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Award Number: DAMD17-98-1-8160

TITLE: Examination of the Role of Membrane Type-1 Matrix Metalloproteinase (MTI-MMP) in Breast Cancer Metastasis

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REPORT DATE: August 2001

TYPE OF REPORT: Annual Summary

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 2001	Annual Summary (1 Aug 00 - 31 Jul 01)

4. TITLE AND SUBTITLE
Examination of the Role of Membrane Type-1 Matrix Metalloproteinase (MTI-MMP) in Breast Cancer Metastasis

5. FUNDING NUMBERS
DAMD17-98-1-8160

6. AUTHOR(S)
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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

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 Words)*

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
9

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

Table of Contents

Principal Investigator: Jian Cao, M.D.
Mentor: Stanley Zucker, M.D.
Grant Number: DAMD17-98-1-8160
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This postdoctoral research training grant entitled "examination of the role of membrane type 1-matrix metalloproteinase (MT1-MMP) in breast cancer metastasis" was initiated in August 1998 and terminated in July 2001. This grant provided Dr. Cao with a great opportunity to be trained to fight the war against breast cancer. Reviewing the progress of the past three years, Dr. Cao reached the goals that he originally proposed. His work during the last three years was highly recognized in the field as demonstrated by several awards including a Young Investigator award and Scholar-in-Training award from American Association for Cancer Research (AACR) and by his publications. During the training period, Dr. Cao was promoted to a Research Assistant Professor which fulfils the goal that Dr. Cao initially proposed of becoming an independent scientist. Dr. Cao is very appreciative of the US Army Medical Research and Material Command committee for providing him with such a training opportunity in a highly competitive field.

As we know, metastasis from breast cancer remains a major stumbling block in treatment. Critical steps in this disseminated process include extracellular matrix degradation and cell migration. Based on the examination of pathologic breast cancer tissues and experimental models, MT1-MMP has been proposed to be involved in breast cancer dissemination. MT1-MMP is able to activate progelatinase A on the surface of tumor cells by producing an initial cleavage in the N-terminal propeptide domain followed by an autolytic cleavage. Because of its central role in cell surface proteolysis, a more complete understanding of the role of MT-MMPs in cancer will expedite progress in therapy of metastasis. To this end, this project has been focused on the role of MT1-MMP in experimental breast cancer invasion and metastasis.

Task 1. Examination of the role of wild type membrane type-1 matrix metalloproteinase (MT1-MMP) in breast cancer metastasis

To directly address the role of MT1-MMP in experimental breast cancer, human breast cancer cell line, MDA-MB 436 that does not express MT1-MMP (confirmed by Western blot and Northern blot), was used in this project. Green fluorescent protein (GFP) was employed as a tumor cell marker at metastatic sites and also as a visible fluorescent tag to localize MT1-MMP fusion molecules within cells. Since a deletion mutant of MT1-MMP lacking the C-terminal cytoplamic tail of MT1-MMP does not lose the function in terms of progelatinase A activation, GFP tag was fused into the C-terminus of MT1-MMP. By gelatin zymography, the MT1-MMP/GFP chimera was able to cleave progelatinase A and further induced gelatinase A activation. Alternatively, the distribution of MT1-MMP\GFP was examined in transiently transfected MDA-MB-436 cells. GFP fluorescence exhibited diffuse distribution in transfected cells. In contrast, MT1-MMP\GFP accumulated primarily in the endoplasmic reticulum and in the perinuclear golgi apparatus of the transfected cells. Fluorescence on the leading edge of plasma membrane was also observed but seems limited in vitro. By

immunofluorescent study using a MT1-MMP antibody and TRITC conjugated anti-mouse IgG, the same profiles of MT1-MMP transfected cells were noted. These data indicated that MT1-MMP primarily accumulated in the perinuclear region of transfected cells and may require a signaling mechanism to facilitate membrane trafficking; GFP does not change the distribution of fused protein.

