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13. ABSTRACT *(Maximum 200*

This grant investigates the role of EMMPRIN, seprase and DPPIV in breast cancer invasion and metastasis. The aims are to evaluate these conceivable invasion-related molecules as prognostic markers for node-negative breast cancer. We identified, cloned, and sequenced a full-length cDNA that encoded for a 58-kDa human tumor derived collagenase-stimulating factor, called EMMPRIN. We have expressed EMMPRIN in COS7 monkey kidney cells and human breast cancer cells in order to test the function of EMMPRIN as a collagenase-stimulating factor or a protease docking protein. To evaluate breast cancer markers, we have (1) established a new mAb screening assay of COS7 expression system for epitope mapping of mAbs, (2) improved the hybridoma protocol by including immunogens isolated directly from breast carcinoma tissues, (3) stained tumor sections of paraffin-embedded materials with mAb D8 and D28 against seprase, and (4) increased our efforts in developing serum tests for detecting breast cancer progression.

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Mark S. Marr 7/28/1996 WT

PI - Signature Date

Molecular diagnosis for breast malignancy.

(5). INTRODUCTION

***Revisions according to Reviewers' Comments & Justification of Changes of Task 1-3 of the Statement of Work:** This is the revised Annual (July 1996) Progress Report. I have made extensive revision according to reviewers' comments, included data obtained, and listed manuscripts under reviewed or in preparation for publication. In addition, based on the progress during the past two years I have included detailed justification throughout the text for changes in the Statement of Work. I have marked "*" at the beginning of the paragraphs that described reasons in details the changes for the Updated Statement of Work. Specifically, the justification is summarized as follows:

(1) Task 1: The cloning work of EMMPRIN "p10 maligrin" shows a newly defined function as a collagenase-stimulating factor or as a co-factor for integrin-matrix adhesion and as a binding protein for maligrin, immune complex (M complex) containing C4BPα (p90 maligrin) and p20 small MW serum protein, and membrane proteases that are localized on invadopodia. Therefore, we have extended our experimental plan in Task 1 to determine other potential roles in the induction of invasiveness and metastatic ability of cancer cells.

(2) Task 2: Because of the difficulty involved in the use of old assay, we established new plan, i.e., breast cancer antigen ELISA, to select existing monoclonal antibodies directed breast cancer antigens. Also we extended the work of producing new antibodies that are useful as prognostic markers. We have done additional hybridoma work, utilizing breast cancer lysates as immunogens instead of cultured tumor cells as originally proposed.

(3) Task 3: We have decreased the work of staining paraffin-embedded sections which were proven to be not productive, and increased the work of breast cancer antigen ELISA to determine prognostic markers of node-negative breast cancer.

Nature of the problem. Recently, EMMPRIN, maligrin, integrins, and integral membrane proteases (membrane type, matrix metalloprotease, and serine proteases seprase and DPPIV), have been shown to localize predominantly to the invading front (invadopodia) of the breast cancer cells and to shed membrane vesicles. These molecules are promising indicators of the metastatic potential of breast cancer cells (malignancy antigens). Ideally, a measurable product of invasive cancer cells either localized at the invading front of the breast cancer tissues or shed in patient's blood would permit objective assessment of micrometastases formation. The purpose of this application is to perform a clinical evaluation of invasion-related tumor cell surface antigens as prognostic markers for node-negative breast cancer.

***Terminology, Changes after 1995 Annual Report, particularly referring to the Statement of Work (SOW).**

EMMPRIN -- previously p10 maligrin.

C4BP α -- previously p90 maligrin.

Seprase – previously seprin. The term "seprase" was used in our recent publication (Monsky et al., 1994).

***Background of previous work; Year 1 Progress Summary; Issues relating Task 1 of the Statement of Work:** During the first year, we have cloned and sequenced a full length cDNA encoding a protein previously named "p10 maligrin". An open reading frame of three overlapping clones was identified as a human tumor derived collagenase-stimulating factor, now called extracellular matrix metalloproteinase inducer (EMMPRIN). Northern analysis of mRNA from six breast carcinoma cell lines showed that they all express high levels of EMMPRIN message. The result is a surprise as we postulated in the original proposal that the protein may be an endogenous inhibitor of cell surface proteases. However, this points to the first possibility that EMMPRIN on breast carcinoma invadopodia, when it contacts the host cells directly, serves as a collagenase-stimulating factor. Thus, we have re-named the protein p10 maligrin as EMMPRIN. Based on this result, our first working hypothesis in Task 1 became that EMMPRIN, instead of serving as a putative invasion-inhibitor, maybe expressed at invadopodia to induce the production of collagenases by targeted cells (either other tumor cells or stromal cells). In addition, recent progress in EMMPRIN research from our laboratory and others has indicated that EMMPRIN can serve as a co-factor for integrin-matrix adhesion and as a binding protein for maligrin, immune complex (M complex) containing C4BPα (p90 maligrin) and p20 small MW serum protein, and membrane proteases that are localized on invadopodia. Therefore, we have extended our experimental plan in Task 1 to determine other potential roles of EMMPRIN associated with maligrin, C4BPα and membrane proteases in the induction of invasiveness and metastatic ability of cancer cells.

***Issues relating Task 2-3 of the Statement of Work: Production of monoclonal antibodies directed against malignancy antigens which are useful for clinical studies.** For the past two years, we have established over 100 hybridoma cell lines that produce mAbs directed antigens localized at the invadopodia of breast cancer cells. The critical tests for pathological utility of these antibodies are two folds, i.e. precise epitope identification of mAbs directed against malignancy-associated antigens derived from cultured tumor cells, and (2) their usefulness for clinical studies, including histochemical staining of breast cancer in paraffin sections (Task 3) and serum ELISA tests (Task 4).

Unfortunately, after extensive trials we found that only mAbs D8, D28 and D43 that recognized seprase could stain paraffin-embedded sections of human melanoma and human breast carcinoma (please see *Body Section* for details). Most hybridoma cell lines that we generated during the past produce antibodies against bovine serum components, a result of creating antibodies against tissue culture cells. Consequently, experiments involving screening of cultured breast cancer cells - due to the presence of bovine serum proteins in culture - with anti-tumor cell surface mAbs had generated several false positives (Task 2a, 2b, please see *Body Section* for details). We have been very careful in analyzing these results of paraffin section staining method. However, we have only limited number of antibodies that recognize human tumor antigens, and worried that our

original approach using paraffin sections of cultured tumor cells to determine tissue prognostic markers for node-negative breast cancer (Task 3) may not be successful. In order to secure Task 3, we have sought several alternative approaches, including ELISA tests for breast cancer tissue lysates and immunoblotting analysis for tumor antigens, as well as RT-PCR for tumor mRNA. In the *Body Section* below, we presented data to show that breast cancer antigen ELISA provides powerful immunological tests that are more sensitive and feasible for determining right monoclonals as breast cancer prognostic markers. Thus, we have changed our original plan: by decreasing the work of staining paraffin-embedded sections in Task 3, and by increasing the work of breast cancer antigen ELISA to determine prognostic markers of node-negative breast cancer.

Updated Statement of Work.

