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ANNUAL PROGRESS REPORT

PROJECT TITLE: Examination of the unique role of membrane type 1-matrix metalloproteinase (MT1-MMP) in prostate cancer invasion and metastasis

PI: Jian Cao, M.D.

AFFILITY: State University of New York at Stony Brook

DATE: Nov. 30, 2002

This grant was initiated on January 1, 2002 with the goal of studying the role of membrane type 1 matrix metalloproteinase (MT1-MMP) in prostate cancer invasion and metastasis. The basic concept of this grant is that the membrane enzyme MT1-MMP plays a central role in prostate cancer metastasis. Although considerable progress has been made in the last decade in our understanding the pathophysiology of cancer invasion and metastasis, the pathobiology of prostate cancer is not well understood. Increasing evidence demonstrated that the ability of prostate cancer cells to migrate across basement membranes and to degrade extracellular matrix (ECM) relies on the activity of matrix metalloproteinases (MMPs). MT1-MMP is considered to be the most important proteinase involved in cancer cell migration, invasion and metastasis. The rational for this grant is based on the fact that production and activation of MMPs, especially proMMP-2 (pMMP-2), in prostate cancer is required for cancer cell invasion and metastasis. Cell motility is a critical determinant of prostate cancer metastasis. MT-MMPs are responsible for cell surface activation of pMMP-2, thereby representing a key factor in cancer progression. Regulation of MT-MMP function, particularly in cancer cell migration, a key step for metastasis is poorly understood; elucidation of this mechanism is important for anti-metastatic drug development. Therefore, the goal of this proposal is to elucidate the role of MT1-MMP in human prostate cancer cell motility, invasiveness, and metastasis and to develop reagents for reducing prostate cancer metastasis.

Task 1: Examine the role of MT1-MMP in experimental prostate cancer metastasis using green fluorescent protein (GFP), Months 1-16:

A: Establishment of a stable prostate cancer cell line expressing MT1-MMP/GFP fusion protein: This task will be documented by gelatin substrate zymography in terms of activation of pMMP-2, Northern blotting and Western blotting techniques.

To address the role of MT1-MMP in prostate cancer progression, LNCaP cells, a less aggressive tumor cell line that does not make any detectable MT1-MMP, were used in this experiment. LNCaP cells were transfected with MT1/GFP chimeric cDNA or GFP cDNA as a control followed by the selection of resistant cells in the presence of G418. Six-weeks later, GFP expressing resistant clones from both GFP and MT1/GFP transfected cells were isolated by cloning cylinder method (Bellco Glass, Inc. Vineland, NJ) based on the green fluorescence and expanded for further their characterization (Fig.1). Based on the intensity of green fluorescence, the clones were classified into three groups, low, medium and high expression for both GFP and MT1/GFP transfectants. Western blotting was employed to characterize the clones using anti-GFP antibody. GFP was identified as a 27kDa protein in cell lysate, while MT1/GFP was detected as 95 and 68 (degraded product) kDa proteins (Fig.2). The similar result was obtained using anti-MT1-MMP antibody.

By taking advantage of the localization capacity, fluorescent tagged MT1/GFP fusion protein was identified at the plasma membrane of stable cells expressing MT1/GFP under fluorescent microscopy as compared to the diffuse distribution of GFP. The plasma membrane localization of MT1/GFP was further conformed by immunofluorescent assay using anti-MT1-MMP hinge antibody (Rabbit) and Texas-Red conjugated anti-rabbit IgG. Co-localization of antibody-recognized MT1-MMP with GFP tagged MT1-MMP was noted under fluorescent microscopy. No MT1-MMP was detected in GFP expressing LNCaP cells by immunofluorescent assay(Fig.3).

To examine the function of stable cell lines expressing MT1/GFP in terms of pro-MMP-2 activation, substrate gelatin zymogram was performed. As expected, LNCaP cells expressing MT1/GFP resulted in pro-MMP-2 activation, but no dramatic difference in proMMP-2 activation was noted among non-MT-MMP clones. This could be explained by the different sensitivity of fluorescence visualization and enzymatic activity. No proMMP-2 activation was found in LNCaP cells expressing GFP.

