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Award Number DAMD17-94-J-4033

TITLE: Molecular Diagnosis for Breast Malignancy

PRINCIPAL INVESTIGATOR: Wen-Tien Chen, Ph.D.

CONTRACTING ORGANIZATION: Georgetown University
Washington, Dc 20057

REPORT DATE: July 1998

TYPE OF REPORT: Final

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

DISTRIBUTION STATEMENT: Approved for Public Release;
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20000828 189

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

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

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 1998	Final (1 Jul 94 – 30 Jun 98)

4. TITLE AND SUBTITLE
Molecular Diagnosis for Breast Malignancy

5. FUNDING NUMBERS
DAMD17-94-J-4033

6. AUTHOR(S)
Wen-Tien Chen, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Georgetown University
Washington, DC 20057

E-MAIL:
wchen@mail.som.sunysb.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

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

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

This report contains colored photos

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

The overall project goal is to identify and evaluate new prognostic markers that can be used to indicate the metastatic potential of node-negative breast cancer. Cell surface antigens in the invading front, invadopodia, of cancer cells are considered as promising diagnostic indicators of metastatic potential. Five invasion-associated proteins are being examined in this project: seprase, dipeptidyl peptidase IV (DPPIV), membrane type-matrix metalloprotease (MT-MMP), fibronectin, and extracellular matrix metalloprotease inducer (EMMPRIN).

14. SUBJECT TERMS biomarker, detection, diagnosis, malignancy, sprase, DPPIV, breast cancer

15. NUMBER OF PAGES
75

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Molecular diagnosis for breast malignancy

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(5). INTRODUCTION

Nature of the problem: Serine integral membrane proteases (seprase and DPPIV), membrane type 1 matrix metalloprotease (MT1-MMP) and EMMPRIN 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 cell surface antigens, including seprase, DPPIV, MT1-MMP, EMMPRIN, and fibronectin, as prognostic markers for node-negative breast cancer.

Background: Metastasizing cancer cells can invade the extracellular matrix using plasma membrane protrusions, termed invadopodia, that contact and dissolve the matrix. Various membrane-bound proteases and their associated proteins such as integrins localized on the invadopodial membranes are responsible for the extracellular matrix degradation and cell invasion. Work from our laboratory shows that secreted proteases including gelatinase A and integral membrane proteases (seprase, dipeptidyl peptidase IV [DPPIV], and membrane type-matrix metalloprotease [MT-MMP]) are associated with cell surface invadopodia and shed membrane vesicles. For example, invasive human breast carcinoma cell line MDA-MB-436 retains the invasive phenotype *in vitro*, constitutively expresses invadopodia-associated proteases, degrades and enters into a fibronectin-rich collagenous matrix. We suggest that invadopodia-associated proteases are ideal targets for the diagnosis and treatment of cancer as their presence in association with primary tumors may signal increased metastatic potential. An approach toward the development of new prognostic markers for breast malignancy involves production of monoclonal antibodies directed against membrane proteases in a mixture of invadopodial glycoproteins. The hybridoma cell lines that produced monoclonal antibodies against invadopodia-associated proteases were selected by antibody localization at invadopodia and antibody immune reactivity against breast cancer antigens. Membrane protease accessibility at the cell surface invadopodia and shed membrane vesicles can therefore be used to detect surface proteases on micrometastases or to detect components shed by micrometastases in serum.

Goals: We have generated several panels of monoclonal antibodies that recognize fibronectin, seprase, DPPIV and EMMPRIN. Using these antibodies we would determine that EMMPRIN and protease molecules were expressed in human breast carcinoma and melanoma cells, when these cells were identified to be invasive using fibronectin- and collagen degradation assays. The cells

made contact with the matrix as well as stromal fibroblasts and endothelial cells, localized protease molecules to invadopodia, activated soluble and matrix-bound gelatinase A, and degraded and invaded the matrix. We established monoclonal antibody-based ELISA and Western blot analyses for quantitative detection of seprase, DPPIV, fibronectin and EMMPRIN in breast cancer and melanoma cells, in tumor tissues, in sera of experimental metastasis mice as well as in sera of node-negative breast cancer patients.

(6). BODY

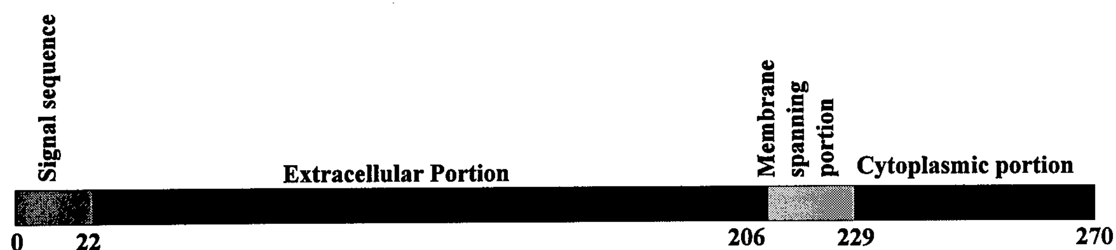
Task 1. Characterization of breast cancer EMMPRIN.

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

In this study, we have used antibody screening of λgt11 cDNA library of MDA-MB-231 cells to isolate a complementary DNA 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) [1]. 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 has following properties:

- The deduced amino acid sequence from EMMPRIN cDNA predicts a type I integral membrane protein. It is a 58-kDa glycoprotein and its non-glycosylated form is a 27-29-kDa protein.
- EMMPRIN, identical to human basigin and M6 antigen, is a member of the immunoglobulin superfamily (e.g. NCAM, ICAM and VCAM), which has been shown to interact with Integrins.
- It is present in human lung and breast carcinoma cells and it functions by stimulating fibroblasts to produce MMP-1, MMP-2, and MMP-3.

EMMPRIN structure is depicted as fellow:



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, C4BPα and membrane proteases (invadopodial activity). **Years 2 & 3.**

We have expressed EMMPRIN in COS7 monkey kidney fibroblastic cells (transient expression). As most transient transfection models, we have obtained 20% expression efficiency. However, COS7 was engineered to exhibit extremely high protein expression, i.e., 100- to 1,000-fold increase in protein expression. In transfected COS7 cells, EMMPRIN is expressed as soluble and cellular forms with M(r) ranging from 20- to 58-kDa, which interacts with untransfected cells to stimulate production of several matrix metalloproteinases by COS7 cells in the medium. We determined the effect of EMMPRIN overexpression in production of gelatinase A (also called MMP-2 and 72-kDa type IV collagenase) in the total 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 in the total COS7 cell-population, a result confirming that of Biswas and colleagues [2]. 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, but it was low in normal epithelial cells and fibroblasts.

In MDA-MB-231 cells, mAb B26 recognizes the native 58-kDa EMMPRIN by both immunoprecipitation and immunoblotting analyses. Antibody B26 was used to isolate a complementary DNA encoding EMMPRIN from a λgt11 cDNA library derived from MDA-MB-231 cells. EMMPRIN protein expression was correspondent with mRNA expression in three breast carcinoma cell lines, MDA-MB-231, MDA-MB-436 and MCF-7, which showed different degrees of cell invasiveness as assayed by *in vitro* fibronectin/gelatin degradation/invasion assay [3]. Expression levels of EMMPRIN in these cells correspond with cell invasiveness: from high to low invasiveness, MDA-MB-231 > MDA-MB-436 > MCF-7.

To examine the role that EMMPRIN may play in the interaction with fibroblasts, immunocytochemical localization of EMMPRIN was performed as described [4]. Using mAb B26, EMMPRIN co-localizes with invadopodial antigens (IA) and a p90 membrane glycoprotein (p90) in invasive breast carcinoma cells (HS578T), demonstrating that EMMPRIN is an invadopodial antigen. In addition, EMMPRIN localized on invadopodia of invasive MDA-MB-231 cells but diffusely on surfaces of non-invasive MCF-7 cells. These 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, possibly integrins.

1c. 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.

We have constructed vectors containing EMMPRIN sense and antisense inserts and transfected them into MCF7 cells (low expresser, non-invasive). Three attempts obtained transient transfectants expressing EMMPRIN mRNA but these transfectants failed to exhibit any detectable activity. In addition, we have used vectors containing EMMPRIN sense and antisense inserts to transfect MDA-MB-231 cells (high expresser, invasive). Again, these transfectants failed to exhibit any detectable activity. We thus terminated this experiment in the last year of this grant period.

1d. Mapping of functional domains of EMMPRIN: mutation (deletion) of cDNA, and expression of mutant EMMPRIN in breast cells and analysis of effect of mutation on invadopodial activity. Years 3 & 4

Originally, we thought that EMMPRIN may associate with integrin for its localization at invadopodia [5]. However, the negative results we obtained above suggested that it is not possible in our hands to identify potential integrins in association with EMMPRIN and putative EMMPRIN domain responsible for this association, as originally proposed.

In addition, we failed to demonstrate an association between EMMPRIN and integrins or integrin-associated proteins, including C4BPα and membrane proteases, in two attempts using cross-immunoprecipitation. Since most of our EMMPRIN work is in confirmatory nature and since the Chen laboratory at Georgetown ended in July 1998 it is unlikely that this work will be written for a publication.

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. Years 1 & 2.

The goal of this originally proposed Task was to select available antibodies that are useful in diagnostic tests of a large set of breast cancer

Paraffin-embedded samples. We obtained only three anti-seprase mAbs, D8, D28 and D43 that could stain breast cancer Paraffin-embedded samples. We have established a new assay for antigen identification of their cognate mAbs based upon the transient expression of COS7 mammalian cells. 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.

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

To produce new monoclonal antibodies directed against breast cancer antigens, 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 initiated new experiments by direct immunization of rats with carcinoma lysates derived from node-**positive** breast cancer patients. 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 that were selected based upon breast cancer antigen ELISA. Unfortunately, these monoclonal antibodies have not been useful due to the failure of repeating our preliminary results of serum ELISA (see Task 4 below).

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

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

Three mAbs D8, D28 and D43 that recognized seprase could stain paraffin-embedded sections. We have used these mAbs to stain paraffin-embedded materials, including lung-micrometastases in experimental metastasis mice, small human melanoma, and human breast carcinoma (Task 3a, 3b, and 3c, respectively). 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.

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. Anti-seprase mAbs D8, D28 and D43 stained paraffin-embedded sections of human melanoma tissues. Antibody D8 gave best staining results: it stains both tumor cells and fibroblasts in 5/8 human skin melanoma samples when keratin mAb AE1/3 staining was used as a positive staining control. Other 5 samples were found to be negative due to poor preservation. Similarly, when mAb HMB45 staining for melanosomes was used as positive staining of samples, mAbs D28 and D43 stained both tumor cells and fibroblasts in 5/8 human skin melanoma samples. These results were published in abstract form. Dr. J.-Y Wang has completed this work at Stony Brook and prepared a manuscript in combination with the work on biochemical identification of melanoma seprase (see list of manuscripts below and manuscripts enclosed in the Appendix).

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

In collaboration with Dr. A.-K. Ng and Dr. Tetsu Yamane, we examined the seprase protein expression *in situ* in small human breast carcinoma (20 samples from Maine and 20 samples from Japan). The addition of new samples from Japanese pathologist group has been most successful in this grant. Because Japanese group has been able to work out a suitable tissue preservation protocol for antibodies available for human seprase and DPPIV, we have achieved the main aim of this grant proposal, clinical evaluation of invasion-related cell surface antigens, including seprase, DPPIV, in malignant breast carcinoma.

Antibody staining of paraffin-sections of breast cancer tissues showed that seprase was expressed in malignant breast ductal carcinoma cells and adjacent fibroblasts but not in distant normal tissue cells. Immunofluorescent staining of frozen sections of breast carcinoma samples showed that seprase was expressed in malignant breast ductal carcinoma and adjacent angiogenic microvessel endothelia.

Importantly, Dr. Wang has found that monoclonal antibody E26 directed against DPPIV and D8 against seprase stain identical cells (activated stromal fibroblasts, angiogenic endothelial cells, and invading carcinoma cells) in malignant carcinoma but not in these cells distant from cancer tissue. Dr. J.-Y Wang has completed this work at Stony Brook. We include these

immunohistochemical data with ELISA and biochemical data to be described below, and prepared a manuscript (see list of manuscripts below and manuscripts enclosed in the Appendix).

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

Originally, our main goal in the updated Task 3 is to perform breast cancer antigen ELISA using breast cancer tissue lysates from node negative- & positive- breast cancer patients and statistical analysis of the results. Because of the limit of number of mAbs available for paraffin-embedded materials, we had added a new breast cancer antigen ELISA to address the role of seprase, DPPIV, EMMPRIN, and fibronectin in node-negative breast cancer (see Task 4b below). However, we were not able to obtain confirmatory results using three mAbs M4, S22 and E96 that recognize breast cancer antigens.

3e. 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.

We have established RT-PCR analyses 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 [6] (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 that could resolve the altered protease mRNA(s) in malignant carcinomas.

We have completed this work at Stony Brook. We include this RT-PCR work with immunohistochemical data, ELISA and biochemical data, and prepared a manuscript (see list of manuscripts below and manuscripts enclosed in the Appendix).

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 ELISA for the quantitative detection of DPPIV and seprase in cultured 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 [8-10]. We collected sera from these mice (0.2-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. 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.

Fibronectin: We have recently developed a panel of mAbs directed against cellular fibronectin, isolated from human term placenta using WGA chromatography. We used this major serum glycoprotein as a target to develop serum ELISA screening of node-negative patients, and examined the idea of "breast cancer serum markers". Cellular fibronectin (with mAb D27) is localized on basement membranes underlying embryonic endothelia 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 Figure 1 below, initially, 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 cellular fibronectin in serum has been considered as a potential breast cancer marker [11], our pilot studies suggest that we need to continue this investigation in order to pinpoint the role of cellular fibronectin in occurrence or recurrence of breast cancer.