To better understanding the role of MT-MMP in human breast cancer metastasis, a stable cell line expressing MT-MMP was established. MDA-MB-436 cells were transfected with MT1-MMP plasmids by calcium phosphate method and stable clones survived in G418 conditioned media were selected by fluorescent microscopy. Fluorescent clones were further examined by gelatin substrate zymography. The clone expressing MT1-MMP/GFP chimera protein and inducing progelatinase A activation was expanded for further experimentation.

Using a FITC-labeled fibronectin (Fn) cross-linked gelatin film, MDA-MB-231 cells transfected with MT1-MMP/GFP cDNA were examined for invasive ability including substrate degradation (demonstrated by loss of fluorescence of FITC-labeled Fn) and migration over the substrates. An antibody to MT1-MMP was used to identify transfected cells (immunofluorescent technique). Consistent with previous reports, MT1-MMP degrades Fn. Interestingly, cells producing MT1-MMP migrated over Fn and presented a coil-like track resembling a finger print. The digestion of Fn was dramatically increased by co-expression of MT1-MMP with pMMP-2 cDNA in cells, but was limited in area to the cell migration track. MDA-MB-231 cells tranfected with MMP-2 cDNA alone produced minimal substrate degradation. This suggests that only cell surface-bound activated progelatinase A along with MT1-MMP digest substrate. Co-transfection of these cells with TIMP-1 cDNA resulted in inhibition (down to the basal digestion level resulting from MT1-MMP) of the digestion of Fn induced by the cells producing both MT1-MMP and MMP-2, but did not interfere with cell migration. In contrast, co-transfection with TIMP-2 cDNA totally abolished both the digestion and the migration of cells expressing MT1-MMP and pMMP-2. Based on these data, I conclude that functionally active MT1-MMP is required for cancer cell migration and contributes to Fn degradation.

Using GFP as a tag molecule to trace the invasive ability of MDA-MB-436 stable expressing MT1-MMP, I found MT-MMP enhanced the invasive ability of breast cancer cells more than two-fold compared with vector transfected cells. Native MDA-MB-436 cells produce slow growing, poorly invasive tumors. Injection of MDA-MB-436 cells stably transfected with MT1-MMP/GFP cDNA into the inferior mammary fat pad of female nude mice resulted in enhancement of tumor growth and local metastasis as compared to GFP alone transfected tumor cells. Thus, MT1-MMP may trigger the invasion of breast cancer cells by activating progelatinase A and thereby enhance tumor metastasis.

Task 2. Determine whether soluble forms of MT1-MMP play a role in experiment breast cancer dissemination (month 18-30)

To examine whether soluble forms of MT1-MMP play a role in experimental breast cancer dissemination, I made a soluble MT1-MMP lacking the C-terminal transmembrane domain and cytoplasmic tail (MTΔTM) fused with GFP cDNA as a fusion protein. Without the transmembrane domain of MT1-MMP, this chimera is secreted into culture medium and is no longer anchored on the plasma membrane (examined by immunofluorescent

assay). To directly investigate the substrate specificity of MTΔTM, I purified the soluble form of MT1-MMP by using a His tag. A cDNA encoding the His tag was fused to the C-terminus of mutant MT1-MMP lacking the transmembrane domain as a chimera MTΔTM/His. MTΔTM/His purified from transfected COS-1 cells appeared as 63kDa and 57 kDa proteins in conditioned medium and both forms of MTΔTM/His (active form of MT1-MMP) have enzymatic activity against gelatin as examined by gelatin zymography. MTΔTM/His is also able to digest type I and type IV collagen using a commercially available fluorescent labeled substrate. It was also noted that purified MTΔTM/His can activate progelatinase A. Therefore, the soluble form MT1-MMP appears to play an important role in the degradation of components of the basement membrane.

Stable cell lines for both MTΔTM/GFP and MTΔTM/His were established for stable expression in MDA-MB-436 cells and CHO-K1 cells, respectively.