Task 1. Characterization of breast cancer EMMPRIN.

a. Screening of MDA-MB-231 cDNA library with anti-EMMPRIN mAbs, and positive clones will be sequenced. Year 1

b. Expression of EMMPRIN: transfection of EMMPRIN expression vector into low expressing cells, and analysis of transfectants for collagenase-stimulating activity or binding to integrins, maligrin, C4BPα and membrane proteases (invadopodial activity). Years 2 & 3.

c. Suppressing EMMPRIN expression: transfection of antisense orientation of mammalian vector into breast cells and confirmation of antisense RNA expression, and analysis of invadopodial activity. Years 3 & 4.

d. Mapping of functional domains of EMMPRIN: mutation (deletion or substitution) of cDNA, and expression of mutant EMMPRIN in breast cells and analysis of effect of mutation on invadopodial activity. Years 3 & 4

Task 2. Production of monoclonal antibodies directed against malignancy antigens which are useful for clinical studies.

a. Screening of breast cancer cells, MDA-MB-231, in paraffin sections with existing mAbs, and selection of diagnostically useful mAbs against malignancy antigens. Years 1 & 2.

b. Production of new monoclonal antibodies directed against breast cancer antigens, and positive hybridoma clones will be established. Year 3.

Task 3. Tissue prognostic markers for node-negative breast cancer.

a. Staining paraffin sections of lung-micrometastasis in experimental invasion and metastasis mice with available mAbs, and analysis of the results. Year 2.

b. Staining paraffin sections of small human melanoma (13) containing invading front (<1 cm) with anti-seprase mAbs or available mAbs, and analysis of the results. Year 3.

c. Staining paraffin sections of human breast carcinoma (20) with anti-seprase mAbs or available mAbs, and analysis of the results. Year 3.

d. ELISA Screening of breast cancer tissue lysates from node negative- & positive- breast cancer patients (each 100), as compared to normal with available mAbs, and statistical analysis of the results. Years 3 & 4.

e. Studies on EMMPRIN, seprase and DPPIV message RNA of 20 representative tumor tissues of node negative breast cancer patients with RT-PCR approach, and analysis of the results. Year 4.

Task 4. Serum prognostic markers for node-negative breast cancer.

a. Establishment of breast cancer antigen ELISA using breast cancer cells in culture (6 cell lines) and in experimental (mouse) melanoma metastasis model. Years 1 & 2.

b. Studies on serum screening of breast cancer patients using same samples (200) at the time of initial surgery, the latest recurrence-free follow-up time, and the first recurrence follow-up time that are available in the Serum Bank of the Lombardi Cancer Center, and statistical analysis of the results. Years 3 & 4.

(6). BODY

Task 1. Characterization of breast cancer EMMPRIN.

1a. Screening of MDA-MB-231 cDNA library with anti-EMMPRIN mAbs, and positive clones will be sequenced. Year 1

Please see 1995 Progress Report and Background of previous work in *Introduction* above.

***EMMPRIN functions as a collagenase-stimulating factor:** During the first year, we have cloned and sequenced a full length cDNA encoding a protein previously named "p10 maligrin". An open reading frame of three overlapping clones was identified as a human tumor derived collagenase-stimulating factor, now called extracellular matrix metalloproteinase inducer (EMMPRIN). EMMPRIN was previously purified from human lung carcinoma cells (Ellis et al., 1989). This protein is present on the surface of several types of human tumor cells in vitro and in vivo and stimulates production of interstitial collagenase in human fibroblasts. It has been shown that EMMPRIN stimulates expression in human fibroblasts of mRNA for stromelysin 1 and 72-kDa gelatinase/type IV collagenase/gelatinase A, as well as for interstitial collagenase (Kataoka et al., 1993). Thus, tumor cell interaction with fibroblasts via EMMPRIN could lead to increased degradation of interstitial or basement membrane matrix components and thus to enhance tumor cell invasion (Kataoka et al., 1993).

EMMPRIN is a M(r) approximately 29-58,000 glycoprotein which is located on the outer surface of human tumor cells and which interacts with fibroblasts to stimulate expression of several matrix metalloproteinases in the fibroblasts. In this study, we have used several approaches, including antibody screening of λgt11 cDNA library of MDA-MB-231 cells, to isolate a complementary DNA encoding a recently reported protein EMMPRIN (Biswas et al., 1995). Computer database searches indicate that EMMPRIN is a member of the immunoglobulin superfamily and that the deduced amino acid sequence of EMMPRIN is identical to that recently reported for human basigin and M6 antigen, molecules of previously undetermined biological function.

***EMMPRIN is recently identified as a member of Tetraspan Membrane 4 (TM4) family which involves in the interaction with integrins to adhere to matrix.** An exciting recent development in studies on EMMPRIN was reported in the recent Gordon Research Conference on Fibronectin, Integrins and Related molecules, January 24-30, 1997. Martin Hamler and his colleagues at Harvard reported that a group of small MW glycoproteins, which contain transmembrane topology with 4 turns of membrane domains, serves as a co-factor with integrins to perform adhesion functions. EMMPRIN is a member of TM4 family. This is an exciting lead for our study of EMMPRIN function. We are now proposing that EMMPRIN may interact with integrins at the leading edge of migratory cells.

***Maligrin and C4BPα, immune complex involved in malignancy:** With the support of an NIH grant (R01CA61274) we found that an 180 kDa cell surface glycoprotein, maligrin, was specifically concentrated at invadopodia of invasive breast carcinoma cells (Fig. 1). In addition, we found that maligrin associated with cellular EMMPRIN and they could be co-isolated by a serine protease inhibitor, *p*-aminobenzamidine, column. Although we are still in the process of identifying molecular nature of maligrin, we have already had three mAbs directed against maligrin, S1, A27 and CP1 which are markers of invadopodia (Fig. 1). Using these mAbs we plan to determine the potential role of EMMPRIN that EMMPRIN interacts with maligrin for the formation of invadopodia in invasive carcinoma cells.

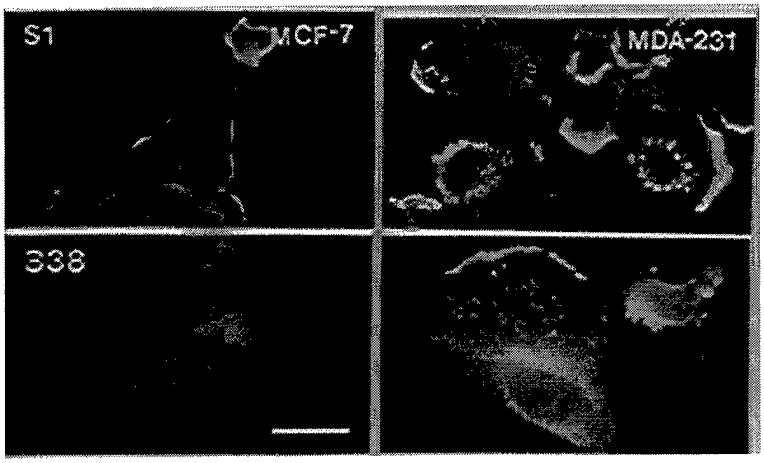

Figure 1. Maligrin localization with C4BPα at invadopodia and surface protrusions of the malignant human breast cancer cell line MDA-MB-231. Cultures of an invasive human breast carcinoma cell line MDA-MB-231 (right panels), and non-invasive MCF-7 cells (left panels) were labeled with the rat mAb S1 against maligrin (upper panels) and mAb B38 against C4BPα (lower panels). The same cultures were viewed with epifluorescence at the focal plane of the ventral cell surface-substratum interface. Invadopodia at cell-substratum contacts under the centers of the MDA-MB-231 cells were labeled by mAbs S1 and B38. The mAbs also label membrane extensions on the upper surface and at the cell periphery. Bar, 20 μm

As stated in the Year 1 progress report, we confirmed that the previously named protein "p90 maligrin" is a bovine serum product, and it has sequence homology with C4BPα (this work was supported by a NIH grant R01CA61274). We discovered that C4BPα is a major component of malignancy-related serum protein complex in adult bovine and human blood termed "M-complex" that interacts with cellular maligrin, by virtue of its localization to invadopodia of cancer cells (Fig. 1), and retention of M complex by a serine protease inhibitor, *p*-aminobenzamidine, column. While EMMPRIN is primarily a key product of breast cancer cells, the p90 appears to be a ubiquitous serum component of adult individual. Thus, the investigation on p90 identity is beyond the scope of this grant and will not be pursued further in this grant.