Since MT1/GFP expressing clones produce fusion protein and have fully enzymatic function as compared to wild type MT1-MMP, Northern blot was not performed as planned in original proposal.

B: Examination of the invasive ability of prostate cancer cells expressing MT1-MMP/GFP fusion protein: Invasion of cells through Matrigel coated filters will be measured.

Using a FITC-labeled fibronectin (Fn) cross-linked gelatin film, the invasive ability of MT1/GFP was examined for substrate degradation (demonstrated by loss of fluorescence of FITC-labeled Fn) and cell migration over the substrate. Consistent with previous reports, MT1-MMP expressing LNCaP cells degrade Fn. Interestingly, cells producing MT1/GFP migrated over Fn and presented a digested track resembling a finger print. Addition of recombinant MMP-2 in MT1/GFP producing cells markedly enhanced Fn degradation, but was limited in area to the cell migration track. The substrate degradation induced by cells expressing MT1/GFP was completely blocked by TIMP-2 or a synthetic MMP inhibitor, CT1746, but not by TIMP-1 (Fig.4). Inhibition of enzymatic activity of MT1/GFP by TIMP-2, however, had no effect on MT1/GFP mediated cells migration examined by phagokinetic assay.

By the Boyden chamber assay, the MT1/GFP invasive ability was conformed. $2X10^5$ LNCaP cells expressing GFP or MT1/GFP were plated onto polycabonate filters with 8 um pore size coated with gelatin for 18 hours in a cell culture incubator. Invasive/migratory cells on the lower surface were determined based on the staining of cells with crystal violet. GFP expressing cells has a basal level in terms of invasive ability while MT1/GFP enhances LNCaP cell invasion by approximately one-fold.

Matrigel invasion assay for LNCaP cells expressing MT1/GFP was unsuccessful because of technique problems with Matrigel coating (protein coating too thick). This assay has been modified and optimized based on the protocol described in Metastasis Research Protocols (Methods in Molecular Medicine, Vol 58, PP61-71). Currently, the invasive ability of cells expressing MT1/GFP is being undertaken by using optimized Matrigel Boyden chamber assay. Recently, I have successfully labeled Matrigel with FITC. FITC labeled Matrigel is being characterized and will be employed in future invasion assays.

C: Examine the effect of MT1-MMP transfection on the metastatic ability of prostate cancer cells: Approximately 10 nude mice in each group will be injected orthotopiclly with LNCaP

prostate cancer cells transfected with MT1-MMP/GFP fusion cDNA and control cDNA. Differences between groups will be assess by Analysis of Variance.

The tumorigenicity of MT1-MMP expressing LNCaP cells was evaluated by injection (s.c.) of MT1/GFP producing LNCaP cells (2 X 10^6/0.1 ml of 50% Matrigel) in NCI nu/nu mice. Visible tumor was noted at two weeks after injection for both MT1/GFP and GFP producing cells. Although no significant size difference of MT1/GFP tumors and GFP tumors was noted, marked weight loss was observed in mice bearing MT1/GFP tumor (Fig.5). The invasive and metastatic abilities of MT1/GFP tumors are under examination.

To ensure the success and accuracy of this study, Surgical Orthotopic Implantation (SOI) technique has been practiced and optimized under the supervised by Dr. H. Adler, a consultant of Urologist. Based on recent report by Chang XH et al. (Anticancer Res 1999 5B:4199-202) with improved metastatic animal model of human prostate carcinoma using SOI technique, we are planning to use tumor grafts for prostate metastasis assay. Since the experimental period for this study is relative long, the expected results may be slightly delayed.

Task 2: Examine the role of MT1-MMP and MMP-2 in prostate cancer cell migration and ECM degradation: Months 6-24:

A: Examine the role of MT1-MMP in ECM degradation and migration: This task will be performed by employing different approaches: a) LNCaP (a MT1-MMP negative cell line) transfected with MT1-MMP cDNA or PC-3/DU-145 (MT1-MMP positive cell lines) treated with or without Con A will be cultured on FITC-labeled type IV collagen or Fibronectin coated gelatin films. The substrate degradation and cell migration will be determined under fluorescent microscopy as described in section D2.1; b) a microcarrier-based chemotaxis assay will be performed to evaluate the specific role of MT1-MMP in prostate cancer cell migration.