The invasive ability of MTΔTM/GFP was examined using in vitro model systems. MDA-MB-436 cells transiently transfected with MTΔTM/GFP, MT-MMP/GFP, MTΔC which lacking only cytoplasmic portion of MT1-MMP and vector control, cDNAs were plated on FITC-labeled Fn-gelatin film followed by immunohistochemistry using anti-MT1-MMP antibody. MT1-MMP and MTΔC producing cells degrade Fn and migrate over digested substrate. In contrary, cells producing soluble MT1-MMP were able to neither digest Fn substrate nor migrate over the substrate. The defects of substrate digestion and cell migration were not due to an effect on protein synthesis (examined by Western blotting). Similar results were obtained using a Boyden chamber cell migration assay. These data clearly demonstrate that membrane anchorage of MT1-MMP is necessary and sufficient for MT1-MMP mediated cancer invasion and metastasis Although soluble MT1-MMP (shed MT1-MMP) maintains some of the biochemical characteristics of wild type enzyme, the cell migration effect is not manifest. These data raise some interesting questions to be answer. Purified soluble MT1-MMP can activate progelatinase A as well as cells producing soluble MT1-MMP, but cells producing soluble MT1-MMP failured to initiate cell migration. What is the role of activated gelatinase A in cell migration? Does the MT1-MMP shedding mechanism represent an inactivation process? Currently, these puzzles are being addressed by making recombinant cDNAs and using in vitro migration model system.

Since soluble MT-MMP did not enhance the invasive ability of MDA-MB-436 cells, it is not necessary to continue performing in vivo studies for examination of soluble form of MT1-MMP in breast cancer metastasis.

Task 3. Investigate the trafficking and localization of MT1-MMP in transfected breast cancer cells (months 25-36).

A) We have demonstrated that Con A induces rapid activation of progelatinase A and rapid appearance of MT1-MMP at the cell surface by gelatin zymogram, cell surface biotinylation techniques, and ^{125}I-TIMP-2 bind assay, respectively in HT1080 cells. By employing techniques for endocytosis , I demonstrated that endocytosis of MT1-MMP occurred in HT1080 cells transfected with MT1-MMP cDNA. Endocytosis of MT1-MMP was also present in MDA-MB-436 cells transfected with MT1-MMP cDNA. However I was not able to detect an effect of Con A, PMA or thrombin on the translocalization of endogenesis MT1-MMP from ER/Golgi to plasma membrane in MDA-MB-231 which was originally proposed in my grant. Although Northern blotting demonstrated the existence of MT1-MMP mRNA, MT1-MMP protein was not detected by Western blotting in MDA-MB-231 cells using different antibodies (i.e. catalytic antibodies, 113 and 114; hinge antibody or propeptide antibody). Consistent with Western blotting, MDA-MB-231 cells treated with

Con A failed to activate progelatinase A, as evaluated by gelatin substrate zymogram. MDA-MB-231 and 436 transfected cells with MT1-MMP cDNA resulted in progelatinase A activation. Using transiently transfected cells expressing MT1-MMP/GFP chimera and GFP as a trace marker, the effect of Con A, PMA and thrombin on MT1-MMP localization was examined. No marked difference of MT1-MMP localization pattern between treated and untreated breast cancer cells was noted.

B) Pro-sequence of MT1-MMP serves as an intramolecular chaperone for the production of active MT1-MMP in transfected COS-1 cells.

The goal of this study is to further clarify the role of the propeptide domain of MT1-MMP in trafficking the molecule to the plasma membrane and in maintaining the function of the plasma membrane-inserted enzyme. We propose that the propeptide sequence of MT1-MMP serves as an intramolecular chaperone in protein folding. To explore the concept, we have co-transfected COS-1 cells with the plasmids encoding the N-terminal propeptide domain of MT1-MMP and the MT1-MMP cDNA lacking the entire propeptide sequence. We have demonstrated reconstitution of function of MT1-MMP. Co-tranfection of COS-1 cells with both expression vectors resulted in reconstitution of MT1-MMP function in terms of facilitating ^{125}I labeled TIMP-2 binding to transfected COS-1 cells and subsequent activation of progelatinase A. Transfection of cells with either cDNA alone or cDNA for the propeptide of collagenase-1 with MTΔpro resulted in non-functional cells. Smaller cDNA mutations of the open reading frame of the N-terminal propeptide of MT1-MMP were employed to delineate critical conserved regions of the molecule required as a membrane-bound enzyme. These data indicated that the MT1-MMP prosequence acts as an intramolecualr chaperone and is necessary for the correct folding of the MT1-MMP *in vivo*.