Invasive breast carcinoma cell lines that express high levels of EMMPRIN bind substantially higher amounts of M-complex containing C4BPα than a less invasive tumor cell line that express low levels of EMMPRIN (Fig. 2). Using a two-site monoclonal antibody (mAb) enzyme-linked immunosorbent assay (ELISA) we detected higher amounts of M-complex in cell lysates of invasive breast carcinoma cells (MDA-MB-231) and malignant melanoma cells (LOX) than non-invasive SKMEL28 melanoma and MCF7 carcinoma cells (not shown), consistent with the result of Western blot analysis (Fig. 2). It is possible that EMMPRIN and maligrin may serve as a part of cellular receptor for M-complex. This seems to be an exciting hypothesis since in separate

studies we found M-complex is a major protease docking vehicle which recruits secreted proteases in body fluid to cancer cell surfaces.

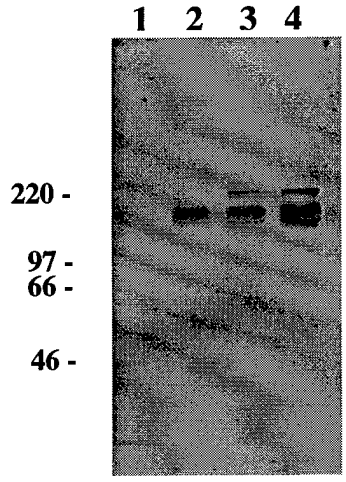

Figure 2. M complex containing C4BPα in malignant human breast cancer cell lines. Western blot analysis using mAb S5 that recognizes M complex under the non-reducing condition was performed on Triton X-100 (1%) cell extracts derived from human breast carcinoma cell lines MCF-7 (lane 1), MDA-MB-436 (lane 2), MDA-MB-231 (lane 3) and HS578T (lane 4). Note that invasive breast carcinoma cell lines shown in lanes 2-4, bind substantially higher amount of M complex containing C4BPα than the non-invasive MCF-7 cells (lane 1). The total amount of protein loaded in each lane was 16 μg.

***Justification for changes in Task 1:** I feel that the progress during the past two years points to an exciting new direction for this grant and I would like to propose changes in Task 1 Years 3 & 4 of this grant: from determining the role of EMMPRIN or "p10 maligrin" as an inhibitor of membrane proteases to the investigation of EMMPRIN function in invadopodial activities, specifically characterizing its association with integrins, maligrin, serum C4BPα or membrane proteases. This re-direction of efforts has significantly increased the scope of investigation and it will require intensive efforts on parts of EMMPRIN expression and suppression experiments. Two additional years have been planned to complete these new experiments. However, the old Task 1e "Assessment of tumorgenicity and metastatic ability of transfectants *in vivo*" with animal models will not be done during this grant period based on following considerations: (1) previous studies found that EMMPRIN seems to be a ubiquitous gene product rather than a malignancy-associated antigen and its functional studies should be re-investigated with extensive *in vitro* studies as in the revised Task 1, (2) stable transfectants that exhibit altered function are not likely to be obtained before early 1998 and there is not enough time for examining their effect in animal models, and (3) revised EMMPRIN

expression/knockout works are labor-intensive and there is not enough person- and money-power during the grant period to carry out animal studies.

1b. Expression of EMMPRIN: transfection of EMMPRIN expression vector into low expressing cells, and analysis of transfectants for collagenase-stimulating activity or binding to integrins, maligrin, C4BPα and membrane proteases (invadopodial activity). Years 2 & 3.

Expression of EMMPRIN in COS7 cells: During the second year, we have expressed EMMPRIN in COS7 monkey kidney fibroblastic cells (transient expression, ~20% expression efficiency) and determined the effect of EMMPRIN overexpression in production of gelatinase A (also called MMP-2 and 72-kDa type IV collagenase) in the fibroblastic cell population. Using gelatin zymography to assess gelatinase A in cell conditioned media, we found that overexpression of EMMPRIN in 20% COS7 cells stimulated the production of gelatinase A two folds, a result confirming that of Biswas and colleagues (Kataoka et al., 1993). Also, we found that EMMPRIN expression alone could not stimulate the formation of invadopodia in fibroblasts. This could be explained by low levels of maligrin in fibroblasts (see *Maligrin section* above).

Expression of EMMPRIN in breast carcinoma cells: During the second year, we investigated the role of EMMPRIN in invadopodial activity of human breast cancer cells. Linda Howard, C.C. Lee, and Jerry Santos have studied EMMPRIN expression in six breast carcinoma cell lines, one normal epithelial line and two fibroblastic lines using Northern blotting analysis as well as RT-PCR of mRNA profile. They found by Northern blotting that while EMMPRIN message was high in breast carcinoma cells, normal epithelial cells and fibroblasts expressed moderate or low levels of EMMPRIN message (data not shown here). We also obtained similar results with RT-PCR which are more sensitive and quicker than Northern.

mRNA Analysis---Northern blotting and RT-PCR were used to determine the levels and alterations of mRNA. Reverse Transcriptase - PCR (RT-PCR), a procedure that we have already tested for seprase mRNA in several *in vitro* cell lines (Goldstein et al., manuscript under reviewing, see *List of Grant Manuscripts*). The method demonstrates the presence of mRNA corresponding to seprase in highly invasive melanoma cell line LOX, moderately invasive melanoma line RPMI7951, moderately invasive breast carcinoma line MDA-MB-436, and embryonic lung fibroblast line WI-38, but not in non-invasive melanoma line SKMEL28. Strikingly, it showed that a truncated seprase mRNA appears in all lines: RMPI7951, LOX, MDA-MB-436 and 231, and HUVEC. We believe that this PCR method will give us a pleasant surprise of finding altered protease mRNA(s) in malignant carcinomas. Thus, we intend to use RT-PCR to investigate EMMPRIN, seprase and DPPIV message RNA of 20 representative tumor tissues of node negative breast cancer patients (Year 4, Task 3e).

Brief RT-PCR protocol for Task 3e--- Total and/or poly A^+ RNA will be isolated from tumor tissues using the RNA Stat - 60 kit (Tel - Test "B", Inc.) and/or Dynabeads oligo$(dt)_{25}$(Dynal), respectively. Also total and/or poly A^+ RNA will be used as a

template for first strand cDNA synthesis with oligo (dT) $_{12\text{-}18}$ as primer. The reaction will be catalyzed by Superscript II RNase H$^-$ reverse transcriptase as directed by the manufacturer (Gibco/BRL). For analyses of potential alternatively processed seprase mRNA, two oligonucleotide primers corresponding to sense 5' - CCACGCTCTGAAGACAGAATT - 3' (# 161-181; 5' UTR) and antisense 5'- ACAGACCTTACACTCTGAC - 3' (# 1863-1845; ORF) orientations will be used. The primers will be utilized for PCR sequence analysis. Polymerase chain reactions will be carried out using the Expand Long Template PCR System kit (Boehringer - Mannheim) and a Perkin Elmer Gene Amp 9600 Cycler.

