsied breast tissues samples. We will concentrate on this part of the project to complete the goals that were outlined in the proposal.

Meaningful statistical analysis of 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. We have more samples to add to our processed

samples and hope to fill in the gaps for restricted number of tissues such colloidal and pappillary carcinomas. Our study definitely suggests that an inhibitor specially designed to knock out the MMP-9 enzyme activity may arrest the invasiveness and metastatic potential of breast cancer cells.

There are several manuscripts in preparation that have been partially completed and will be submitted by the end of 1997.

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STATEMENT OF WORK

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

Task 1. Gather additional histo-pathological information on well-characterized specimens that have been analyzed. Additional specimens will be obtained to complete a group for statistical analysis of enzymes and inhibitors..

a. Year 4: consider further division of tissues into groups based on criteria suggested by initial studies - e.g., nuclear grade.

c. Year 4: collect data to complete all subdivisions for all samples.

Task 2. Collect additional enzymological immunohistochemical and mRNA data.

a. Year 4: Analyze the same specimens for which immunohistochemical data are available. Analyze mRNA content by quantitative RT-PCR method.

Task 3. Fresh tissues will be collected from patients for explant epithelial culture studies. This task will be initiated in the fourth year. Enzyme and inhibitor studies will continue into the final year.

Task 4. Correlate the findings obtained from the immunohistochemical analyses, quantitation of MMPs and TIMPs and their mRNAs, pinpoint the over- or under-expression of MMPs or imbalance between the enzymes and inhibitors. Design further experiments to bolster or refute the underlying hypothesis. This task will be ongoing throughout the grant period, but adequate data will probably be available in the final year.