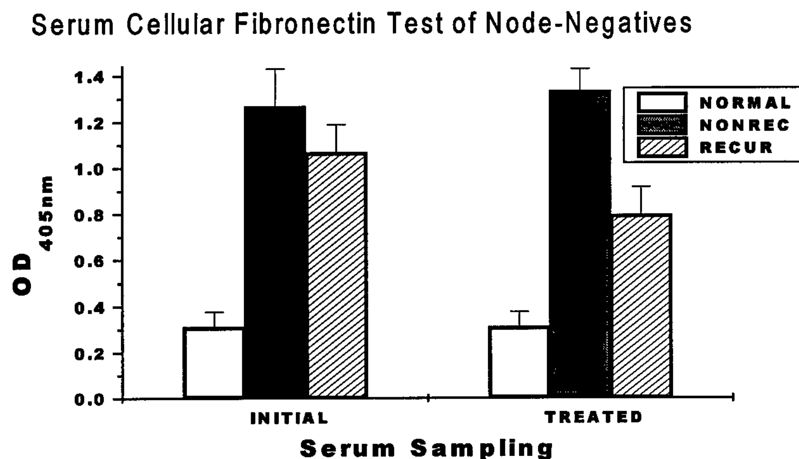


Figure 1. Result of a pilot serum test showing serum cellular fibronectin levels of paired serum samples (NONREC and RECUR) from 10 patients taken at the time of initial therapy (INITIAL) and at the first follow-up visit where recurrence was detected (TREATED), as compared to serum cellular fibronectin 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.

Three attempts of fibronectin ELISA in sera of breast cancer patients available in the Serum Bank of the Lombardi Cancer Center were disappointing and conflicting with our initial data shown above. We are confusing with the outcome but a possible explanation could be the sample issue of sera in the Bank. Fibronectin is a cold precipitation immunoglobulin protein. Long storage and frequent freezing/shawing in blood banking may generate experimental inconsistency that is beyond our control.

ELISA tests for other breast cancer antigens: We have found that seprase and DPPIV are potential serum antigens for diagnosing breast cancer. Studies in Task 4b used available mAbs against seprase and DPPIV to evaluate

if they are serum prognostic markers for node-negative, malignant breast cancer. As we have concerned about the serum sample available in the Serum Bank of the Lombardi Cancer Center for this type of study shown above, we changed our protocol to quantitate differences in seprase and DPPIV expression between breast carcinoma and adjacent normal tissues, and to correlate this data with the corresponding qualitative data shown in Western blot and enzymatic activity analyses. We utilized an ELISA to determine the levels of seprase and DPPIV expression in the two sample groups. ELISA analysis showed that seprase extracts from tumor tissues exhibited a 6-fold increase over extracts from adjacent normal tissues (1.251±0.308 versus 0.196±0.070) (Wang et al., the manuscript on breast cancer enclosed), and differences were statistically significant ($p<0.05$). On the other hand, there was no significant difference in DPPIV levels between the two groups ($p>0.05$).

(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 both stromal and tumor cell invasion.
- Northern and Western blot analyses demonstrate that while EMMPRIN message and protein were high in breast carcinoma cells, but they were low in normal epithelial cells and fibroblasts.
- EMMPRIN is expressed at invadopodia of invasive breast carcinoma cells where it interacts with membrane proteases and integrins.
- We failed to demonstrate an association between EMMPRIN and integrins or integrin-associated proteins, including C4BPα and membrane proteases, in two attempts using cross-immunoprecipitation. Since most of our EMMPRIN work is in confirmatory nature and since the Chen laboratory at Georgetown ended in July 1998 it is unlikely that this work will be written for a publication.

Under the support of this grant, we have cloned and sequenced a full length cDNA encoding a 20-58 kDa 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. We show that a human tumor derived collagenase-stimulating factor, now called extracellular matrix metalloproteinase inducer (EMMPRIN), is associated in membrane

protrusions of carcinoma cells where matrix degradation occurs. Northern analysis of mRNA from six breast carcinoma cell lines showed that they all express high levels of EMMPRIN message. We suggest that EMMPRIN on carcinoma invadopodia may activate stromal fibroblasts and angiogenic endothelia, which express SIMP members (seprase & DPPIV) and MT1-MMP. Thus, EMMPRIN may play an important role in the activation of fibroblast/endothelial invasion during breast cancer progression.

Task 2.

- To produce new monoclonal antibodies directed against breast cancer antigens, 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.
- At completion of this grant, we have established panels of mAbs directed against seprase and DPPIV that are potentially useful as tissue or serum prognostic markers for breast cancer.

Task 3.

- We examined the expression of seprase and DPPIV in melanoma using three systems: *in vitro* human malignant melanoma cell lines, *in vivo* human melanoma tissues, and LOX human melanoma tumors in athymic nude mice. mRNAs for these two proteases were shown to be present in the malignant melanoma cell line RPMI-7951 using RT-PCR. Additionally, we investigated the expression of these two proteases in malignant melanoma and adjacent normal tissues using Western blot analysis and the seprase gelatinase activity using proteolytic assays. Both the 170-kDa gelatinase activity of seprase and 220-kDa exopeptidase activity of DPPIV were identified in extracts of human malignant melanoma tissues, and the expression of seprase was also detected in extracts of LOX tumors from nude mice.
- Immunohistochemical staining of lung-micrometastases in experimental invasion and metastasis mice as well as malignant melanoma tissues with monoclonal antibodies against seprase showed that the immunoreactivity of the malignant cells was more intense than the adjacent fibroblasts in the same tissue. The expression of seprase is considerably higher in malignant melanoma tissues than adjacent normal tissues; this was confirmed by a sandwich enzyme-linked immunosorbent assay ($p<0.05$). However, we could not detect a significant difference in DPPIV expression between melanoma and normal tissues ($p>0.05$).

Our findings indicate that malignant melanoma cells *in vitro* and *in vivo* expression of these integral membrane proteases, especially seprase, may participate in the regulation of the invasion and spread of melanoma cells. The differential expression of seprase in melanoma tissues may suggest this membrane-bound protease as a potential marker of invasiveness for malignant melanoma. Also, we suggest that that seprase may be an important cell activation protease involved in cancer invasion.

- We examine the possibility that the expression of seprase and DPPIV in cells of tumor invading front may contribute to the invasive behavior of malignant human breast cancer. Immunofluorescence microscopy of the Hs578T and the MDA-MB-436 invasive breast carcinoma cell lines revealed that both proteases are expressed at unique surface structures, invadopodia, where degradation of collagenous substrata occurs. These cell lines have detectable seprase and DPPIV protein and mRNA, as well as enzymatic activities.
- In paraffin sections of human breast carcinoma, both seprase and DPPIV are co-localized in carcinoma cells at the invasion front, as well as in the endothelial cells and fibroblasts immediately adjacent to ductal carcinoma *in vivo*. There is lack of seprase and DPPIV stains in normal tissue cells distanced from tumor site and in tumor cells within tumor mass. In addition, both the 170-kDa gelatinase activity of seprase and 220-kDa exopeptidase activity of DPPIV were identified in extracts of breast cancer tissues. The expression of seprase is considerably higher in breast carcinoma tissues than adjacent normal tissues; this was confirmed by a sandwich ELISA (see Task 4 below).

The inducible cell surface enzyme seprase is a serine integral membrane protease (SIMP), highly homologous to a 220-kDa exopeptidase DPPIV (CD26) that exhibits peptidase activities on T-cells and brush borders of epithelial cell types. Our findings indicate that both seprase and DPPIV are implicated in the formation of invadopodia in both breast cancer and stromal cells (fibroblasts and endothelial cells) at sites of breast carcinoma invasion, and that seprase may participate in activation of both cancer and host cells to invade surrounding tissue during breast cancer progression.

Consistently, molecular cloning of melanoma seprase [6] shows that melanoma seprase is identical with fibroblast activation protein α and it is expressed in breast carcinoma cells as well as activated fibroblasts and endothelial cells [7]. Thus, the data suggest that DPPIV is also a cell activation protease.

Task 4.

- A breast cancer serum ELISA was initially established to address potential prognostic roles of seprase, DPPIV, EMMPRIN, and fibronectin in sera of node-negative breast cancer patients. Although pilot studies using anti-cellular fibronectin mAbs and mAbs against seprase and DPPIV showed promise of this assay in a large scale study as described in Task 3 above, the sample issue of sera available in the Bank was found to be inconsistent. Three attempts of fibronectin and breast cancer antigen ELISA in sera of breast cancer patients available in the Serum Bank of the Lombardi Cancer Center were disappointing and conflicting with our initial data shown previously.
- Using a newly designed protocol, the expression of seprase is considerably higher in breast carcinoma tissues than adjacent normal tissues; this was confirmed by a sandwich ELISA ($p<0.05$). However, we could not detect a significant difference in DPPIV expression between breast cancer and adjacent normal tissues. Our findings indicate that both seprase and DPPIV are implicated in the formation of invadopodia in both breast cancer and stromal cells (fibroblasts and endothelial cells) at sites of breast carcinoma invasion, and that seprase may participate in activation of both cancer and host cells to invade surrounding tissue during breast cancer progression.

Studies in Task 4 used available mAbs against seprase and DPPIV to evaluate if they are prognostic markers for node-negative, malignant breast cancer that have potential to release into blood as the assay was performed on buffer-extract tissues that contained blood components. Recent studies indicate that tumor surface antigens can act as possible circulating tumor markers. For example, serum DPPIV activity has been suggested to be useful in the diagnosis of benign versus malignant tumors [12,13]. Furthermore, we will use a combinations of these molecules as potential serological markers as previously suggested [4,14,15].

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Two partially completed manuscripts are listed below. Three copies of current version of manuscripts are enclosed in the Appendix.

Wang, J.Y., L. A. Goldstein, G. Ghersi, Y. Yeh, T. Yamane, Y Mori, N. Hirashima, M. Mitsumata, A.-K. Ng, L. Ford-Davey, M. Jones, and W.-T. Chen. (2000). Seprase and DPPIV are co-cell activation marker for invasiveness in malignant human breast ductal carcinoma (in preparation).

Wang, J.Y., L. A. Goldstein, G. Ghersi, Y. Yeh, T. Yamane, Y Mori, N. Hirashima, M. Mitsumata, A.-K. Ng, L. Ford-Davey, M. Jones, and W.-T. Chen. (2000). The role of cell invasion marker seprase in malignant human melanoma (in preparation).

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(10). A LIST OF PERSONNEL

Wen-Tien Chen, Ph.D./P.I.

Bruce Trock, Ph.D./Co-P.I.

Chong-Chou Lee, Ph.D., Research Assistant Professor

Linda Howard, Ph.D., Postdoctoral Fellow

Denise Flessate, B.S., Research Assistant

Cancer Res. (January 14, 2000; p64Wang.doc)

The role of cell invasion marker, seprase, in malignant human melanoma

Jaw-Yuan Wang [1], Leslie A. Goldstein [1], Giulio Ghersi [1], Yunyun Yeh [1], Tetsu Yamane [2], Yoshiyuki Mori [2], Naoko Hirashima [2], Masako Mitsumata [2], Ah-Kau Ng [3], L. Ford-Davey [3], Michael Jones [3], and Wen-Tien Chen [1, 4, 5]

1. Department of Medicine/Division of Oncology, State University of New York, Stony Brook, NY 11794-8160.
2. Department of Pathology, Yamanashi Medical University, 1110 Shimokato Tamaho Nakakoma, Yamanashi Japan 409-3898.
3. Department of Applied Medical Science, S117, University of Southern Maine, Portland ME 04104-9300.
4. Lombardi Cancer Center & Department of Cell Biology, Georgetown University Medical Center, Washington, DC 20007.
5. Correspondence addressed to: **Wen-Tien Chen, Ph.D.,** Department of Medicine/Medical Oncology, HSC T-16, Rm. 020, State University of New York, Stony Brook NY 11794-8160. T: (516) 444-6948; 444-7594 (lab); F: (516) 444-2493; Email: wchen@mail.som.sunysb.edu

Running Title: Seprase and DPPIV in Breast Cancer.

Key Words: Invadopodia, serine integral membrane protease; seprase; dipeptidyl peptidase IV; malignant melanoma; invasion

ACKNOWLEDGMENTS: We thank Dr. Kenneth A. French for the critical review this article. This work was supported by USPHS grant number R01 CA-39077, DOD US Army DAMD17094-J-4033 and Susan Komen Breast Cancer Foundation Research Award to W.-T.C.

The abbreviations used are: **DPPIV,** dipeptidyl peptidase IV; **ECM,** extracellular matrix; **FAP**α**,** fibroblast activation protein α; **MMP,** matrix proteinases; **MT1-MMP,** membrane-type 1 matrix metalloproteinase; **mAb,** monoclonal antibody.

Abstract

The invasion of human malignant melanoma cells into extracellular matrix (ECM) has been correlated with accumulation of enzymes at sites of the ECM degradation. Serine integral membrane proteases (SIMP), such as seprase and dipeptidyl peptidase IV (DPPIV), may play an important role in tumor invasion and metastasis. Little information is available regarding the roles of these two membrane proteases in human malignant melanoma. To resolve this issue, we examined both proteolytic enzymes using three systems: *in vitro* human malignant melanoma cell lines, *in vivo* human melanoma tissues, and LOX human melanoma tumors in athymic nude mice. mRNAs for these two proteases were shown to be present in the malignant melanoma cell line RPMI-7951 using RT-PCR. Additionally, we investigated the expression of these two proteases in malignant melanoma and adjacent normal tissues using Western blot analysis and the seprase gelatinase activity using proteolytic assays. Both the 170-kDa gelatinase activity of seprase and 220-kDa exopeptidase activity of DPPIV were identified in extracts of human malignant melanoma tissues, and the expression of seprase was also detected in extracts of LOX tumors from nude mice. Immunohistochemical staining of malignant melanoma tissues with monoclonal antibodies against seprase showed that the immunoreactivity of the malignant cells was more intense than the adjacent fibroblasts in the same tissue. The expression of seprase is considerably higher in malignant melanoma tissues than adjacent normal tissues; this was confirmed by a sandwich enzyme-linked immunosorbent assay ($p<0.05$). However, we could not detect a significant difference in DPPIV expression between melanoma and normal tissues ($p>0.05$). Our findings indicate that malignant melanoma cells *in vitro* and *in vivo* expression of these integral membrane proteases, especially seprase, may participate in the regulation of the invasion and spread of melanoma cells. The differential expression of seprase in melanoma tissues may suggest this membrane-bound protease as a potential marker of invasiveness for malignant melanoma.