EMMPRIN is co-localized with maligrin at invadopodia---EMMPRIN protein expression was analyzed by immunoblotting and mAb sandwich ELISA, and these measurements were described in our original proposal. During the second year we found that EMMPRIN protein expression was in general correspondent with mRNA expression in three breast carcinoma cell lines, MDA-MB-231, MDA-MB-436 and MCF-7 (not shown). However, three cell lines showed different degrees of cell invasiveness as assayed by *in vitro* fibronectin/gelatin degradation/invasion assay (Chen et al., 1994b), and from high to low invasiveness: MDA-MB-231 > MDA-MB-436 > MCF-7. To examine the potential different role that EMMPRIN plays in different cell lines, immunocytochemical localization of EMMPRIN was performed as described (Chen et al., 1994a). We found that, using mAb B26, EMMPRIN co-localized with maligrin (Fig. 1 above) at invadopodia of invasive MDA-MB-231 cells but diffusely on surfaces of non-invasive MCF-7 cells (co-localization data will be shown in 1997 Progress Report). This results shed light on the function of EMMPRIN in tumor invasion. EMMPRIN may exist its cellular function by association with specific molecules localizing at invadopodia, including membrane proteases, maligrin, C4BPα and integrins. We have available mAb and cDNA reagents to investigate membrane protein-protein interaction, specifically using mutant gene transfection experiments, protein co-localization and co-immunoprecipitation as described in this updated Task 1.

Task 2. Production of monoclonal antibodies directed against tumor cell surface antigens which are useful for clinical studies.

2a. Screening of breast cancer cells, MDA-MB-231, in paraffin sections with existing mAbs, and selection of diagnostically useful mAbs against malignancy antigens. <u>Years 1 & 2</u>.

Monoclonal antibodies available in the laboratory. Table I represents the production of mAbs and characterization of mAbs with regard to antibody reactivity to paraffin embedded materials. The goal of this Task is to select available antibodies that are useful in diagnostic tests of a large set of breast cancer Paraffin-embedded samples from the Armed Forces Institute of Pathology. The ratios of antibody reactivity of mAbs that stained paraffin-embedded sections from a node-positive cancer tissue over that of MDA-MB-231 cells in culture in the presence of bovine serum are shown in the right

column. Note that we initially proposed to use the later test and we obtained extremely high numbers of false positives. The problems involved were discussed below.

Table I. Monoclonal antibodies generated from the laboratory.

Hybridoma	# Rats/# die	Immunogens	# Hybridoma	# Positives
S1 - S23	2/6	Cultured tumor cell membranes	23	0/11
R1 - R53	2/2	SDS denatured cultured tumor cell membranes	53	0/9
B1 - B53	4/2	SDS denatured cultured tumor cell membranes	53	0/15
D1 - D47	4/0	Placental membranes	47	3/?
E1 - E37	4/0	Placental membranes	37	0/?
F1 - F24	2/0	Placental membranes	24	0/?
C1 - C48	2/4	Invadopodial complex from cultured tumor cells	48	0/?
M1 - M4	2/4	Breast tumor membranes	4	?/?

Problems involving the screening of breast cancer cells in paraffin sections with mAbs and establishment of a new assay for screening and epitope identification of mAbs. From the table above, we have been very disappointed of the results of screening of mAbs using cultured breast cancer cells in paraffin sections that was proposed in our original application. The most serious problem we encountered was high number of false positives using mAbs produced from S, B and R hybridomas. To determine if epitopes of these mAbs could be serum components used in the tissue culture condition, a potential artifact, we used these mAbs positive for cultured cells to stain breast tumor sections. None of these mAbs stained breast tumor sections!! Using other approaches, including ELISA and Western blotting, we found that these mAbs listed in Table I are indeed directed against serum components. We had thus abandoned the cell-paraffin screening and adapted the tumor tissue sections instead.

The second major problem of this study is that many mAbs against tumor surface antigens gave us interesting results in immunoblotting, localization, and ELISA assays (see below) but the molecules these mAbs recognized remained to be determined. To do so, we have established the transient expression of COS7 mammalian cells in order to definitively identify the antigen that our mAbs recognize, and in the future, mapping antibody epitopes by antibody staining of cells expressing mutants of various molecules (see the protocol below).

Protocol of epitope identification using transient expression of COS7 mammalian cells--- COS7 cell line was derived from the African green monkey kidney cell line CV-1 by transformation with an origin-defective SV40 virus. This cell line produces wild-type SV40 large T antigen but no viral particles. When pcDNA3 or pCR3.1 plasmids containing an SV40 virus-derived *ori* and cDNA insert - *EMMPRIN, seprase or DPPIV* - are transfected into COS7 cells, the plasmid is replicated to a high copy number 48 hr posttransfection (10,000 to 100,000 copies/cell). This high-level replication allows for amplification of all DNA templates available for transcription and super immunoreactivity of COS7 cells with mAbs directed against their protein products. Briefly, 10^8 COS7 cells were transfected with pcDNA3- *EMMPRIN, seprase or DPPIV* - by electroporation or LipofectAMINE method (Life Technologies, Inc.). COS7 transfectants were cultured for two days and passaged to 96-well or 24-well microtiter plates. Cells in microtiter plates were cultured for another 24 hours and fixed for immuno-peroxidase staining (to be visualized in brown color) with individual mAbs. This protocol allows us to definitely identify mAb epitope on a specific molecule when a full-length cDNA insert was used. It can also map the epitope on a specific domain of the molecule when a truncated cDNA was used. In addition, for each transfection we were able to screen over 100 mAbs. We believed that this protocol is the most powerful procedure for antigen identification of mAbs although other procedures proposed in proposal such as immunoblotting and immunostaining of tissue sections may work well for other purposes.

The COS7 expression system for epitope mapping of mAbs has been established in our laboratory for determining mAbs that recognize seprase, DPPIV (see color Figure 5 in *Appendices* & Goldstein et al., 1997 in *List of Manuscripts*) or EMMPRIN. Using this assay, we have confirmed the identity of mAb B26 antigen as EMMPRIN, since EMMPRIN could be overexpressed in COS7 cells and immuno-stained with mAb B26. During the second year, we have confirmed the antigens of several mAbs generated from our laboratory: mAb B26 directed against EMMPRIN, 6 mAbs against seprase, and 22 mAbs against DPPIV. Also, we found that all of these mAbs could work on other immuno-assays such as ELISA, immunoblotting, and immunofluorescence on frozen tissues.

2b. Production of new monoclonal antibodies directed against breast cancer antigens, and positive hybridoma clones will be established. <u>Year 3</u>.

*** Targeted production of new monoclonal antibodies directed against breast cancer antigens:** As described in the progress of screening existing mAbs above, we had not obtained mAbs specific for breast cancer. To do so, we have developed a new

strategy of mAb production: the use of breast cancer lysates as immunogens and selection of hybridoma cell lines by breast cancer antigen ELISA.