C) Intracellularly trafficking of MT1-MMP along with TIMP-2.

C1) Colocalization of MT1-MMP with TIMP-2 on the cell surface of MDA-MB-436 cells. MDA-MB-231 cells were co-transfected with MT1-MMP cDNA and TIMP-2 or TIMP-1 cDNA followed by immunofluorescent technique without permeablization using both anti-MT1-MMP and anti-TIMP-2 or TIMP-1 antibodies. Under fluorescent microscopy, MT1-MMP was detected on the plasma membrane of transfected cells as well as TIMP-2, but not TIMP-1. Both TIMP-2 and MT1-MMP were colocalized on the cell surface.

C2) Endocytosis of TIMP-2 along with MT1-MMP. MT1-MMP severs as a receptor for TIMP-2 as demonstrated in Section C1. Following trafficking of MT1-MMP to the cell surface, endocytosis of MT1-MMP occurs (task 3 A). Since MT1-MMP assembles a complex with TIMP-2 on the cell surface, it is reasonable to ask if TIMP-2 will undergo endocytosis along with MT1-MMP. To this end, HT1080 cells transfected with MT1-MMP cDNA and TIMP-2 cDNA were examined by endocytosis assay. The data demonstrated that both MT1-MMP and TIMP-2 were colocalized in the endocytosis pathway. TIMP-2 was not found to change the distribution of MT1-MMP on the cell surface.

D) Cell-cell interaction did not change the distributions of MT1-MMP/GFP.

Task 4. This postdoctoral fellowship grant provides Dr. Cao with an important experience of studying experimental animal models of breast cancer and in evaluating the effect of mutating MT1-MMP cDNA on the frequency of experimental metastasis. More importantly, Dr. Zucker meets Dr.Cao for 2 and a half hours every week to discuss experimental plans

and to analyze the results from completed experiments. This experience gave Dr.Cao the opportunity to learn how to become an independent investigator using molecular biology techniques to study the role of MMPs in tumor invasion and metastasis.

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2. **J. Cao**, M. Hymowitz, C. Conner, W. Bahou and S. Zucker: The propeptide domain of membrane type 1-matrix metalloproteinase acts as an intramolecular chaperon when expressed *in trans* with the mature sequence in COS-1 cells (2000), **J.Biol.Chem.**, Vol.275:29648-29653
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6. Zucker S, Hymowitz M, Rollo EE, Mann R, Conner CE, **Cao J**, Foda HD, Tompkins DC, Toole BP.
Tumorigenic potential of extracellular matrix metalloproteinase inducer. **Am J Pathol**. 2001 Jun;158(6):1921-8.
7.Zucker S, **Cao J**. Imaging metalloproteinase activity in vivo. **Nat Med**. 2001 Jun;7(6):655-6.

Book Chapters:
S. Zucker, **J. Cao**, C. Molloy, (2001), Role of matrix metalloproteinases and plasminogen activators in cancer invasion and metastasis: Therapeutic potential in anticancer drug development, edited by Bruce C. Baguley and Avid Kerr. **Academic Press** (in press)

Oral Presentations:
1. J. Cao, et al. The propeptide domain of membrane type 1-matrix metalloproteinase is required for progelatinase A activation and binding of TIMP-2. (1998) 89th Annual Meeting of the American Association for Cancer Research. New Orleans, LA
2. J. Cao, et al. The propeptide domain of membrane type 1-matrix metalloproteinase acts as an intramolecular chaperone. (2000) 91th Annual Meeting of the American Association for Cancer Research. San Francisco, CA
3. J. Cao, et al. The structure-functional relationship of membrane type 1-matrix metalloproteinase in cancer cell migration and substrate degradation. (2001) Membrane Bound Proteinase in Cancer, Palermo, Italy

Awards:
1. 1998 American Association for Cancer Research-Bristol-Myers Seuibb Young Investigator Award, New Orleans
2. 2001 American Association for Cancer Research Scholar-in-Training Award, New Orleans

3. 2001 Gorden Research Conference (MMPs) travel award, Italy