Based on my recent data using FITC labeled substrate assay, I demonstrated that cell migratory ability induced by MT1-MMP is coordinated with cell surface localization of MT1-MMP. The more MT1-MMP on the cell surface, the stronger the substrate degradation and long migration track noted. These conclusions were further confirmed by examination of prostate cancer cells expressing endogenous MT1-MMP. Con A stimulated native PC-3 and Du-145 prostate cancer cells initiated substrate degradation resulted in the visible digested migratory tracks because of up-regulation and cell surface trafficking of endogenous MT1-MMP in spite of relative weak substrate degradation compared to cells transfected with MT1-MMP cDNA. In contrast, Con A was not able to initiate cellular invasion (substrate degradation and cell migration) for LNCaP cells because of lack of endogenous MT1-MMP.

A microcarrier-based chemotaxis assay will be employed in year 2 as planned.

B: Examine the role of cell surface-bound aMMP-2 as compared to soluble MMP-2 in substrate degradation and cell:
a)Determine the role of plasma membrane-bound aMMP-2 in substrate degradation and cell migration using the hemopexin-like domain of MMP-2 as a competitive inhibitor;

This experiment will be performed in year 2 as planned.

b) Utilize recombinant techniques to examine the role of surface-bound aMMP-2 in substrate degradation: surface-bound aMMP-2 will be first constructed by a two step PCR and resulted PCR fragment will be inserted into pcDNA3 expression vector. DNA sequencing analysis, gelatin substrate zymogram and Western blotting will be used to determine the new construct and FITC-labeled ECM substrate will be employed to evaluate the role of cell surface-bound aMMP-2 vs soluble MMP-2 on substrate degradation and cell migration.

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To examine the role of surface-bound, activated MMP-2 in MT1-MMP mediated substrate degradation and cell migration, a membrane anchored and theoretically active MMP-2 cDNA (aMMP2/TM) was constructed and confirmed by DNA sequencing. By Western blotting using anti-MMP-2 antibody, a major aMMP-2/TM product migrated as a 68 kDA protein band in cell lysate as compared to wild type MMP-2, but not in culture medium indicating membrane anchor of the fusion protein (Fig.6A). Zymogram of transfected COS-1 cell lysates demonstrated that aMMP2/TM retained gelatinolytic activity. To examine the conformation of aMMP2/TM, the distribution of aMMP2/TM and TIMP-2 in transfected COS-1 cells was determined by immunofluorescent assay using anti-MMP-2 and TIMP-2 antibodies. Co-localization of aMMP2/TM with TIMP-2 was noticed on the cell surface (Fig.6B). The invasive ability of aMMP2/TM expressing cells is currently being examined.

Task 3: Investigate endocytosis of MT1-MMP in prostate cancer cells, Months 20- 36:

A: The endocytosis of MT1-MMP in prostate cancer will be examined by immunofluorescent techniques and ^{125}I-TIMP-2 binding assay, a surrogate marker for the endocytosis of MT1-MMP.

B: The relationship between endocytosis of MT1-MMP and cell migration will be evaluated using different reagents and migratory model system described in this proposal.

As originally planned, these experiments will be initiated in year 2.

Task 4: Isolate selective peptide inhibitors of MT1-MMP using a phage display library, Months 24-36:

A: Selection of MT1-MMP binding phages by screening phage display peptide libraries;

B: Confirmation of binding activity of selective phage clones by ELISA and Immunofluorescent assay.

C: Characterization of the candidates by examining the inhibitory effect of candidates on MT1-MMP-induced pMMP-2 activation using gelatin substrate zymogram.

D: Determine the effect of the selected peptides on MT1-MMP induced prostate cancer cell migration using the migratory model system.

As originally planned, these experiments will be initiated in year 3.

Invited lectures: Invited speaker on MMPs

1) Oral presentations at American Association for Cancer Research, 93th annual meeting in San Francisco, 04/02

2) NYU, Department of Surgery, School of Medicine, New York, NY 05/02

3) VA Medical Center, Northport, NY, 09/02

4) Oral presentations at American Association for Cancer Research special meeting entitled "Proteases, ECM, and Cancer" at Hilton Head Island, SC, 10/02

Legends:

Fig 1:Establishment of prostate cancer cell lines stably expressing MT1/GFP chimera or GFP. LNCaP cells were transfected with MT1/GFP chimeric cDNA or GFP cDNA as a control, followed by the selection of resistant cells in the presence of G418. Six weeks later, G418 resistant clones were formed and isolated by cloning cylinder method based on the green fluorescence.