Breast cancer lysates as immunogens for mAb production--- To produce new mAbs that are useful for diagnosis of breast cancer, we have initiated new experiments by direct immunization of rats with carcinoma lysates derived from node-positive breast cancer patients (see Table II M hybridoma below). We used malignant breast cancer tissues, ~15 gm in weight, as starting material and, after homogenization in PBS, membrane glycoproteins were subjected to purification by WGA-agarose chromatography. We have immunized 6 rats with WGA binding proteins and established 4 hybridoma cell lines (Table II).

Table II. Monoclonal antibodies generated from the laboratory that are reactive in breast cancer antigen ELISA.

Hybridoma	# Rats/# die	Immunogens	# Cell ELISA +/-	# Tumor ELISA +/-
S1 - S23	2/6	Cultured tumor cell membranes	19/4	1/22
R1 - R53	2/2	SDS denatured cultured tumor cell membranes	26/27	0/53
B1 - B53	4/2	SDS denatured cultured tumor cell membranes	22/31	0/53
D1 - D47	4/0	Placental membranes	31/16	34/13
E1 - E37	4/0	Placental membranes	25/12	26/11
F1 - F24	2/0	Placental membranes	18/6	20/4
C1 - C48	2/4	Invadopodial complex from cultured tumor cells	37/11	8/40
M1 - M4	2/4	Breast tumor membranes	4/0	4/0

Breast cancer antigen ELISA--- Antigen ELISA, as compared with "sandwich" ELISA, involves direct coating/drying of antigen on microtitered wells and single-step mAb detection of the amount of antigens. Other screening methods such as "sandwich" ELISA and staining paraffin-embedded materials were too stringent to assess antibody reactivity. We have therefore directed our effort to develop the breast cancer antigen ELISA for selecting existing as well as new monoclonals. The antigen ELISA (described in details in the legend of Fig. 3 below) is a rapid and quantifiable assay and it have been used as a primary screening for new mAbs generated. We have established three hybridoma cell lines, M4, S22 and E98, that produce mAbs useful in breast cancer antigen ELISA. Figure 3 shows that mAbs M4, S22 and E98, direct against antigens breast cancer tissues (6 samples mixed) that are significantly higher than that in normal (6 samples mixed), while mAbs C14 and C19 recognize antigens present in high levels in normal breast tissues.

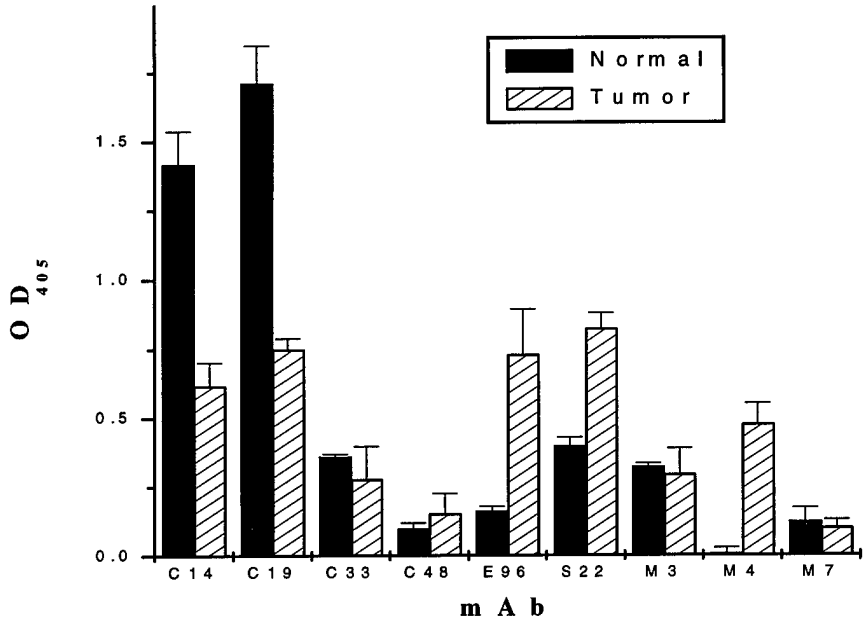

Fig. 3. Breast cancer antigen ELISA has selected mAbs M4, S22 and E96 that are promising prognostic markers for breast cancer. A 96-well U-shaped Microtiter vinyl plate (Dynatech Immulon I, Alexandria, VA) was pre-coated with 20 mg of PBS soluble proteins from either normal or breast tumor tissue in CBC buffer. Hybridoma cells secreting mAbs C14, C19, C33, C48, E96, S22, M3, M4 and M7 were used for antigen detection. Quantitation was performed using an alkaline phosphatase-conjugated goat anti-rat IgG (*Sigma Chemical Co., St. Louis, MO) followed by color reaction using an alkaline phosphatase substrate kit (BioRad, Richmond, CA). The color reaction product was measured at 405 nm using an ELISA reader (Model 2550, BioRad, Richmond, CA). The graph shows readings at one hour except for mAbs C14 and C19 for which 30 min readings are shown. Readings for those antigens recognized by mAbs C14 and C19 are two fold higher in the normal tissue as compared to the breast tumor tissue. No significant difference was observed for those antigens recognized by mAbs C33, C48, M3 and M7. Those antigens recognized by mAbs E96, S22 and M4 appear to be four, two and seventeen fold higher in the breast tumor tissue than in the normal tissue, respectively.

Task 3. Tissue prognostic markers for node-negative breast cancer.

***Justification of Changes in Task 3:** Three mAbs D8, D28 and D43 that recognized seprase could be reactive to paraffin-embedded sections. We have used these mAbs to stain paraffin-embedded materials, including lung-micrometastases in experimental invasion and metastasis mice, small human melanoma, and human breast carcinoma (Task 3a, 3b, and 3c, respectively). Because of the limit of number of mAbs

available for paraffin-embedded materials and our concerns about the potential of seprase as a breast cancer marker, we had made changes in original Task 3 by reducing the number of paraffin-embedded samples and by inserting a new breast cancer antigen ELISA (see Task 2b above). Now, we have obtained three mAbs M4, S22 and E96 that recognize breast cancer antigens and these mAbs are potentially useful tools for diagnosis of breast cancer. Specifically, main goal in the updated Task 3 is to perform a larger scale of breast cancer antigen ELISA using breast cancer tissue lysates from node negative- & positive- breast cancer patients and statistical analysis of the results.

Could membrane proteases alone serve as breast cancer antigens? Based on antibody reactivity to paraffin-embedded materials, we found that mAbs D8, D28 and D43 recognize human melanoma and carcinoma seprase. Also, we found that seprase might share same gene as that of fibroblast activation protein α (Scanlan et al., 1994) (see manuscript by Goldstein et al., 1997) and it was expressed highly in activated stromal fibroblasts as well as some melanoma and carcinoma cells *in situ* (Figures 6-7 in *Appendices*, Manuscript by Ng et al., 1997). These results suggest that seprase is a cell activation protease (Scanlan et al., 1994; Rettig et al., 1988; Garin-Chesa et al., 1990) and it may not be an ideal breast cancer marker.

3a. Staining paraffin sections of lung-micrometastases in experimental invasion and metastasis mice with available mAbs, and analysis of the results. Year 2.

We found that mAb D8, D28 and D43 against seprase stained only LOX human melanoma cells in primary tumors as well as lung metastases but they did not label any host (mouse) stromal cells in the experimental metastasis model. The result is very interesting as metastasizing cells seem to maintain seprase expression and anti-human seprase mAbs do not recognize mouse antigens.

3b. Staining paraffin sections of small human melanoma (13) containing invading front (<1 cm) with anti-seprase mAbs, and analysis of the results. Year 3.