1. Introduction

The production and local release of various proteolytic enzymes, either by tumor cells or tumor-associated stromal cells, is thought to facilitate cell invasion into the extracellular matrix (ECM). Degradation of ECM and basement membranes is a key step for tumor progression and the development of metastasis, and is mainly mediated by several classes of proteases including matrix metalloproteinases [1], membrane-bound proteases [2], as well as cathepsins [3]. Membrane-bound proteases may contribute significantly to ECM degradation by metastatic cancer cells by virtue of their localization at plasma membrane protrusions (invadopodia) that contact the ECM [4-6]. Recently, several plasma membrane-associated proteases have been described on neoplastic cells as well as their association with ECM degradation [1,6-10]. Seprase is a homodimeric 170-kDa integral membrane gelatinase whose expression correlates with the invasiveness of the human melanoma cell line LOX, which exhibited aggressive behavior in experimental metastasis [11,12], by an *in vitro* ECM degradation / invasion assay [7,8]. Dipeptidyl peptidase IV (DPPIV) is also a nonclassical serine integral membrane protease (SIMP) highly expressed at cell surfaces of many solid tissues and different subtypes of lymphocytes in mammals [13-15]. From previous findings, we have proposed that seprase and DPPIV represent a new subfamily of nonclassical SIMPs [16,17], which is consistent with the observations from Bermpohl and his coworkers [18]. Furthermore, expression of DPPIV was found to be correlated with some types of malignant cells, and DPPIV is thought to participate in the pathogenic mechanisms used by the neoplastic cells [19-21].

Malignant melanomas are cutaneous neoplastic tumors characterized by a high capacity for invasion and metastasis. The incidence of melanoma is increasing at a rate faster than that for any other cancers in the United States and worldwide [22-24], and no effective treatment of the disseminated disease exists. Significant improvement in survival rates might be achieved by earlier diagnosis before the onset of the invasion process and metastatic spread of this disease.

Previously, we have reported that seprase was identified *in vitro* from extracts of the invasive human melanoma cell lines LOX and RPMI-7951 [7,8]. However, it remains unknown whether seprase and DPPIV were expressed in *in vivo* human malignant melanoma tissues. In the present study, we have examined the presence of seprase and DPPIV mRNAs in three human malignant melanoma cell lines LOX, RPMI-7951, and SK-MEL-28 by reverse transcription–polymerase chain reaction (RT-PCR). In addition to detection of seprase expression in LOX human melanoma tumors of nude mice, we also investigated the expression of seprase and DPPIV in human malignant melanoma tissues using Western blot analysis. Enzyme-linked immunosorbent assay (ELISA) was performed to quantify both membrane-bound proteases in melanoma and adjacent noncancerous tissues. Immunohistochemical analysis of seprase expression in human melanoma tissues revealed preferential immunoreactivity with the malignant cells, which is in concordance with higher levels of seprase determination in malignant tissues than adjacent normal tissues by ELISA. Our data demonstrated that seprase is overexpressed in malignant melanoma tissues; thus, it might provide novel clinical tools for malignant melanoma detection and metastasis.

2. Materials and methods

Samples of malignant melanoma tissues were kindly provided by Dr. Michael Jones from the Department of Pathology at Maine Medical Center. Tumor samples from patients with malignant melanoma were frozen in liquid nitrogen following surgery, and then stored at -80°C until analyzed. The human amelanotic melanoma cell line LOX was obtained from Professor Oystein Fodstad, Institute for Cancer Research, The Norwegian Radium Hospital, Oslo, Norway. The melanotic melanoma SK-MEL-28, the melanotic melanoma RPMI-7951, and the human embryonic lung fibroblast WI-38 were purchased from the American Type Culture Collection (Rockville, MD). All cell lines were cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and RPMI1640 medium supplemented with 10% calf serum, 5% Nu-serum

(Collaborative Research Inc., Bedford, MA), 2 mM L-glutamine, 1 unit/ml penicillin, and 10 μg/ml streptomycin in a humidified 5% atmosphere of CO_2 at 37°C. Cells were confirmed as *Mycoplasma*-free using the Genprobe kit (Gen-Probe, San Diego, CA). Rat mAbs D8, D28, E19, E97, and F4 were obtained as described previously [8,17]. The mAbs D8, D28 and E97 were directed against seprase, and E19 and F4 were raised anti-DPPIV antibodies.

For our animal model, female BALA/c athymic nude mice (n=60) were purchased from The Jackson Laboratory (Bar Harbor, ME). They were maintained under specific pathogen-free conditions and used for experiments at the age of 8-12 weeks. All experiments were performed according to the NIH Guidelines for Care and Use of Laboratory Animals. LOX cells were scraped from 80% confluent monolayer cultures with a rubber policeman, and injected (2×10^6 cells/site) s.c. into each flank of BALA/c athymic mice. Mice bearing LOX tumors were sacrificed on day 22 and primary tumors were excised for further analyses.

2.1. Tissue extraction

One g of each frozen malignant melanoma tissue sample and its matched normal tissue were sectioned, and quickly homogenized in a Polytron tissue grinder with 20 strokes and then mixed in RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate] at 4°C overnight. The homogenates were cleared by centrifugation for 20 min at 10000 × g at 4°C, and the total protein in the extracts was determined. The protein sample was further purified by wheat germ agglutinin (WGA)-agarose affinity chromatography according to a previously described method [7], and subsequently concentrated up to threefold by a Centricon-30 concentrator (Amicon Inc., Beverly, MA). Protein determinations were done using the Bio-Rad DC protein assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin as the standard protein. The LOX tumors excised from nude mice were processed similarly to the human malignant melanoma tissues in the extraction. The procedure of isolating seprase from LOX human melanoma tumors was prepared as previously described [25,26].

Briefly, tumor tissues were homogenated in 200 ml of Tris-buffered saline (TBS: 50 mM Tris-HCl, pH 7.4/150 mM NaCl), and particulates were removed by 2,000 × g centrifugation for 5 min. The supernatant was further spun at 100,000 × g for 1 h at 4°C to collect the vesicles. The vesicles were then homogenized and extracted in 50 ml of 1% Triton X-114 in TBS. The total Triton X-114 detergent extracts of LOX tumors were partitioned into detergent (DT) and aqueous phases (AQ). The detergent fraction was further enriched for the 170-kDa protease using WGA-agarose affinity chromatography.

2.2. RT–PCR

The total cell RNA was isolated from LOX, SK-MEL-28, RPMI-7951 malignant melanoma cells, and WI-38 fibroblasts using the RNA Stat-60 kit (Tel-Test Inc., Friendswood, TX) and quantified spectrophotometrically. Reverse transcription-PCR for seprase and DPPIV were performed as previously described [16,17]. Five μg of total RNA served as the template for cDNA synthesis. The oligonucleotide primer pair used for the PCR of seprase was 5'-CCACGCTCTGAAGACAGAATT-3' (FAP 1; nt 161-181) as an upstream primer, and 5'-TCAGATTCTGATACAGGCT-3' (FAP 6; nt 2526-2505) as a downstream primer [27]. The primers specific for the PCR of DPPIV were the forward primer, 5' -ACTTCTGCCTGCGCTCCTT-3' (DPPF-2; nt 24-43) and the reverse primer, 5'-GAGCTGACAGTAGCCTGC-3' (DPPR-2; nt 2798-2781). The Expand Long Template PCR System (Boehringer and Mannheim, Indianapolis, IN) and a Perkin Elmer GeneAmp 9600 thermal cycler (Perkin Elmer, Branchburg, NJ) carried out the PCR amplification. PCR products were analyzed by 1% agarose gel electrophoresis and detected with ethidium bromide staining.

2.3. Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue samples were sectioned in 5-μm-thick pieces using a microtome. The samples were fixed to clean poly-L-lysine (Sigma Chemical Co., St.

Louis, MO) coated slides and dried at 60°C overnight. The slides were cooled and deparaffinized through three changes of Americlear (Baxter, Deerfield, IL). Then the slides were rinsed in 100% ethanol twice and 95% ethanol twice and rehydrated with distilled water. The tissue was digested with Protease XXIV enzyme (Biogenex, San Ramon, CA) for 5 min and then washed with Tris buffer. After that, the anti-seprase hybridoma supernatants containing mAbs D8, D28 or D43 were added at a dilution of 1:10 to each tissue section and incubated at room temperature for 30 min in a humidity chamber. The slide was then incubated with biotin-conjugated goat anti-rat antibody (Jackson Immunoresearch, Wet Grove, PA) at a 1:500 dilution for 20 min. And strepavidin/alkaline phosphatase conjugate (Jackson Immunoresearch) was used at a 1:500 dilution to detect the reactive antibodies. Fast red chromogen in napthol phosphate reconstitution buffer (Biogenex) was used for color development. Histologic sections were counterstained with hematoxylin. Negative control sections were processed with PBS or irrelevant antibodies instead of specific antibodies. The anti-keratin mAb AE1/AE3 and anti-melanosomes were used as positive controls for keratinocytes and melanocytes, respectively.

2.4. Western blot analysis

For immunoblotting, tissue extracts containing 7.5 μg of proteins were separated by SDS-PAGE in 7.5% polyacrylamide gels, and electrotransferred onto nitrocellulose sheets (Schleicher & Schuell, Keene, NH) as previously described [28]. Briefly, the membranes were blocked with 5% nonfat dry milk in TBS containing 0.1 % Tween 20 (TBS-T) overnight at 4°C and subsequently incubated with distinct mAbs: mAbs D8 and E97 directed against seprase, and E19 against DPPIV at a dilution of 1:30 in TBS-T for 1 h at room temperature. Immunoreactive polypeptides were visualized by incubation with horseradish peroxidase-conjugated anti-rat immunoglobulin at a 1:4000 dilution followed by the enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL).

2.5. Gelatin zymography

To identify the gelatinase activity of seprase in melanoma and adjacent normal tissues, gelatin zymography was carried out in 7.5% SDS-polyacrylamide gel electrophoresis as previously described [7]. Aliquots from concentrated tissue sample extracts were electrophoresed on SDS-polyacrylamide gels containing 3 mg/ml gelatin. After electrophoresis, gels were incubated in activation buffer [50 mM Tris-HCl (pH 8.0), 0.2 mM $CaCl_2$, 1.5% Triton X-100, 0.02% sodium azide] or inhibition buffer [50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 1% Triton X-100, 0.02% sodium azide] at 37°C overnight, respectively. The gels were then stained with Coomassie Brilliant Blue, and the gelatinase activity was revealed by negative staining of transparent bands.

2.6. Enzyme-linked immunosorbent assay

Using a two-antibody sandwich ELISA, seprase and DPPIV were quantified in malignant melanoma and adjacent normal tissues. A 96-well ELISA plate (Dynatech Immulon I, Alexandria, VA) was precoated with 100 μl/well of mAb D8 or E19 (10 μg/ml; protein free supernatant in TBS-T) and held at 4°C overnight to capture the antigen. Nonspecific binding sites were blocked with 200 μl/well of 1% BSA in TBS-T at room temperature for 1 h. After washing with TBS-T three times, 2 μg/100 μl/well of WGA-purified antigen was loaded and incubated at 4°C for 1 h. After three washes, the captured antigens were incubated with 100 μl/well of biotinylated mAb D28 (1 μg/ml) that is directed against seprase or F4 (1 μg/ml) that is directed against DPPIV at room temperature for 1 h. The plate was washed with TBS-T. Then the immobilized biotinylated mAbs were detected by alkaline phosphatase-conjugated goat anti-biotin IgG (Sigma Chemical Co., St. Louis MO), followed by the color reaction using the alkaline phosphatase substrate kit (Bio-Rad Laboratories). The plates were developed and measured at

405 nm in a microplate spectrophotometer (SPECTRAmax™ 250, Molecular Devices Co., Sunnyvale, CA).

2.7. Statistical Analysis

All values presented were means ± SE. The significance of the difference was determined by the Mann-Whitney *U* test for unpaired observations, and a *P* value of < 0.05 was considered statistically significant. All statistical analyses were performed using SPSS statistical software (Version 8.0, SPSS, Chicago, IL).

3. Results

To detect the specific mRNAs of seprase and DPPIV in malignant melanoma cells, we carried out RT-PCR of LOX, SK-MEL-28, RPMI-7951 and WI-38 total RNAs using oligonucleotide primers that correspond nucleotide sequences within the 5' untranslated region (FAP 1) and the 3' untranslated region (FAP 6) of the FAPα cDNA [16,27] and the DPPIV specific primers of DPPF-2 (sense) and DPPR-2 (antisense), respectively. A seprase PCR product of ~2.4 kb which encodes the 97-kDa subunit was detected in LOX and RPMI-7951 malignant melanoma cells (Fig. 1A), whereas a DPPIV PCR product of approximately 2.8 kb which encodes the 110-kDa subunit was found in only the RPMI-7951 cells (Fig. 1B). Both seprase and DPPIV PCR products were detected in WI-38 fibroblasts, which were used as a positive control. Fig. 2A and 2B revealed the results of isolation of the 170-kDa membrane proteases from LOX human melanoma tumors of nude mice using Western blot analysis and gelatin zymography in Triton X-114 (TX), aqueous phase (AQ), and detergent phase (DT) of Triton X-114 extract, as well as that purified from a WGA-column. Membrane vesicles collected from LOX tumor of nude mouse contain a distinct but limited protein profile in the aqueous phase after partitioning (AQ). A similar profile is obtained in the detergent phase (DT), whereas

detergent extracts of LOX tumors purified by WGA-column (WGA) contains two major bands having apparent molecular weights at 170 and 120 kDa (Fig. 2A). The 170-kDa gelatinase bands were also found by gelatin zymography (Fig. 2B). Therefore, seprase is demonstrated to be present in the tissue extracts of LOX human melanoma tumors of nude mice.