Fig 2: Expression of MT1/GFP in LNCaP transfectants. LNCaP cells or cells stably expressing MT1/GFP or GFP were lysed. The proteins in the cell lysates were separated on SDS-PAGE followed by standard Western blotting using anti-GFP antibody. GFP was identified as a 27 kDa protein, while MT1/GFP was detected as 95 kDa fusion protein with a 68 kDa degraded product. The degraded form of MT1/GFP is being examined.

Fig 3: Plasma membrane localization of MT1/GFP chimera in LNCaP cells. LNCaP cells stably expressing MT1/GFP or GFP were grown on cover-slips followed by immunofluorescent staining with anti-MT1-MMP antibody (hinge) and Texas-Red conjugated anti-rabbit IgG. The localization of MT1/GFP and GFP in cells were observed under fluorescent microscopy. Co-localization of antibody-recognized MT1-MMP (red) with GFP tagged MT1-MMP (green) at the plasma membrane of stably LNCaP cells expressing MT1/GFP was noted. GFP presented as diffused distribution throughout the stable cells expressing GFP. No MT1-MMP was detected at the plasma membrane of GFP expressing LNCaP cells.

Fig. 4: Initiation of cellular invasion (substrate degradation and cell migration) of LNCaP cells by expressing MT1-MMP in the cells. LNCaP cells stably transfected with MT1/GFP chimeric cDNA were plated on FITC labeled Fn coverslips followed by adding buffer control, recombinant GLA, TIMP-1 and TIMP-2 in serum free conditioned medium, respectively. MTI-MMP expressing cells degrade Fn and migrated over digested substrate as a finger print. The digestion of Fn was dramatically increased by incubating cells with recombinant GLA. TIMP-1 inhibited the digestion of Fn induced by both MT1-MMP and MMP-2 down to the basal digestion level resulting from stable LnCap cells with MT1-MMP alone, but did not interfere with cell migration. In contrast, TIMP-2 totally abolished the digestion of LnCap cells expressing MTI-GFP in the presence of rGLA.

Fig. 5: Weight loss of nu/nu mice bearing LNCaP tumor expressing MT1/GFP as compared with that of GFP. 2X 10^6 LNCaP cells expressing GFP or MT1/GFP in 0.1 ml 50% Matrigel/RPMI1640 medium (5.5 mg/ml Matrigel) were injected s.c in the back of mice. Visible tumor was noted at two weeks after injection for both MT1/GFP and GFP producing cells. Although no significant size difference of MT1/GFP tumors and GFP tumors was noted, marked weight loss was observed in mice bearing MT1/GFP tumor. The figure and pictures show the weight gain of mice after 6-weeks inoculation.

Fig. 6: Plasma membrane localization of engineered active surface-bound MMP-2. By employing two-step PCR approach, active MMP-2/$TM_{MT/Cyto}$ was constructed by deleting propeptide domain of MMP-2 followed by fusing the C terminal tail of MT1-MMP including transmembrane and cytoplasmic domains at the C-terminal aMMP-2. The serum free conditioned medium and cell lysates of COS-1 cells transfected with pcDNA3 control, MMP-2(pGLA), engineered active MMP-2 (aGLA), and surface-bound active MMP-2 (aGLA/$MT_{TM/Cyto}$) were analyzed by Western blotting using anti-MMP-2 antibody.

aGLA/$MT_{TM/Cyto}$, appearing as a protein band of 68 kDa, was only detected in cell lysate whereas pGLA and aGLA were detected in both conditioned medium and cell lysates. To examine the conformation of surface-bound active MMP-2, immunofluorescent staining was performed. Transfected COS-1 cells with aGLA/$MT_{TM/Cyto}$ cDNA were co-stained with anti-MMP-2 antibody (rabbit) and anti-TIMP-2 antibody (mouse) followed by reacting with secondary antibodies, i.e. Texas-red conjugated anti-rabbit Ig G and FITC conjugated anti-mouse Ig G. Plasma membrane co-localization of aGLA/$MT_{TM/Cyto}$ with TIMP-2 was observed under fluorescent microscopy indicating proper conformation of aGLA/$MT_{TM/Cyto}$ presented on the cell surface.