3c. Staining paraffin sections of human breast carcinoma (20) with anti-seprase mAbs or available mAbs, and analysis of the results. Year 3.

We have now identified anti-seprase mAbs D8, D28 and D43 that stained paraffin-embedded sections of tumor tissues. In collaboration with Dr. A.-K. Ng of Maine Blood Research Foundation, we have examined the seprase protein expression *in situ* in small human melanoma containing invading front (Figure 6 in *Appendices*) and human breast carcinoma (Figure 7 in *Appendices*). Part of this result was published in abstract form and the whole manuscript is in preparation (see Task 3 and list of manuscripts below).

This work on seprase expression in lung-micrometastases in experimental invasion and metastasis mice, small human melanoma, and human breast carcinoma (Task 3a, 3b, and 3c, respectively) has been partially completed and we are preparing a

manuscript disclosing the results of these studies (see the manuscript by Ng et al., 1997 and Figures 6-7 in *Appendices*).

Task 4. Serum prognostic markers for node-negative breast cancer.

4a. Establishment of breast cancer antigen ELISA using breast cancer cells in culture (6 cell lines) and in experimental (mouse) melanoma metastasis model. Years 1& 2.

As described previously in 1996 Progress Report, we established mAb-based "sandwich" ELISA for the quantitative detection of DPPIV and seprase in culture media conditioned by breast cancer and melanoma cells as well as in sera of experimental metastasis mice. Serum ELISA tests have been conducted using serum samples from 100 mice injected s.c. with LOX cells. Serum samples were taken at the time of initial cell inoculation (day 1) and at the day (day 36) where lung metastases were detected. We performed three times of this experiment (each with 33 mice) and conformed metastases that were originally described by Fodstad and colleagues (Fodstad et al., 1988b; Fodstad et al., 1988a; Fodstad and Kjonniksen, 1994). We collected sera from these mice (0.5-ml each bleeding each mouse) and tested for the presence of human melanoma seprase using "sandwich" ELISA. Unfortunately, "sandwich" ELISA for human melanoma seprase did not detect significant shed seprase in mouse sera. As discussed in Tasks 2b & 3 above, seprase alone may not be effective as a prognostic markers of breast cancer and we have established several mAbs and new antigen ELISA for detecting breast cancer antigens. This new development will be also applied to Task 4, i.e. evaluation of these breast cancer antigens as serum prognostic markers in node-negative breast cancer patients. We concluded that the animal work should be ended Year 2.

4b. Studies on serum screening of breast cancer patients using same samples (200) at the time of initial surgery, the latest recurrence-free follow-up time, and the first recurrence follow-up time that are available in the Serum Bank of the Lombardi Cancer Center, and statistical analysis of the results. Years 3 & 4.

Breast Cancer Serum Markers. The use of circulating tumor markers to develop serum tests for screening breast cancer patients have been suggested (Schwartz et al., 1993; Mansour et al., 1994; Chen et al., 1994a). For example, it was found that increases in the serum concentrations of carcinoembryonic antigen (CEA) associated with progression of cancers of the gastrointestinal tract, lung, and breast (Beard and Haskell, 1986). Recent studies indicate that cell surface proteases can act as possible tumor markers. For examples, DPPIV activity is reduced in the blood of patients with various malignant tumors, and it has been suggested to be useful in the diagnosis of benign versus malignant tumors (Maes et al., 1994; Kubota et al., 1994; Atherton et al., 1994; Johnson et al., 1993). Cellular fibronectin in serum has also been considered as a potential breast cancer marker (Ylatupa et al., 1995). They are not, however, specific markers that precisely pinpoint the occurrence or recurrence of breast cancer. Currently, combinations of potential serological markers that are theoretically independent of one another are used

to screen patients, in the hope that one or more of the combination of the markers will detect the patients with the disease (Schwartz et al., 1993; Mansour et al., 1994; Chen et al., 1994a).

Because of our progress in obtaining mAbs useful for breast cancer antigen ELISA, we have moved forward to perform pilot serum studies during Year 2. We have recently developed a panel of mAbs directed against membrane glycoproteins, including cellular fibronectin, isolated from human term placenta using WGA chromatography. We used this major serum glycoprotein as a target to develop mAb-based ELISA screening for serum antigen levels in sera of node-negative patients, and examined the idea of "breast cancer serum markers". Cellular fibronectin is localized on basement membranes underlying embryonic endothelia with mAb D27 and it is found in high levels in human sera - estimated 0.1 to 6 μg per ml of serum - using quantitative ELISA. A pilot cellular fibronectin serum test was established to determine whether serum antigen levels change during breast cancer progression and near the time of recurrence as compared to the serum antigen level of health women. Initial serum ELISA tests were conducted using paired serum samples from 10 normal individuals and from 20 patients taken at the time of initial therapy and at the first follow-up visit where recurrence or nonoccurrence were detected. As shown in Fig. 4 below, we performed variance and paired t-tests to determine the relationship of recurrence and serum cellular fibronectin levels. We did find a significant difference between these of healthy women and patients, however, we did not find any significant difference in the serum cellular fibronectin of same pair of patients from the time of the initial sample to the time of recurrence ($p=.85$). However, there was a significant difference in cellular fibronectin between recurrent and nonrecurrent subjects regardless of the time of measurement ($p=.002$). The association between cellular fibronectin and recurrence did not vary according to the time at which the sample was taken.

As discussed above, we have obtained three mAbs M4, S22 and E96 that recognize breast cancer antigens. Future studies in Task 4 will use these mAbs to evaluate if they are serum prognostic markers for node-negative breast cancer.

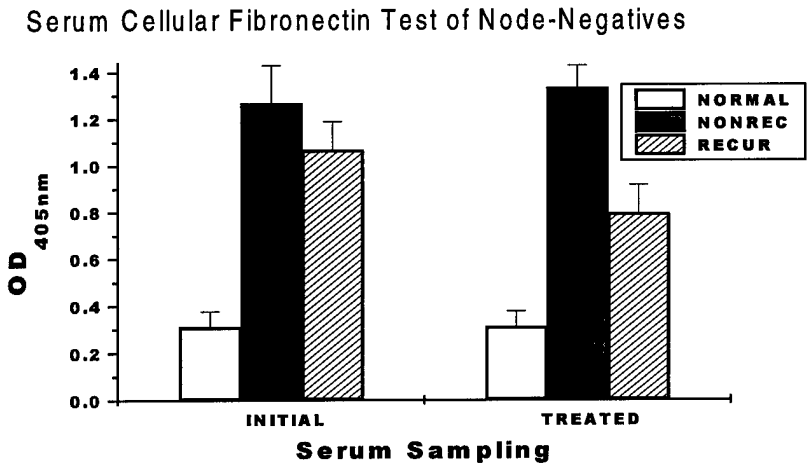

Figure 4. Result of a pilot serum test showing serum cellular fibronectin levels of paired serum samples (NONREC and RECUR) from 10 patients taken at the time of initial therapy (INITIAL) and at the first follow-up visit where recurrence was detected (TREATED), as compared to serum cellular fibronectin levels of 10 health women (NORMAL). We did not find any significant difference in the serum cellular fibronectin of same pair of patients from the time of the initial sample to the time of recurrence (p=.85). However, significant difference occurs between these of healthy women and patients.