Immunohistochemical technique is performed to evaluate the expression of seprase in malignant melanoma tissues using mAbs directed against affinity-purified seprase. Positive immunostaining is indicated as a pink or red cytoplasmic staining and blue cellular nuclei. Non-tumor keratinocytes of the stratum corneum and stratum granulosom were stained with mAb AE1/AE3 raised against keratin (Fig. 3A). Using specific mAbs D8 (Fig. 3B), D28 (Fig. 3G), or D43 (Fig. 3 C and F) for seprase, a predominant red cytoplasmic staining was seen in both the nests of tumor cells (open arrows) and adjacent fibroblasts (closed arrows), and the immunoreactivity of the neoplastic cells was more intense than that of the stromal cells in the same specimen (Fig. 3G). No reactivity was observed in the negative control (Fig. 3E) as compared to the mAb HMB45 (anti-melansomes) staining in tumor cells and stromal fibroblasts of the positive control (Fig. 3D). From the above observations, it is evident that seprase exists in malignant melanoma cells, as well as in stromal fibroblasts of tumor tissues.

To assess a comparable seprase expression in the tumor and adjacent normal tissues of identical specimens, we investigated these protein extracts by Western blot analysis. Immunoblotting analysis revealed that mAb D8 recognized the 170-kDa active seprase dimer and its 97-kDa subunit (Fig. 4A), while mAb E97 labeled only the monomeric 97-kDa subunit (Fig. 4B). Seprase was more prominent in tumor tissues than in adjacent normal tissues, though the level of its expression was not quantified. Immunoblotting analysis of DPPIV showed that mAb E19 could recognize the 220-kDa dimeric form (Fig. 4C). However, the amount of DPPIV expression was variable between the tumor and adjacent normal tissues. To detect the proteolytic activity of seprases in malignant melanoma tissues and adjacent normal tissues, we assayed their detergent lysates using gelatin zymography. Seprase gelatinase

activity was observed with the presence of a lytic band at 170-kDa (Fig. 5B) under inactivation buffer, but without detection of this lytic band in activation buffer (Fig. 5A). All tumor tissues exhibited endogenous seprase activity. This result corresponds to the immunoblot results for 170-kDa form of seprase in Fig 4A. To quantify differences in seprase between malignant melanoma and adjacent normal tissues, and correlate this data with the corresponding qualitative data shown in Western blot analysis, we used an ELISA to determine the levels of seprase and DPPIV expression in the two sample groups. ELISA showed that seprase extracts from tumor tissues exhibited a threefold increase over extracts from adjacent normal tissues (0.835 ± 0.152 versus 0.273 ± 0.082) (Fig. 5C), and differences were statistically significant ($p<0.05$). On the other hand, there was no significant difference in DPPIV levels between the two studied groups ($p>0.05$).

4. Discussion

Neoplastic cells possess integrated surface activity necessary for ECM degradation and adhesion that appear at unique surface, termed invadopodia, that contact and proteolytically digest ECM [6,29]. Various membrane-bound proteases localized on the invadopodial membranes may contribute significantly to ECM degradation and tumor progression [4,29,30]. Based on previous *in vitro* studies, we showed that seprase was expressed and localized on invadopodia during invasion into the ECM by human malignant melanoma cell lines [7,8] as well as breast carcinoma cells [6]. The level of expression of this 170-kDa membrane-bound protease is known to be associated with the invasive phenotype of the LOX cell line. Consistent with this observation, overexpression of this proteolytic enzyme was demonstrated in invasive ductal carcinoma cells of human breast cancers by Kelly *et al.* [31] and our unpublished data [ ]. DPPIV, which is highly homologous to seprase, is variably expressed by melanoma cell line, is

proposed to form with seprase a new subfamily of nonclassical SIMP [16,18]. Therefore, DPPIV and seprase may be participants in the pathogenic mechanisms of human malignant melanoma invasion and metastasis.

This current study was undertaken to determine the presence of seprase and DPPIV in various melanoma cell lines and human malignant melanoma tissues, as well as the expression of seprase in LOX human melanoma tumors of nude mice. Our RT-PCR data showed the presence of seprase mRNAs in the melanoma cell lines LOX and RPMI-7951, in accordance with the invasive characteristics of malignant melanoma cells (Table I), and the simultaneous presence of seprase and DPPIV mRNAs in the cell line LOX. Consequently, together with our previous descriptions [7,8], the present information affords additional evidence that the expression of seprase correlates with the invasive phenotype of human melanoma. Besides, these results indicate, for the first time, the expression of seprase and DPPIV in both human melanoma and adjacent non-tumor tissues, in addition to presence of seprase in LOX tumors of nude mice. Furthermore, the measurement of the expression levels of the two proteolytic enzymes using an ELISA revealed the significant increase in seprase level in tumor tissues compared to their adjacent normal tissues ($P<0.05$). However, no significant difference was found in DPPIV levels between the two groups ($P>0.05$). Our previous studies suggested that seprase overexpression might be a marker of an early event in the process of progression from the noninvasive, premalignant to the invasive, malignant phenotype [7,8]. Thus, with our present observations, the increased seprase activity in melanoma tissues might facilitate the invasion and metastasis of human malignant melanoma. Despite the result that the level of DPPIV in tumor tissues is not significantly higher than that in adjacent normal tissues; we could not exclude its relation to melanoma invasion with our limited number of analyzed samples. DPPIV has been proposed to be associated with the stage of malignant melanoma progression [15]. Furthermore, DPPIV expressed on rat lung endothelial cells is also shown to promote adhesion and metastasis in rat breast cancer cells [32]. In addition, immunohistochemical staining of melanoma tissues showed that both melanoma cells and intratumoral fibroblasts are immunoreactive against seprase,

which is consistent with our results of seprase mRNAs in both melanoma cells and fibroblasts by RT-PCR analysis. It has been speculated that normal stromal cells would become activated during tumor invasion and metastasis to produce proteases, tissue inhibitors of metalloproteinases and receptors [33,34]. Therefore, our findings of co-localization of seprase in tumor cells and stromal cells suggest that stromal fibroblast-epithelial cell interactions would activate seprase expression in melanoma tissues. Though this complex modulation of seprase in neoplastic cells and stromal cells is not well understood, our hypothesis may explain the fact that the lower levels of seprase in adjacent normal tissues is due to the absence of any stimulatory effect of activated tumor cells.

In conclusion, we have shown that both seprase and DPPIV are expressed in melanoma tissues of cancer patients and mice inoculated with LOX cells, as well as demonstrating the differential expression of seprase in tumor tissues and adjacent normal tissues. Thus, seprase determination may be helpful in novel diagnostic approaches for malignant melanoma before its establishment of secondary tumors. Additional studies with a large number of serum and tissue samples from patients with malignant melanoma are clearly warranted in order to further elucidate the role of seprase in metastasis.

Table I *Invasive characteristics of human malignant melanoma cell lines*

Cell line	Tumor[1] type	Degradation of FN, MG-Gel film[2]	Expression of seprase/Localization of invadopodia
LOX	A MEL	++++	+
RPMI-7951	A MEL	+++	+
A-3755M	A MEL	+	–
SK-MEL-28	M MEL	0	–
A2058	A MEL	NA	–

[1]Tumor type by histolopathologic diagnosis: A MEL, amelanotic melanoma; M MEL, melanotic melanoma. NA, not analyzed.

[2]Degradation of gelatin film covalently coupled to fibronectin that is labeled with rhodamine and degradation of fluorescein conjugated matrigel. Levels of degradation are qualitative with LOX as the standard.

Figure Legends

Fig. 1. RT-PCR analysis of seprase and DPPIV expression in malignant human melanoma cells LOX, RPMI-7951, SK-MEL-28, and human WI-38 fibroblasts.

RT-PCR was carried out on LOX, RPMI-7951, SK-MEL-28 and WI-38 RNAs using seprase and DPPIV specific primers, respectively. A PCR product ~2.4 kb amplicon of seprase was detected in LOX and RPMI-7951 malignant melanoma cells on agarose gels containing ethidium bromide (Panel A). However, only RPMI-7951 cells could be shown to produce the DPPIV PCR product of ~2.8 kb (Panel B). Human WI-38 fibroblast RNA, used as a positive control, generated an identical amplicon pattern as the RPMI-7951 melanoma cells.

Fig. 2. Isolation of invadopodia-associated membrane protease from LOX tumors of nude mice was verified by Western blot analysis and gelatin zymography.

Western blot analysis (Panel A) and gelatin zymography (Panel B) of membrane-bound proteins derived from LOX tumors in Triton X-114 (TX), aqueous phase (AQ), and detergent phase (DT) of Triton X-114 extract, as well as that purified from a WGA-column (WGA). Membrane vesicles collected from LOX tumors of nude mice contain a distinct but limited protein profile in the aqueous phase after partitioning (AQ). A similar profile is obtained in the detergent phase (DT), whereas detergent extracts of LOX tumors purified by WGA-column contains two major bands having apparent molecular weights at 170 and 120 kDa (Panel A, closed arrows). The 170-kDa gelatinase bands were also visualized by gelatin zymography (Panel B, closed arrow).

Fig. 3. Detection of seprase in malignant melanoma cells (open arrows) and adjacent fibroblasts (closed arrows) in formalin-fixed, paraffin embedded melanoma tissues by immunohistochemistry ($\times$ 400).

Keratin staining in keratinocytes of the stratum corneum and stratum granulosom was detected by mAb AE1/AE3 (Panel A). Photomicrographs showed that mAbs D8

(Panel B), D43 (Panels C and F) and D28 (Panels G) detected the expression of seprase in melanoma cells (open arrows) and adjacent fibroblasts (closed arrows) in tumor samples. The nests of melanoma cells stained bright red in cytoplasm. The tumor cells are concentrated in the stratum spinosum and the stratum basale areas of the epidermis. Melanosomes were positively stained by anti-melanosomes mAb HMB45 in melanocytes. And no staining of tumor cells was noted in the negative control substituted with an irrelevant antibody or PBS (Panel E).

Fig. 4. Expression of seprase and DPPIV in melanoma and adjacent normal tissues by Western blot analysis.

Immunoblot analysis of 170-kDa dimeric seprase and its monomeric 97-kDa subunit derived from tissue extracts using mAb D8 and E97, respectively. The mAb D8 recognized both seprase and the 97-kDA subunit (Panel A), whereas mAb E97 recognized only its monomeric 97-kDa subunit (Panel B). Immunoblot analysis of DPPIV derived from tissue extracts using mAb E19. The mAb E19 could recognize the native 220-kDa protein (Panel C).

Fig. 5. Proteolytic activity of seprase was assayed by gelatin zymography. Additionally, measurement of seprase and DPPIV expression by enzyme-linked immunosorbent assay using mAbs D8 or E19 to capture seprase or DPPIV antigens, respectively, with biotinylated D28 (anti-seprase mAb) and F4 (anti-DPPIV mAb) used for detection of the captured antigens.

Seprase gelatinase activity was detected with the appearance of a lytic region at ~ 170 kDa under inactivation buffer containing EDTA to suppress any gelatinase activity generated by metalloproteases (Panel B). Conversely, several other prominent proteolytic enzymes were found under activation buffer (Panel A). Results of seprase and DPPIV determined in tumor tissues and its corresponding normal tissues by an ELISA (Panel C). The graph shows the reading obtained two hours after the color reaction was initiated. The reading for seprase was threefold higher in malignant

melanoma tissues (■) as compared to their adjacent normal tissues (□) ($p<0.05$). No significant difference was observed in the expression of DPPIV between malignant melanoma tissues and adjacent normal tissues ($p>0.05$). Results represent the mean optical density ± SE (n = 4) as described in "Materials and Methods". *, statistically significant between the two groups ($p<0.05$).