Fig. 1

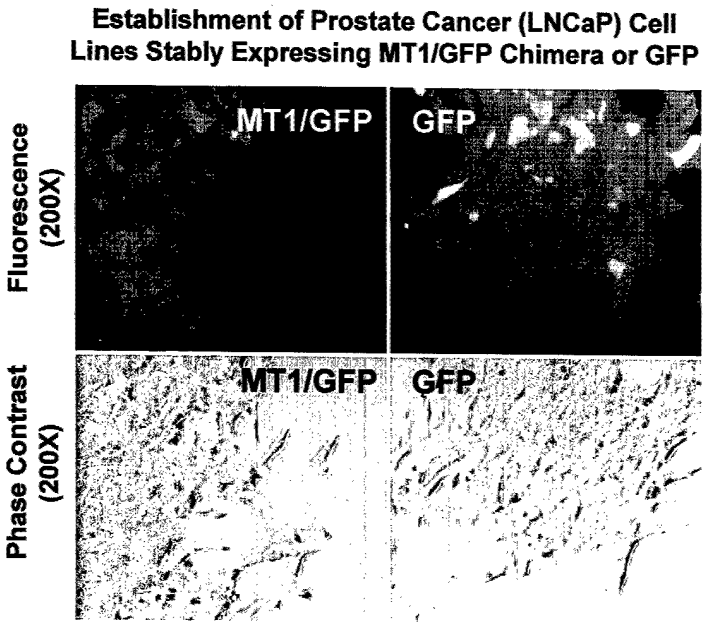

Fig. 2

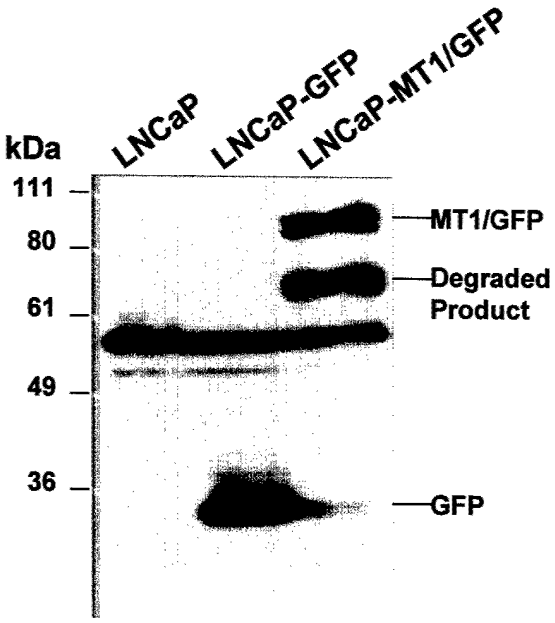

Fig. 3