(7). CONCLUSIONS

Task 1:

- Transient expression of EMMPRIN in COS7 monkey kidney fibroblastic cells stimulates production of gelatinase A (also called MMP-2 and 72-kDa type IV collagenase) in the fibroblastic cell population. This result suggests that EMMPRIN could serve as a collagenase-stimulating factor, leading to increased degradation of interstitial or basement membrane matrix components and thus to enhance tumor cell invasion.
- EMMPRIN is expressed at invadopodia of invasive breast carcinoma cells where other invasion-associated surface proteins concentrated. EMMPRIN may exist its cellular function by association with specific molecules localizing at invadopodia, including membrane proteases, maligrin, C4BPα and integrins.

Task 2.

- We have established the transient expression of COS7 mammalian cells in order to definitively identify the antigen that mAbs recognize. Using this assay, we have

confirmed the antigens of several mAbs generated from our laboratory: mAb B26 directed against EMMPRIN, 6 mAbs against seprase, and 22 mAbs against DPPIV.

- We have developed a new strategy of mAb production: the use of breast cancer lysates as immunogens and selection of hybridoma cell lines by breast cancer antigen ELISA.
- We have established three hybridoma cell lines, M4, S22 and E98, that produce mAbs potentially useful in diagnosis of tissue or serum prognostic markers using breast cancer antigen ELISA.
- The new antibodies obtained may have long-term prognostic significance in axillary node-negative breast cancer.

Task 3.

- We have partial completed the work on seprase expression in lung-micrometastases in experimental invasion and metastasis mice, small human melanoma, and human breast carcinoma (Task 3a, 3b, and 3c, respectively) and we are preparing a manuscript disclosing the results of these studies (see the manuscript by Ng et al., 1997 and Figures 6-7 in *Appendices*).
- We have obtained three mAbs M4, S22 and E96 that recognize breast cancer antigens and we have initiated a larger scale study of breast cancer antigens using breast cancer tissue lysates from node negative- & positive- breast cancer patients.

Task 4.

- We established mAb-based "sandwich" ELISA for the quantitative detection of DPPIV and seprase in culture media conditioned by breast cancer and melanoma cells as well as in sera of experimental metastasis mice. Unfortunately, "sandwich" ELISA for human melanoma seprase did not detect significant shed seprase in mouse sera.
- We developed new antigen ELISA for detecting breast cancer antigens. This new development will be also applied to Task 4, i.e. evaluation of these breast cancer antigens in sera of node-negative breast cancer patients.

List of grant manuscripts

Chen, W.-T. C.-C. Lee, L. Goldstein, S. Bernier, C. H.L. Liu, C.-Y. Lin, Y. Yeh, W. L. Monsky, T. Kelly, M. Dai, J.-Y. Zhou, and S. C. Mueller. 1994. Membrane proteases as potential diagnostic and therapeutic targets for breast malignancy. *Breast Cancer Research and Treatment.* 31:217-226.

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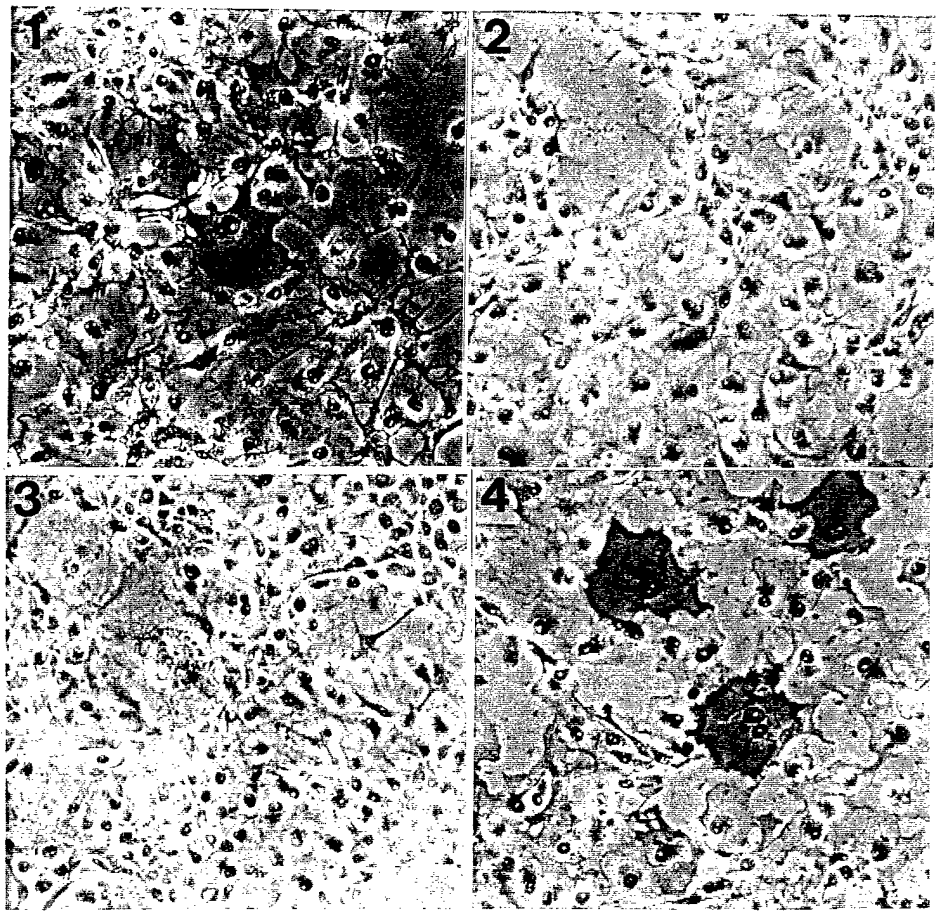
Appendices

Figure 5. COS-7 cells transiently transfected with pA15 express seprase. Immunostaining of COS-7 cells that were transiently transfected with recombinant plasmids containing seprase (pA15) and human DPPIV (pCD26) cDNA were stained with the antiseprase mAb D8 and the class matched (IgG2a) antiDPPIV mAb F4. **1:** pA15 transfected cells stained with D8. **2:** pCD26 transfected cells stained with D8. **3:** pA15 transfected cells stained with F4. **4:** pCD26 transfected cells stained with F4. Primary mAbs D8 and F4 were detected by the StrAviGen Multilink Detection System and AEC substrate (BioGenex).