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Figure 1

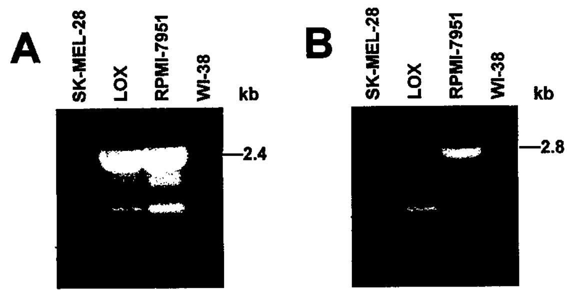


Figure 2

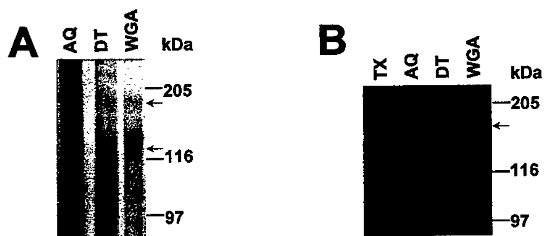


Figure 4

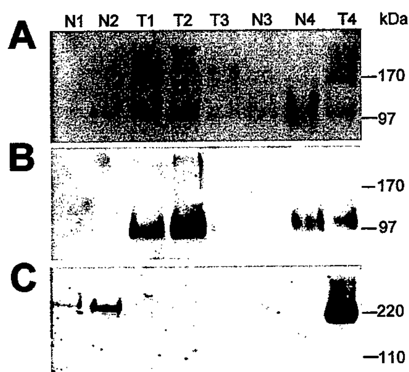
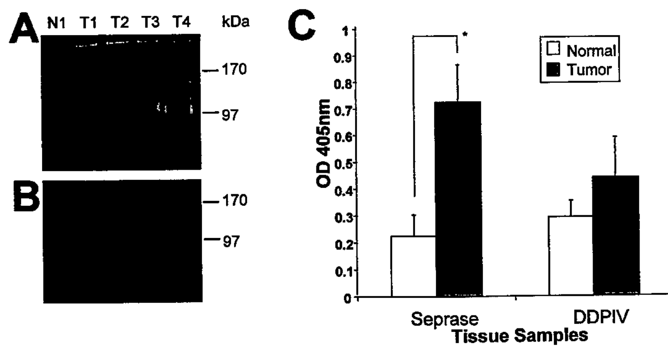


Figure 5



Cancer Res. (January 17, 2000; p65Wang.doc)

Seprase and DPPIV are co-cell activation markers for invasiveness in malignant human breast ductal carcinoma

Jaw-Yuan Wang [1], Leslie A. Goldstein [1], Giulio Ghersi [1], Yunyun Yeh [1], Tetsu Yamane [2], Yoshiyuki Mori [2], Naoko Hirashima [2], Masako Mitsumata [2], Ah-Kau Ng [3], Michael Jones [3], and Wen-Tien Chen [1, 4, 5]

1. Department of Medicine/Division of Oncology, State University of New York, Stony Brook, NY 11794-8160.
2. Department of Pathology, Yamanashi Medical University, 1110 Shimokato Tamaho Nakakoma, Yamanashi Japan 409-3898.
3. Department of Applied Medical Science, S117, University of Southern Maine, Portland ME 04104-9300.
4. Lombardi Cancer Center & Department of Cell Biology, Georgetown University Medical Center, Washington, DC 20007.
5. Correspondence addressed to: **Wen-Tien Chen, Ph.D.,** Department of Medicine/Medical Oncology, HSC T-16, Rm. 020, State University of New York, Stony Brook NY 11794-8160. T: (516) 444-6948; 444-7594 (lab); F: (516) 444-2493; Email: wchen@mail.som.sunysb.edu

Running Title: Seprase and DPPIV in Breast Cancer.

Key Words: Invadopodia, Membrane Protease, Seprase, DPPIV, Breast cancer, Invasion.

ACKNOWLEDGMENTS: This work was supported by USPHS grant number R01 CA-39077, DOD US Army DAMD17094-J-4033 and Susan Komen Breast Cancer Foundation Research Award to W.-T.C.

The abbreviations used are: **DPPIV,** dipeptidyl peptidase IV; **ECM,** extracellular matrix; **FAPα,** fibroblast activation protein α; **MMP,** matrix proteinases; **MT1-MMP,** membrane-type 1 matrix metalloproteinase; **mAb,** monoclonal antibody.

ABSTRACT

The inducible cell surface enzyme seprase is a serine integral membrane protease (SIMP), highly homologous to a 220-kDa exopeptidase DPPIV (CD26) that exhibits peptidase activities on T-cells and brush borders of epithelial cell types. Here, we examine the possibility that the expression of seprase and DPPIV in cells of tumor invading front may contribute to the invasive behavior of malignant human breast cancer. Immunofluorescence microscopy of the Hs578T and the MDA-MB-436 invasive breast carcinoma cell lines revealed that both proteases are expressed at unique surface structures, invadopodia, where degradation of collagenous substrata occurs. These cell lines have detectable seprase and DPPIV protein and mRNA, as well as enzymatic activities. In human tissues of invasive ductal carcinoma, both seprase and DPPIV are co-localized in carcinoma cells at the invasion front, as well as in the endothelial cells and fibroblasts immediately adjacent to ductal carcinoma *in vivo.* There are lack of seprase and DPPIV stains in normal tissue cells distanced from tumor site and in tumor cells within tumor mass. In addition, both the 170-kDa gelatinase activity of seprase and 220-kDa exopeptidase activity of DPPIV were identified in extracts of breast cancer tissues. The expression of seprase is considerably higher in breast carcinoma tissues than adjacent normal tissues; this was confirmed by a sandwich enzyme-linked immunosorbent assay ($p<0.05$). However, we could not detect a significant difference in DPPIV expression between breast cancer and adjacent normal tissues. Our findings indicate that both seprase and DPPIV are implicated in the formation of invadopodia in both breast cancer and stromal cells (fibroblasts and endothelial cells) at sites of breast carcinoma invasion, and that seprase may participate in activation of both cancer and host cells to invade surrounding tissue during breast cancer progression.

INTRODUCTION

Invasion of tumor cells through the surrounding extracellular matrix (ECM) is an important process for cancer metastasis [1]. Several classes of proteases including matrix metalloproteinases (MMPs), serine proteases, cysteine proteases (cathepsin B and cathepsin L), and aspartic acid proteases (cathepsin D) can degrade proteins in the ECM [2]. And invading cancer cells possess ECM degrading proteolytic enzymes that are concentrated at specialized plasma membrane protrusions, termed invadopodia [3]. Recent studies showed that integral membrane proteases may contribute significantly to ECM degradation and ultimately tumor invasion by virtue of their location at invadopodia [4].

Previously, we reported that a cell surface protease, seprase, is a homodimeric 170-kDa integral membrane gelatinase whose expression correlates with the invasiveness of human melanoma cell line LOX [5]. Seprase is expressed and localized on invadopodia during invasion into the ECM by chicken embryo fibroblasts transformed by Rous sarcoma virus, human malignant melanoma and breast carcinoma cells [3,5,6]. We have isolated the active enzyme from the cell membranes and the shed vesicles of LOX cells [7]. The proteolytic activity of seprase is associated with its 170-kDa form, which consists of two proteolytically inactive 97-kDa subunits. The deduced amino acid sequence of a seprase cDNA clone revealed that seprase 97-kDa subunit is essentially identical to the 95-kDa fibroblast activation protein α (FAP α) [8] and is highly homologous to the 110-kDa subunit of DPPIV [9-11]. Furthermore, the deduced amino acid sequence of seprase revealed a 68% amino acid sequence identity to DPPIV in their catalytic regions and a remarkably similar structural organization of both proteases [12]. From the above findings, we have proposed that seprase/FAPα and DPPIV represent a new subfamily of serine integral membrane proteases (SIMPs). This conclusion is in accordance with Bermpohl *et al.*, who demonstrated that a common gelatinolytic activity is detected in seprase and DPPIV [13].

Although experimental studies initially suggested that cancer cells themselves produce the matrix metalloproteinases (MMP) required for degradation of the ECM, recent studies using *in situ* hybridization techniques, have indicated that most MMPs are produced by stromal cells within human tumors, rather than the cancer cells themselves [14]. Interestingly, a recent study showed that seprase was expressed at high level in carcinoma cells alone of human breast cancer [15]. We thought that SIMPs may be closely associated with the invasiveness of both tumor and stromal cells in breast cancer tissue. Consequently, knowledge of these enzymes expression in breast carcinoma would help to elucidate their roles in this disease. Our data demonstrated that seprase and DPPIV were expressed in invasive tumor and stromal (endothelial and fibroblastic) cells in malignant breast carcinoma tissues; thus, they might provide novel clinical applications for breast cancer detection and control of metastasis.

MATERIALS AND METHODS

Materials. Dr. Michael Jones, Department of Pathology at Maine Medical Center, provided twenty samples of breast carcinoma tissues (Maine samples). According to the histopathologic analysis all cancers examined in this study were infiltrating ductal carcinomas, and their corresponding adjacent normal tissues were also examined. Dr. Tetsu Yamane and Dr. Masako Mitsumata, Department of Pathology at Yamanashi Medical University, provided ten samples of breast infiltrating ductal carcinomas and their corresponding adjacent normal tissues (Yamanashi samples). Other forty sets of frozen breast infiltrating ductal carcinomas and their corresponding adjacent normal tissues were obtained from National Disease Research Interchange (Philadelphia).

The human embryonic lung fibroblast line WI38, and breast carcinoma cell lines Hs578T, MDA-MB-231, MDA-MB-436, as well as MCF7 were obtained from American Type Culture Collection (Rockville, MD). Cells were routinely cultured in DMEM supplemented with 10% FCS

(GIBCO-BRL, Gaithersburg, MD), 2 mM L-glutamine, 1 unit/ml penicillin, and 10 μg/ml streptomycin in a humidified 5% atmosphere of CO_2 at 37°C. Cells were confirmed as *Mycoplasma* free using the Genprobe kit (Gen-Probe, San Diego, CA). Rat mAbs were prepared according to a previously described method [7,16]. Rat mAbs E19, E26, and F4 (all subclass IgG 2a) are directed against human placental DPPIV, and rat mAbs D8, D28, D43 and E97 (all subclass IgG 2a) are against human placental seprase [7,12]. Rabbit polyclonal antibodies (pAb) against the peptide CDGNFDTVAMLRGEM (residues 310-333) of MT1-MMP were kindly provided by Hiroshi Sato (Kanazawa, Japan).

Immunofluorescence microscopy. Immunofluorescence microscopy was used to determine seprase and DPPIV distribution in cells in culture and these in frozen tissue sections as described previously [16,17]. Specimens were visualized with a Planapo 63/1.4 objective on a Zeiss Photomicroscope III (Carl Zeiss, Inc., Thornwood, NY) by transmitted illumination for phase contrast microscopy and then by epi-illumination for rhodamine and fluorescein labeling. Endothelial cells in culture or in cancer tissues were verified immunofluorescently using goat anti-human factor VIII antigen antibodies (Atlantic Antibodies, Windam, NE).

RT–PCR of breast carcinoma cell lines. Total cellular RNA was isolated from MDA-MB-231, MDA-MB-436 and MCF7 breast carcinoma cells using the RNA Stat-60 kit (Tel-Test Inc., Friendswood, TX). Reverse transcription-PCR reactions for seprase and DPPIV were performed as previously described [7,12]. The oligonucleotide primer pair used for the PCR of seprase was 5'-CCACGCTCTGAAGACAGAATT-3' (FAP 1; nt 161-181) as an upstream primer, and 5'-TCAGATTCTGATACAGGCT-3' (FAP 6; nt 2526-2505) as a downstream primer [8]. The primers specific for the PCR of DPPIV were the forward primer, 5' -ACTTCTGCCTGCGCTCCTT-3' (DPPF-2; nt 24-43) and the reverse primer, 5'-GAGCTGACAGTAGCCTGC-3' (DPPR-2; nt 2798-2781). PCR products were analyzed by 1% agarose gel electrophoresis and detected with ethidium bromide staining.

Preparation of tissue extracts. One g of each frozen breast cancer tissue or its adjacent normal tissue was homogenized in a Polytron tissue grinder with 20 strokes and then mixed with RIPA buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate] at 4°C overnight. The homogenates were cleared by centrifugation for 20 min at 10,000 × g at 4°C, and the total protein in the extracts was determined. Surface glycoproteins were isolated from the protein sample by wheat germ lectin (WGA) Sepharose 6 MB (Pharmacia LKB, Piscataway, NJ) as previously described [5], and subsequently concentrated up to 3-fold by a Centricon-30 concentrator (Amicon Inc., Beverly, MA). Protein determinations were done using the Bio-Rad DC protein assay (Bio-Rad, Richmond, CA) with bovine serum albumin as the standard protein.

Immunohistochemistry. The Yamanashi procedure (results shown in Fig. 3) involves fixation of invasive breast cancer tissues with 4% paraformaldehyde in PBS for 2-4 h at 4°C, followed by paraffin embedding. Paraffin-embedded tissue blocks were sectioned in 4-μm-thick pieces using a microtome. The samples were adhered to poly-L-lysine (Sigma Chemical Co., St. Louis, MO) coated slides and dried at 42°C overnight. The slides were cooled and de-paraffinized through three changes of Americlear (Baxter, Deerfield, IL). Then the slides were rinsed in 100% ethanol twice and 95% ethanol twice and re-hydrated with distilled water. Anti-seprase mAbs D8 or D28 or anti-DPPIV mAbs E19 or E26 in serum-free supernatant form were added at a dilution of 1:10 to each tissue section and incubated at 4°C overnight in a humidity chamber. Bound primary antibody was then detected by streptavidin-biotin-peroxidase technique (DAKO) according to the manufactory's instructions using diaminobenzidine (3,3'-diaminobenzidine tetrahydrochloride, Sigma) as a chromogen and counter-staining was performed with hematoxylin.

The Maine procedure (results shown in Fig. 4) involves routine pathological fixation of invasive breast cancer tissues with 10% formalin in PBS for 1-7 days at 25°C. Paraffin-

embedded tissue blocks were sectioned, mounted on glass slide, and tissue sections were treated and labeled with primary mAbs as described above. Antigen retrieval for seprase was performed for these sections before antibody incubation. Briefly, hydrated tissue sections were digested with Protease XXIV (Biogenex, San Ramon, CA) for 5 min and then washed with Tris buffer. Following antibody incubation and wash, the slide was then incubated with biotin-conjugated goat anti-rat antibody (Jackson Immunoresearch, Wet Grove, PA) at a 1:500 dilution for 20 min. Strepavidin-alkaline phosphatase conjugate (Jackson Immunoresearch) was used at a 1:500 dilution to detect the reactive antibodies. Fast red chromogen in napthol phosphate reconstitution buffer (Biogenex) was used for color development, and the section was counterstained with hematoxylin. Negative control sections were processed with PBS instead of specific antibodies. The anti-cytoskeleton mAb BNH9 (Immunotech) was used as a positive control staining for carcinoma cells.