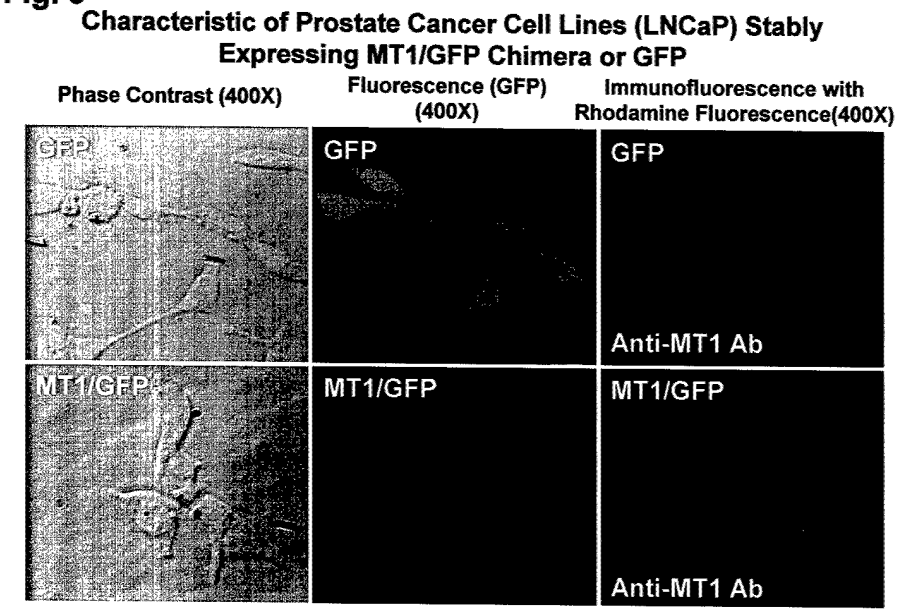

Fig. 4

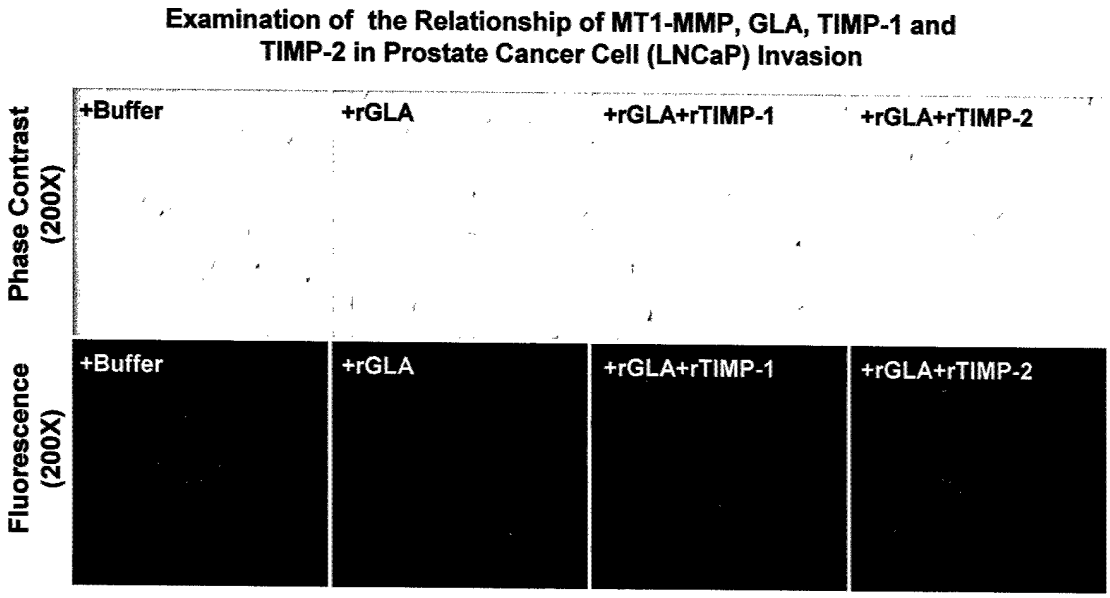

Fig. 5

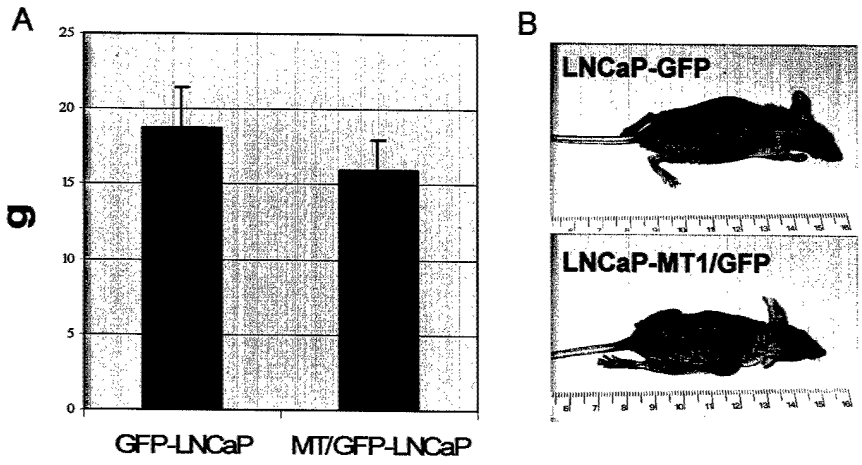

Fig. 6