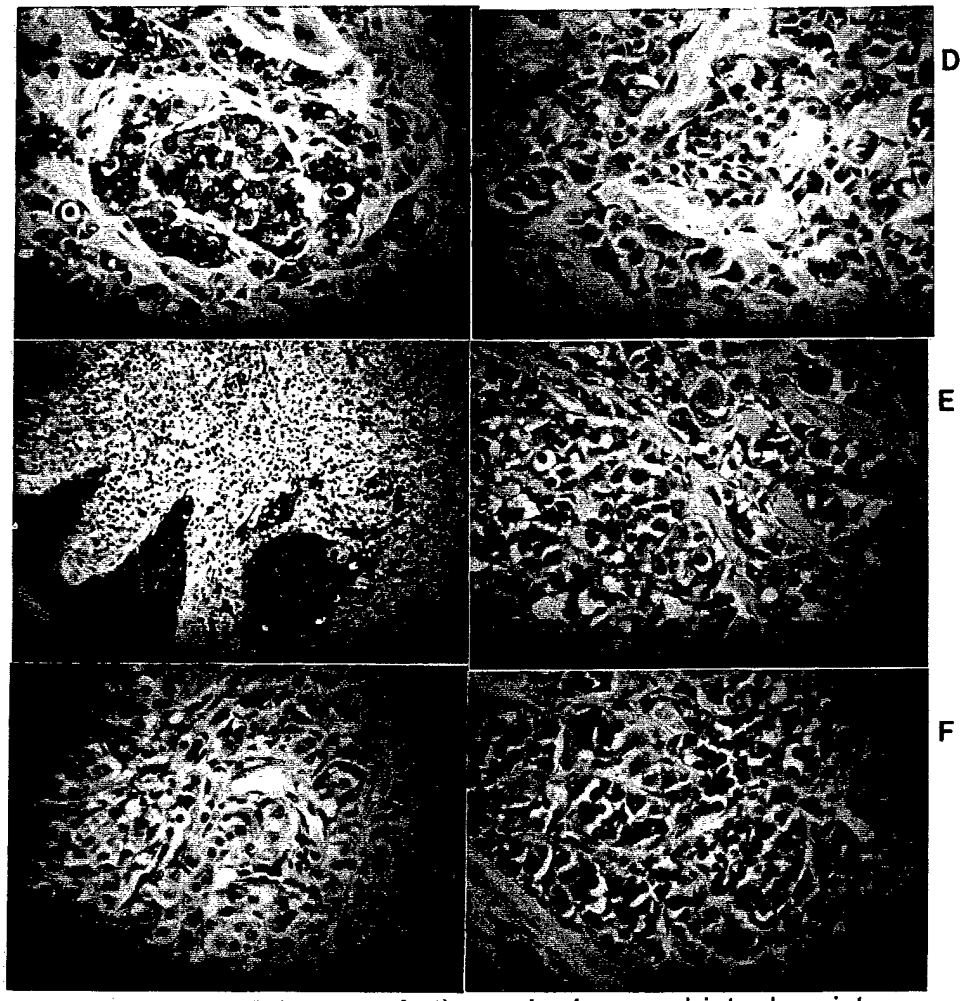


Figure 6. Detection of Melanoma Antigens by Immunohistochemistry

Formalin-fixed, paraffin-embedded melanoma tissue sections were cut at 4 microns and immunostained. The slides were incubated with the TCSNs indicated, washed, and incubated with a species-specific biotin-conjugated antibody. The slides were washed again and incubated with alkaline-phosphatase-conjugated streptavidin. Fast Red in Napthol Phosphate was applied as a substrate/chromagen. Nuclei of each cell are stained blue. A positive reaction is indicated by bright red cellular staining. Tissue sections were provided by the Histology Laboratory, Maine Medical Center. The tumor cells are concentrated in the stratum spinosum and the stratum basale areas of the epidermis. Photographs of sample S93-3671 were taken at 400X by Dr. Michael Jones, MMC at a magnification of 400 X. Panels (A) HMB-45 and (B) AE1/AE3 were treated for 5 minutes with Protease Type XXIV. AE1/AE3 stains the stratum corneum and stratum granulosum. Samples (C) 4B3/E2, (D) 5C8/F2, (E) D8, (F) D28, (G) D43, (H) 1B5, (I) Nambi were first blocked with goat serum before immunostaining. Panel (J) goat serum is the negative control.

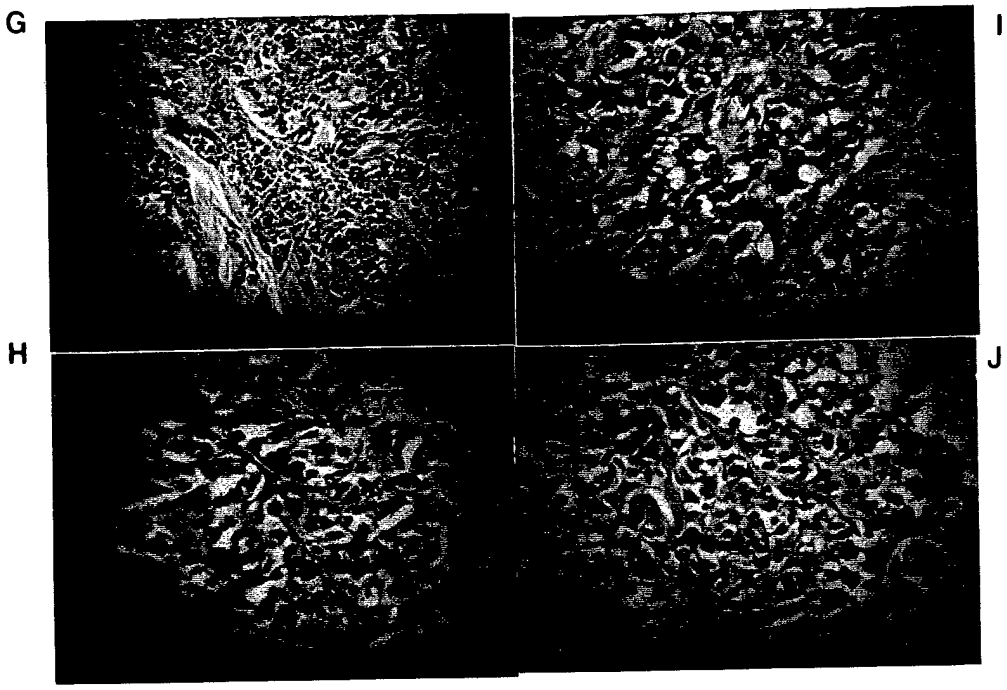


Figure 6 (Continued) Detection of Melanoma Antigens by Immunohistochemistry

ADDENDA B.

Illustrations

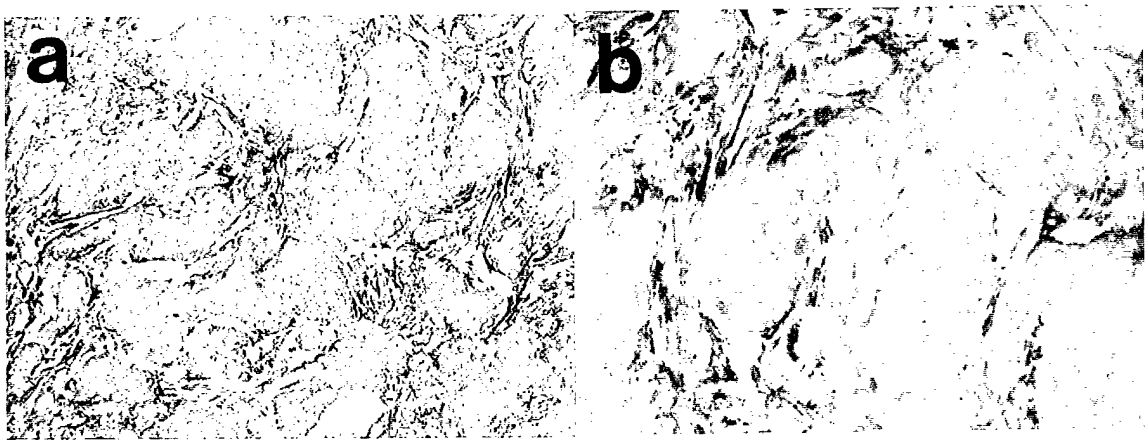


Figure 7. Immunohistochemical localization of mp 170 seprase in malignant human breast carcinoma. mp 170 expression is predominantly detected in stromal fibroblast surrounding clusters of epithelial carcinoma cells in a frozen tissue section of an infiltrating ductal carcinoma (a, 6X; b, 25X). Avidin-biotin immunoperoxidase staining using mAb D8 specific for mp 170 with hematoxylin counterstaining.