Immunoblotting analysis. For immunoblotting, 7.5 μg of proteins were separated by SDS-PAGE in 7.5% polyacrylamide gels, and transferred to nitrocellulose sheets (Schleicher & Schuell, Keene, NH) by electro blotting as described previously [18]. The membranes were blocked with 5% nonfat dry milk in TBS containing 0.1% Tween 20 (TBS-T) overnight at 4°C and subsequently probed with three mAbs: D8 and E97 directed against seprase and E19 against DPPIV, each hybridoma supernatant diluted at 1:30 in TBS-T for 1 h at 25 °C. Immunoreactive polypeptides were visualized by incubation with horseradish peroxidase-conjugated anti-rat antibodies (1:4000) followed by the enhanced chemiluminescence detection system (Amersham Corp.) as previously described [18].

Gelatin zymography and DPPIV substrate membrane overlay assay. Aliquots from concentrated tissue or cell extracts were diluted in SDS sample buffer and analyzed for gelatinase activity by gelatin zymography as previously described [5], and for DPPIV proline-specific peptidase activity using a substrate overlay membrane coupled with the fluorescent

substrate Ala-Pro-7-amino-4-trifluoromethyl coumarin (Enzyme Systems Products, Dublin, CA) as described [7,19].

Enzyme-linked immunosorbent assay. Using a two-antibody sandwich ELISA, seprase and DPPIV were quantified in breast cancer and adjacent normal tissues. A 96-well ELISA plate (Dynatech Immulon I, Alexandria, VA) was precoated with 100 μl/well of mAb D8 or E19 (10 μg/ml; protein free supernatant in TBS-T) to capture the antigen. Nonspecific binding sites were blocked with 200 μl/well of 1% BSA in TBS-T at room temperature for 1 h. After washing with TBS-T three times, 2 μg/100 μl/well of WGA-purified glycoproteins were loaded and incubated at 4°C for 1 h. After three washes, captured antigens were incubated with 100 μl/well of biotinylated mAb D28 (1 μg/ml) or F4 (1 μg/ml) at 4°C for 1 h. The plate was washed with TBS-T, and labeled with alkaline phosphatase-conjugated goat anti-biotin IgG (Sigma Chemical Co., St. Louis MO). Color reaction used the alkaline phosphatase substrate kit (Bio-Rad Laboratories, CA), and it was measured at 405 nm in a microplate spectrophotometer (SPECTRAmax™ 250, Molecular Devices Co., CA).

Statistical Analysis. All values were presented as means ± SE. The significance of the difference was analyzed using the Mann-Whitney *U* test for unpaired observations, and a *P* value of < 0.05 was considered statistically significant. All statistical analyses were performed using statistical software (SPSS for Windows; SPSS, Chicago, IL).

RESULTS

To investigate the expression of seprase and DPPIV on the invadopodia of breast cancer cells, the invasive breast carcinoma cell lines Hs578T and MDA-MB-436 were examined utilizing phase contrast and immunofluorescence microscopy. Hs578T and MDA-MB-436 cells are malignant cells with a high metastatic potential in nude mice. Table 1 compared the results from the invasion assay of various human breast cancer cell lines and the malignant melanoma

cell line LOX using the method described [16] to those from experimental metastasis models described previously [20]. Hs578T and MDA-MB-436 (data not shown) cells cultured on crosslinked gelatin films were observed using phase contrast microscopy (Fig. 1 *A* and *D*), while the distribution of the membrane-bound proteases: seprase, DPPIV, and MT1-MMP were observed using immunofluorescence microscopy (Fig. 1 *B*, *C*, *E* and *F*). Both seprase and MT1-MMP show intense localization on invadopodia at the leading edge and under the cell body (Fig. 1, *B* and *C*). Similarly, DPPIV and MT1-MMP stained with mAb E19 and pAb, respectively, were also observed under immunofluorescence microscopy (Fig. 1, *E* and *F*). To our best knowledge, the expression of seprase and DPPIV in these breast cancer cell lines has not been previously reported.

Furthermore, RT-PCR analysis was performed to confirm the presence of seprase and DPPIV mRNAs in breast cancer cell lines. We carried out RT-PCR of MDA-MB 231, MDA-MB-436, and MCF7 total RNAs using oligonucleotide primers that correspond nucleotide sequences within the 5' untranslated region (FAP 1) and the 3' untranslated region (FAP 6) of the FAPα cDNA [8,12]. A seprase PCR product of approximately 2.4 kb which encodes the 97-kDa subunit was detected in only the MDA-MB-436 breast carcinoma cells (Fig. 2*A*). Using the DPPIV specific primers of DPPF-2 (sense) and DPPR-2 (antisense), a ~ 2.8 kb amplicon which encodes the 110-kDa subunit was found in MDA-MB-436 and MCF7 breast carcinoma cells (Fig. 2*B*). Also, RT-PCR generated identical products for seprase and DPPIV, respectively, from MDA-MB-436 and WI-38 fibroblast mRNAs. To examine whether seprase and DPPIV are expressed in breast carcinoma cells, we used the anti-seprase mAbs D8 and E97 as well as anti-DPPIV mAb E19 to detect these antigens by immunoblotting. Confirming the findings by RT-PCR, both MDA-MB-436 and MCF7 breast carcinoma cells expressed DPPIV (Fig. 2*E*), whereas only the MDA-MB-436 breast carcinoma cells expressed seprase (Fig. 2, *C* and *D*). Additionally, the mAb D8 recognizes the 170-kDa seprase and its 97-kDa subunit in the MDA-

MB-436 breast carcinoma cells under nonreduced and nonboiled conditions (Fig. 2*F*, lane 1), while only 97-kDa protein was observed under reduced and boiled conditions (Fig. 2*F*, lane 2).

We performed the morphological analysis of seprase and DPPIV distribution in malignant breast ductal carcinoma by immunohistochemistry. Using mAbs D8 (Fig. 3 Panels *A, C, E*) and E26 (Fig. 3 Panels *B, D, F*) that direct against seprase or DPPIV, respectively, a predominant brown cellular staining was seen in the infiltrating sheets and cords of poorly differentiated tumor cells (Fig. 3 open arrows), as well as microvessel endothelial cells (open arrowheads) and adjacent fibroblasts (Fig. 3 arrows). The immuno-reactivity of the neoplastic cells was more intense than the stromal cells in the same specimen. No reactivity could be observed in stromal fibroblasts and microvessel endothelial cells in tissues distant from tumor site (Fig. 3, *A, B*). In addition, strong stains were found in tumor cells of infiltrating sheets and poorly differentiated tumor mass, but there was no staining of seprase and DPPIV in these cells in the center of tumors (Fig. 3, *E* and *F*).

We then used another set of malignant breast ductal carcinoma samples using different procedure of immuno-staining. Using mAbs D8 (Fig. 4*A*) D28 and or D43 dierected against seprase, a predominant red cytoplasmic staining was seen in the infiltrating sheets and cords of poorly differentiated tumor cells (Fig. 4*A* open arrows) and adjacent fibroblasts (Fig. 4*A* arrows), and the immunoreactivity of the neoplastic cells was more intense than the stromal cells in the same specimen. No reactivity could be observed in the negative control (Fig. 4*B*) as compared to the mAb BNH9 (anti-cytokeratin) staining of tumor cells and stromal fibroblasts in the postive control (Fig. 4*C*). In addition, we demonstrated the expression of seprase on microvessel endothelial cells in breast cancer stroma using the mAb D28 in conjunction with factor VIII staining of these endothelial cells by immunofluorescence microscopy (Fig. 5, *A* and *B*), but there was no evidence of seprase expression on the microvessel endothelial cells in normal breast tissue adjacent to the tumors (Fig. 5, *C* and *D*). Taken together, our morphological study

demonstrates that seprase and DPPIV are expressed in stromal fibroblasts and angiogenic endothelial cell as well as tumor cells at the invasion front in malignant breast carcinoma.

Immunoblotting analyses of tumor and adjacent normal tissues show that mAb D8 recognized seprase (170 kDa) and its 97-kDa subunit (Fig. 6*A*), while mAb E97 labeled only the monomeric 97-kDa subunit (Fig. 6*B*). Seprase was more prominent in tumor tissues than in adjacent normal tissues, though the level of its expression was not quantitated. Immunoblotting analysis of DPPIV revealed that mAb E19 could recognize the 220-kDa dimeric form (Fig. 6*C*). However, the amount of DPPIV expression was variable between the tumor and adjacent normal tissues. To detect seprase activity in breast cancer tissues and adjacent normal tissues, we assayed their detergent lysates using gelatin zymography. Seprase gelatinase activity was observed with the presence of a lytic band at 170 kDa, but without detection of a lytic band at 220 kDa (Fig. 7*A*). The enzyme activity of DPPIV was confirmed with the appearance of a specific band at 220 kDa (Fig. 7*B*) using the substrate membrane overlay assay. Except for the normal tissue sample N3, all normal tissues exhibited endogenous seprase activity. This result corresponds to the immunoblot results for 170-kDa form of seprase in Fig 6*A*. Also, DPPIV activity was displayed in the two tumor tissue samples tested but in only one of the two normal tissue samples. Thus, except for the lack of DPPIV activity in normal tissue sample N1, no significant increase of seprase or DPPIV activity was found in tumor tissues than in adjacent normal tissues by the proteolytic assays. The expression of seprase and DPPIV in normal and cancerous tissues was summarized in Table 2. To quantitate differences in seprase and DPPIV expression between breast carcinoma and adjacent normal tissues, and to correlate this data with the corresponding qualitative data shown in Western blot and enzymatic activity analyses, we utilized an ELISA to determine the levels of seprase and DPPIV expression in the two sample groups. ELISA analysis showed that seprase extracts from tumor tissues exhibited a 6-fold increase over extracts from adjacent normal tissues (1.251 ± 0.308 versus 0.196 ± 0.070)

(Fig. 8), and differences were statistically significant ($p<0.05$). On the other hand, there was no significant difference in DPPIV levels between the two groups ($p>0.05$).

DISCUSSION

Tumor invasion and metastasis represent multi-step processes that depend on the proteolytic degradation of the ECM which is brought about by various proteases. Among the variety of proteases with potential involvement in tumor invasion and metastasis, integral membrane proteases, because of their ability to degrade the matrix and because of their location on invadopodia that directly contact the ECM, are of particular interest. The 170-kDa membrane-bound gelatinase, seprase, was originally identified from a human malignant melanoma cell line LOX [5,21,22]. DPPIV, an integral membrane serine protease that is variably expressed by melanoma cell lines, is proposed to form with seprase a subfamily of membrane-bound proteases [12,13].

This present study demonstrated the co-expression of seprase and DPPIV in breast cancer cell lines Hs 578T and MDA-MB-436 by immunofluorescence microscopy, as well as the expression of MT1-MMP. Moreover, using RT-PCR and the immunoblotting analysis we have detected the simultaneous presence of seprase and DPPIV mRNAs as well as both proteolytic enzymes in the MDA-MB-436 cell line, respectively. In addition, breast cancer tissues also exhibited seprase and DPPIV expression. Using Western blot analysis, we found that the level of seprase expression was higher in tumor tissues than in adjacent normal tissues. However, only one out of the four tumor tissues showed a markedly higher expression of seprase as determined by gelatin zymography. Possible causes for the gelatin zymography results might be that there are activated fibroblasts present within the adjacent "normal tissues" and/or that the gelatin zymography assay is more sensitive. In contrast to the result from Bermpohl et al. [13], apparently, we could not detect the gelatinase activity of DPPIV in our gelatin zymography assay. One explanation might be that seprase and DPPIV do not form distinct bands in gelatin

zymography and thus form a single diffuse band of lysis. Unlike seprase, Western blot analysis of DPPIV expression in tumor and adjacent normal tissues was variable. By means of immunohistochemical study of *in vivo* tissues, we have observed that not only is seprase expressed in stromal fibroblast cells that are adjacent to epithelial tumor cells but also by the tumor cells themselves, which is in accordance with the presence of seprase mRNA in *in vitro* breast cancer cells and fibroblasts. Furthermore, seprase was also shown to be expressed by endothelial cells within tumor stroma by immunofluorescence microscopy. Finally, after a quantitative assessment by ELISA, we have determined that there is an increased level of seprase expression in breast cancer tissues compared to their adjacent normal tissues. However, the expression of DPPIV was found not to be significantly different between the two groups. During the preparation of this manuscript, Kelly *et al.* [15] have reported that seprase is overexpressed in invasive ductal carcinoma cells of human breast cancers as determined by immunohistochemistry, which is consistent with our results determined by ELISA analysis.

Our findings showed that the expression of seprase in breast carcinomas appeared to be predominately associated with epithelial tumor cells, stromal fibroblasts, and endothelial cells. The co-expression of seprase and DPPIV in tumor and stromal cells also suggested that stromal-epithelial interactions by direct cell-cell contact might induce expression of these proteases in breast carcinoma. Consistent with this observation, Himelstein *et al.* presented evidence that expression of fibroblast 92-kDa gelatinase/type IV collagenase could be induced by direct contact with metastatic tumor cells [23]. It was suggested that the amount of its expression was associated with the activity of the individual tumor cells [24]. Kelly *et al.* also indicated that the immuno-reactivity of seprase in stromal cells and morphologically normal cells was lower than that in breast cancer cells [15]; thus, Himelstein's observations might explain the different levels of seprase expression between cancer and adjacent normal tissues. In our studies, the localization of seprase on the invadopodia of breast cancer cells further confirmed that tumor cells directly contact the ECM and proteolytically degrade the underlying adhesion

molecules. Therefore, it seems likely that increased production of seprase in cancer tissues may be closely related to tumor invasiveness. On the other hand, the presence of seprase in activated normal tissues suggests that it has a role in normal tissue remodeling or wound healing. Additionally, the significance of seprase expression in the endothelium is unclear. It has been pointed out that endothelial cells undergo rapid proliferation during angiogenesis of human breast cancer [25]; thus seprase expression may be correlated with multiple functions in tumor metastasis: activation of invasive carcinoma cells and stromal fibroblasts, and with tumor blood vessel development. Despite the result that the level of DPPIV expression in cancer tissues is not significantly higher than that in adjacent normal tissues, we could not exclude its participation in breast cancer invasion and metastasis with our limited number of analyzed samples. DPPIV was proposed to participate in final collagen degradation, which is generally initiated by interstitial collagenase [13]. And Van den Oord *et al.* [26] has indicated that DPPIV may play a part in the early invasion of malignant melanoma. Moreover, the presence of MT1-MMP, seprase and possibly DPPIV in breast cancer cells suggests the potential cooperation of these proteases in regulating matrix proteolysis and hence facilitating invasion.

In summary, our data suggests that the association of seprase with tumor cell invasiveness is extended from tissue culture to malignant breast cancer tissues in situ. And we detected its expression not only in tumor cells but also in stromal cells as well. Thus seprase might be a prognostic marker of an early event in the progression of tumor invasion and metastasis, and it could provide a new direction for the design of a therapeutic strategy for the control of cancer spread.

The membrane type MMPs (MT-MMPs) are a subclass of the MMP family which uniquely possess a C-terminal transmembrane domain and are initiators of an activation cascade for progelatinase A (MMP-2) [17,27]. Recently, several research groups have reported that MT-MMPs can efficiently degrade a number of matrix macromolecules [28-31]. It has been suggested that MT-MMPs have to be present on tumor cells for the binding and activation of

MMP and then for subsequent coordination of tissue proteolysis and cell movement through the matrix [32]. Membrane type 1-matrix metalloproteinase (MT1-MMP), like seprase, is an integral membrane protease known to be overexpressed in carcinoma cells and on surrounding stromal fibroblasts [33,34]. And there is increasing evidence which indicates that expression of MT1-MMP in tumor cells plays an important role in tumor invasion, as well as in metastasis [33-35].

Table 1. ***Invasive and metastatic potentials of selected human breast cancer cells.***

Cell lines	Tumor[1] type	Degradation of FN, MG-Gel film[2]	Expression of seprase/Localization of invadopodia	Nude mouse metastasis[3]
LOX	A MEL	++++	+	HM
Hs578T	CS	++	+	HM
MDA-MB-436	AC	+	+	LI
SK-Br-3	AC	+	–	N
MDA-MB-435	IDAC	+	–	LI
MCF7	IDAC	–	–	P
BT549	PIDC	–	–	N

[1]Tumor type by histolopathologic diagnosis: A MEL, amelanotic melanoma; AC, adenocarcinoma; IDAC, infiltrating ductal carcinoma; CS, carcinosarcoma; PIDC, papillary infiltrating ductal carcinoma.

[2]Degradation of gelatin film covalently coupled to fibronectin that is labeled with rhodamine and degradation of fluorescein conjugated matrigel. Number of degradation spots by each cell line was compared with that of LOX cells shown as "++++".

[3]From Thompson *et al.* (1992). Activity in the athymic nude mouse: N, non-tumorigenic; P, primary tumor formation, no local invasion or metastasis; LI, local invasion through the peritoneum, colonization of visceral organs; HM, hematogenous metastasis to the lungs. NA, not available.

Table 2 *Summary of seprase and DPPIV expression in breast cancer and adjacent normal tissues*

	Immunoblotting analysis		Proteolytic activity	
Tissue no.	Seprase	DPPIV	Seprase	DPPIV
T1	+	++	++	+
T2	+++	+++	++	++
T3	+	–	++	NA
T4	+	+	++	NA
N1	+	++	++	–
N2	++	+++	++	+
N3	–	–	–	NA
N4	+	++	++	NA

T, tumor; N, adjacent normal tissue; –, no expression of the protein; +, expression of the protein; NA, not analyzed.

FIGURE LEGENDS

Fig. 1. Co-localization of seprase, DPPIV and MT1-MMP on invadopodia of breast carcinoma Hs 578T cells.

A and *D*, are images observed using phase contrast microscopy. *B* and *C*, seprase and MT1-MMP, respectively, on invadopodia of the cell shown in panel A were detected at the cell body (closed arrows) and at the leading edge (open arrows) of an Hs 578T cell using immunofluorescence microscopy. Cells were doubly labeled with rat anti-seprase mAb D28 and rabbit anti-MT1-MMP peptide pAbs, and detected with fluorescent conjugates of secondary antibodies against rat and rabbit, respectively. *E* and *F*, DPPIV and MT1-MMP, respectively, co-localized at invadopodia of the cell shown in panel D were detected at the cell body (closed arrows) and at the leading edge (open arrows) of an Hs 578T cell using immunofluorescence microscopy. Scale bars: 25 μm.

Fig. 2. RT-PCR analysis of seprase and DPPIV expression in breast cancer cells MDA-MB-231, MDA-MB-436 and MCF7, and the detection of seprase and DPPIV expression in these cells by Western blot analysis.

A and *B,* RT-PCR was carried out on MDA-MB-231, MDA-MB-436 and MCF7 RNAs using DPPIV and seprase specific primers, respectively. Only MDA-MB-436 cells could be shown to produce the seprase PCR product of ~2.4 kb which encodes 97-kDa subunit (Panel *A*). Human WI-38 fibroblast RNA, used as a positive control, generated an identical amplicon pattern as the MDA-MB-436 breast carcinoma cells. A DPPIV PCR product of ~2.8 kb which encodes the 110-kDa subunit was detected in the postive control WI-38, and from MDA-MB-436 and MCF7 RNAs on agarose gels containing ethidium bromide (Panel *B*). The two intense lower bands detected in the MCF7 lane were also present in the minus reverse transcriptase control (data not shown). *C-E*, Western blot analysis of seprase and DDPIV expression in breast carcinoma cells using the mAbs: D8 and E97 directed against seprase and E19 against DPPIV. The mAb D8 recognizes both seprase and the 97-kDA

subunit, but mAb E97 recognizes only the monomeric 97-kDa subunit. Similar to RT-PCR analysis, only MDA-MB-436 cells expressed seprase (Panel *C* and *D*), while both MDA-MB-436 and MCF7 breast carcinoma cells expressed DDPIV (Panel *E*). The mAb D8 recognized the 170-kDa seprase and its 97-kDa subunit in the MDA-MB-436 breast carcinoma cells under nonreduced and nonboiled conditions (Panel *F*, *lane 1*), whereas only 97-kDa protein was observed under reduced and boiled conditions (Panel *F*, lane *2*).

Fig. 3. Detection of seprase and DPPIV in malignant breast ductal carcinoma cells and adjacent stromal cells in formalin-fixed, paraffin embedded neoplastic breast tissue samples by immunohistochemistry.

mAbs D8 (Panels *A, C, E*) or E26 (Panels *B, D, F*) were used for the immunohistochemical analysis of seprase or DPPIV expression. *A, B,* tissues approximately 2 cm distance from tumor sites that were stained with mAbs D8 or E26. Negative seprase or DPPIV stains of tumor cells were indicated by black open arrows; endothelial cells by black open arrowheads; and fibroblasts by black arrows. *C, D,* infiltrating sheets of poorly differentiated (high-grade) tumor cells with predominant brown cellular stains of seprase and DPPIV in tumor cells (orange open arrows), in fibroblasts (orange arrows), and in some endothelial cells (orange open arrowheads) but not in some larger vessel lining cells (black open arrows). A – D Scale bar = 100 μm. *E, F,* low magnification view of infiltrating sheets of poorly differentiated (high-grade) tumor cells with predominant brown cellular stains of seprase and DPPIV in tumor cells (orange open arrows), in fibroblasts (orange arrows), and in some endothelial cells (orange open arrowheads) but not in some larger vessel lining cells (black open arrows). Note that seprase and DPPIV are specifically expressed in tumor cells at the invasion front as indicated by most tumor cells in the field but are absent in these in the center of tumors as indicated in the center of the field. Scale bar = 800 μm.

Fig. 4. Detection of seprase in malignant breast ductal carcinoma cells and adjacent fibroblasts in formalin-fixed, paraffin embedded neoplastic breast tissue samples by immunohistochemistry.

mAbs D8 (Panel *A*) D28, or D43 (not shown) were used for the immunohistochemical analysis of seprase expression in breast carcinoma tissues. *A*, infiltrating sheets of poorly differentiated (high-grade) tumor cells with predominant red cytoplasmic staining of seprase (open arrows). Seprase was also detected in stromal fibroblasts (arrow) within tumor tissues. Cell nuclei are stained blue. *B*, Negative control shows infiltrating cords of cancer cells without red staining. *C*, Positive control utilizing mAb BNH9 (anti-cytokeratin) shows clusters and cords of infiltrating tumors cells with strong red staining. Scale bars = 100 μm.

Fig. 5. Immunofluorescent localization of seprase in microvessel endothelial cells of infiltrating ductal breast carcinoma but not in that of adjacent normal tissues.

A and *B*, tumor microvessel endothelial cells were identified using goat anti-human factor VIII related antigen pAb (Panel *A*, arrows) and are also labeled by anti-seprase mAb D28 (Panel *B*, arrows) using immunofluorescence microscopy. *C* and *D*, microvessel endothelial cells in normal tissues adjacent to tumors were stained with goat anti-human factor VIII pAb (Panel *C*, arrows). In contrast, seprase was not found in microvessel endothelial cells of adjacent normal tissues (Panel *D*, arrows). Scale bars = 100 μm.

Fig. 6. Detection of seprase and DPPIV expression in breast cancer and adjacent normal tissues by Western blot analysis.

A and *B*, immunoblot analysis for seprase (170 kDa) and its monomeric 97-kDa subunit derived from cancerous and adjacent normal tissue extracts using mAbs D8 and E97, respectively. The mAb D8 recognizes both seprase and the 97-kDA subunit, but mAb E97 recognizes only the monomeric 97-kDa subunit. The tissue extracts were purified by WGA-Sepharose chromatography. *C*, immunoblot analysis

for DPPIV derived from tissue extracts using mAb E19. The mAb E19 recognizes the native 220-kDa protein.

Fig. 7. Detection of tumor-associated seprase and DPPIV proteolytic activities using gelatin zymography and the substrate membrane overlay assay, respectively.

A, WGA-purified tissue extracts were assayed for seprase proteolytic activity by gelatin zymography in the presence of a buffer containing EDTA to suppress any gelatinase activity generated by metalloproteases. Seprase gelatinase activity was detected with the appearance of a lytic region at ~170 kDa. *B*, DPPIV proteolytic activity was observed with the production of a diffuse band at 220 kDa with the substrate membrane overlay assay.

Fig. 8. Quantitation of seprase and DPPIV levels of expression utilizing the enzyme-linked immunosorbent assay.

ELISA was carried out using mAbs D8 or E19 to capture seprase or DPPIV antigens, respectively, with biotinylated mAbs D28 (anti-seprase) and F4 (anti-DPPIV) used for detection of the captured antigens.The graph shows the reading obtained two hours after the color reaction was initiated. The reading for seprase was six-fold higher in breast cancer tissues (■) as compared to their adjacent normal tissues (□) ($p<0.05$). No significant difference was observed in the expression of DPPIV between breast cancer tissues and adjacent normal tissues ($p>0.05$). Results represent the mean optical density ± SE (n = 4) as described in "Materials and Methods". *, statistically significant between the two groups ($p<0.05$).

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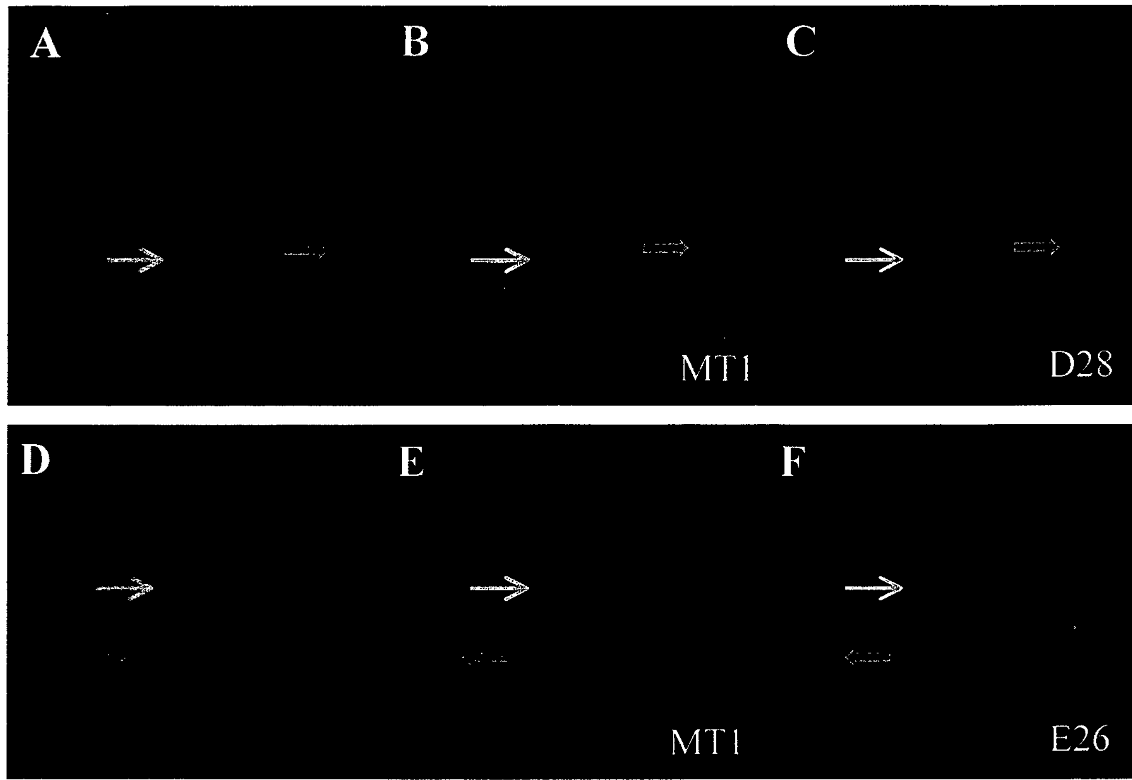
Figure 1



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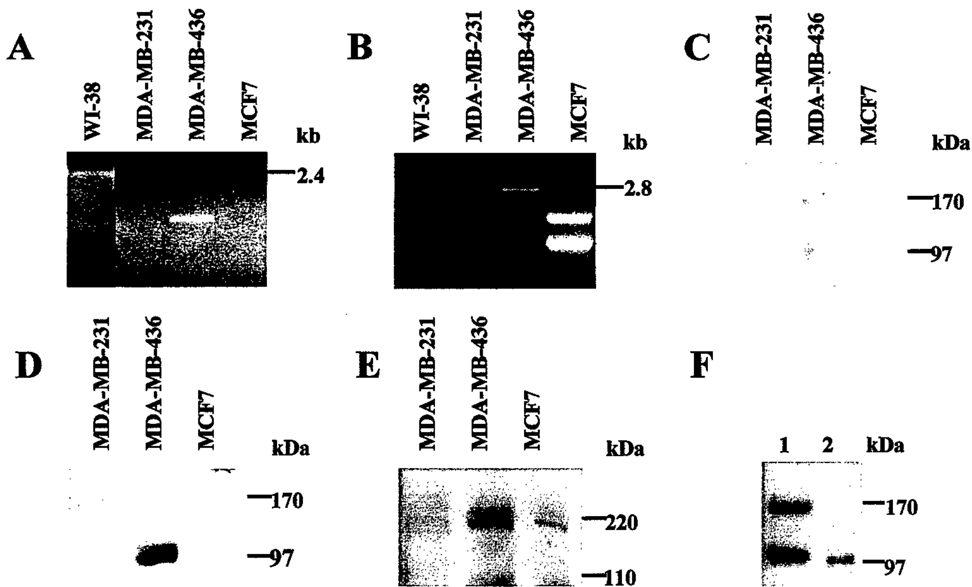

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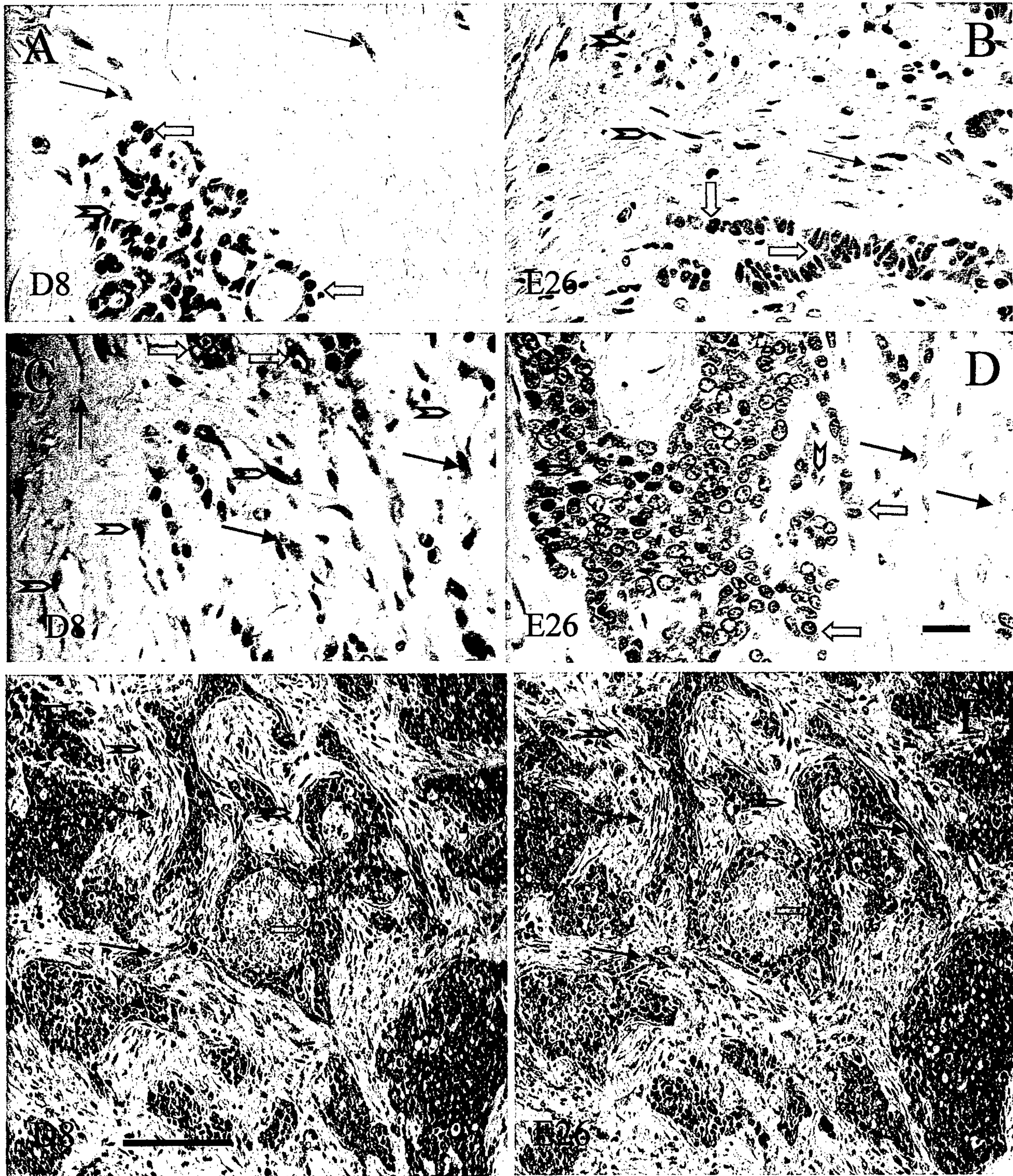

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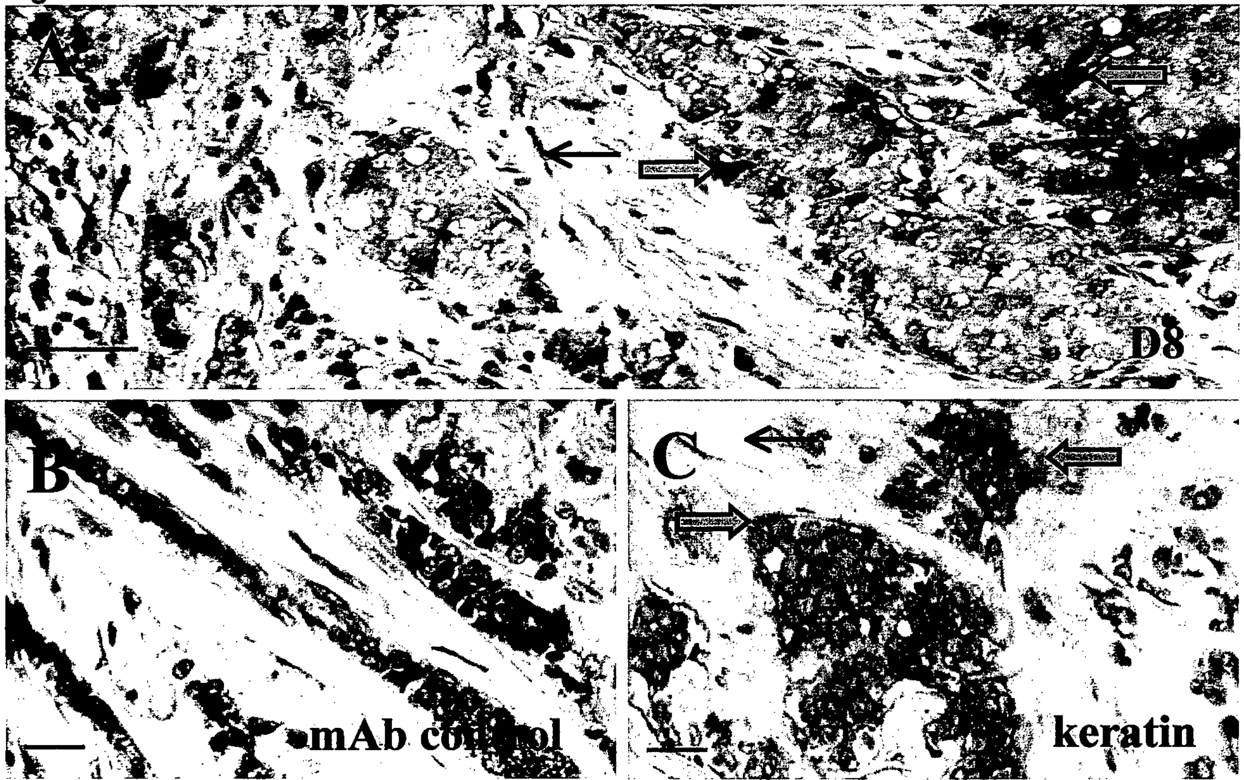

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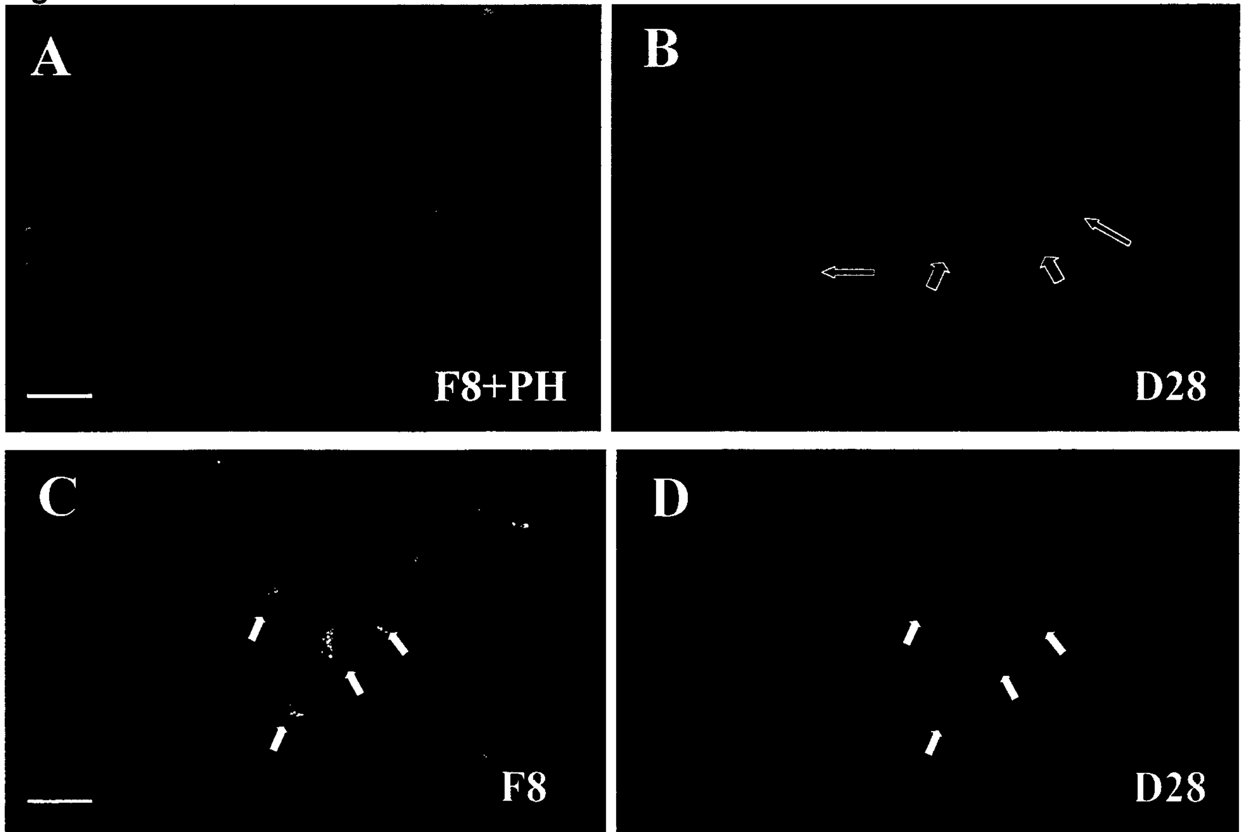

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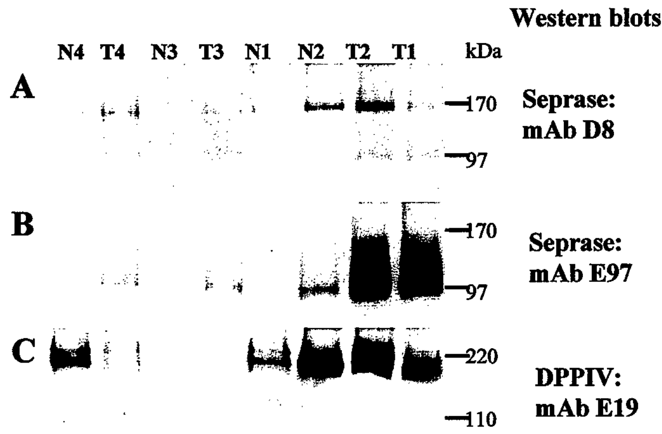

Figure 7

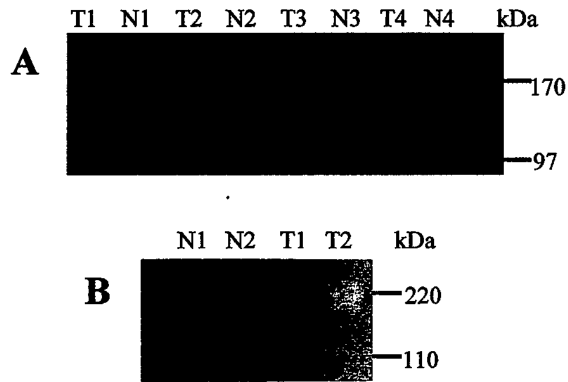

Figure 8