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14. ABSTRACT: This project investigated a biomedical problem related to huamn prostate cancer invasion and a possible biomarker for cancer diagnosis. We reported the identification and characterization of human matrix metalloproteinase-26 **(MMP-26/endometase/matrilysin-2)**. We have tested three specific hypotheses: 1) The expression levels of MMP-26 is correlated with the metastatic potentials and the degrees of malignancy of human prostate cells; 2)MMP-26 has unique structure and enzymatic function; 3) MMP-26 enhances prostate cancer invasion by digesting extracellular matrix proteins and inactivating serine proteinase inhibitors, and specific inhibitors of MMP-26 block prostate cancer invasion. We report that levels of MMP-26 protein in human prostate carcinomas and high-grade prostate intraepithelial neoplasia from multiple patients were significantly higher than those in prostatitis, benign prostate hyperplasia, and normal prostate glandular tissues. Prostate cancer cells transfected with MMP-26 gene are more invasive and with an inactive mutant are less invasive than the parental cell lines. MMP-26 promoted prostate cancer invasion via activation of pro-gelatinase B/pro-MMP-9. Biochemical studies indicated that endometase active site has an intermediate S1' pocket. Multiple novel synthetic MMP inhibitors are designed, synthesized, and characterized, and they are able to block the invasion of prostate cancer cells. Sixteen papers are attached as part of this final report.

15. SUBJECT TERMS
Matrix metalloproteinase-26, endometase, advanced human prostate cancer, novel proteinases, cell biology and biochemistry.

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DAMD17-02-1-0238 Final Report
(2/25/2002-7/31/2006)

Qing-Xiang Amy SANG, Ph.D., Associate Professor. July 31, 2006
Florida State University

Endometase in Androgen-Repressed Human Prostate Cancer

Introduction

Our long term goal is to understand the biochemical and cellular functions of matrix metalloproteinases (MMPs, matrixins) so that we may reveal the molecular steps and pathways of cancer angiogenesis (new blood formation to provide nutrients, oxygen, and passages for cancer cell growth and spread) and metastasis (the spread of cancer). We discovered a novel matrix metalloproteinase (MMP-26, endometase). Endometase is a special biological catalyst that specifically digests some of the connective tissue barrier proteins and may facilitate tumor growth, invasion, and new blood vessel formation. *Endometase* was found to be specifically associated with the androgen-repressed human prostate cancer *(ARCaP)* cells. It was *not* expressed by normal human prostate tissues. ARCaP cells were isolated from a human patient who died of metastasis of prostate cancer. Most importantly, endometase gene was turned on in human prostate cancer tissues from patients. This project has investigated a role of endometase in advanced human prostate cancer and provided knowledge for new strategies to detect and attack prostate cancer. To understand the functions of endometase, we have identified some of its physiological and pathological substrates and developed potent proteinase inhibitors targeting this protein. We have tested the *hypothesis* that this unique endometase is partially responsible for promoting cancer cell growth and invasion because of its activity as "a molecular power drill" that breaks down connective tissue barriers. Together with our collaborators and lab members, we have been developing and testing new MMP inhibitors and identify potent and selective compounds to target endometase. More than 20 novel synthetic inhibitors with better water-solubility and serum stability have been designed, synthesized, and characterized. The inhibitors are useful tools for the investigations of the endometase active site structure and functions, and more importantly, the prostate cancer invasion. We have revealed that the active site of endometase has an intermediate S1' pocket using enzyme inhibition kinetic studies, protein sequence analyses, and homology modeling. Some of these inhibitors have been tested for their stability in cell culture media and ability in invasion through extracellular matrix proteins using modified Boyden invasion chamber model. We have also examined the endometase expression pattern in human prostate and breast cancer tissues and clinical specimens and studied its pathological role in human prostate and breast cancers. Interestingly, high grade prostate intraepithelial neoplasia (HGPIN) has the highest expression levels of MMP-26, indicating that MMP-26 may play a significant role in the conversion of non-invasive tumor to invasive cancer. This project may have identified a novel marker for prostate cancer early detection and a new target for prostate cancer treatment.

Body

Statement of Work

Endometase in Androgen-Repressed Human Prostate Cancer

Task 1. **To examine the endometase (matrix metalloproteinase-26, MMP-26) expression pattern in normal and malignant prostate cell lines and to correlate the endometase protein expression levels with the known malignancy and metastatic potentials of the cells (Months 1-8):**

a. Culture normal and malignant prostate cells.
b. Measure the endometase expression levels by enzyme-linked immunosorbent assay (ELISA) and immunoblot using enhanced chemiluminescence (ECL). There are four specific endometase antibodies (Abs) available at the P.I.'s lab; three Abs are against the catalytic domain, one Ab is against the pro-domain.

This task has been accomplished. For more details please see Fig. 2 and Fig. 3 of the following published paper: Y.-G. Zhao, A. Xiao, R.G. Newcomer, H.I. Park, T. Kang, L.W.K. Chung, M.G. Swanson, H. E. Zhau, J. Kurhanewicz, and **Q.-X. Sang*** (2003) Activation of Pro-Gelatinase B by Endometase/Matrilysin-2 Promotes Invasion of Human Prostate Cancer Cells. *J. Biol. Chem.* **278**, 15056-15064.

Our accomplishments are beyond the originally proposed tasks. Coordinated peak expression of MMP-26 and its most potent tissue inhibitor (TIMP-4) in preinvasive human prostate tumor has been observed. The identification of novel biomarkers for early prostate cancer diagnosis is highly important because early detection and treatment are critical for the medical management of patients. Disruption in the continuity of both the basal cell layer and basement membrane is essential for the progression of high-grade prostatic intraepithelial neoplasia (HGPIN) to invasive adenocarcinoma in human prostate. The molecules involved in the conversion to an invasive phenotype are the subject of intense scrutiny. We have previously reported that matrix metalloproteinase-26 (MMP-26) promotes the invasion of human prostate cancer cells via the cleavage of basement membrane proteins and by activating the zymogen form of MMP-9. Furthermore, we have found that tissue inhibitor of metalloproteinases-4 (TIMP-4) is the most potent endogenous inhibitor of MMP-26. Here we demonstrate higher ($p<0.0001$) MMP-26 and TIMP-4 expression in HGPIN and cancer, compared to non-neoplastic acini. Their expression levels are highest in HGPIN, but decline in invasive cancer ($p<0.001$ for each) in the same tissues. Immunohistochemical staining of serial prostate cancer tissue sections suggests colocalization of MMP-26 and TIMP-4. The present study indicates that MMP-26 and TIMP-4 may play an integral role during the conversion of HGPIN to invasive cancer and may also serve as markers for early prostate cancer diagnosis. Please see the following paper for more detailed results and discussions.

S. Lee[‡], K. K. Desai[‡], K.A. Iczkowski[‡] ([‡]shared first authors), R.G. Newcomer, K.J. Wu, Y.-G. Zhao, W.W. Tan, M.D. Roycik, and **Q.-X. Sang*** (2006) Coordinated peak expression of MMP-26 and TIMP-4 in preinvasive human prostate tumor. *Cell Res.* 16, in press. Advance online publication Aug. 22, 2006; doi: 10.1038/sj.cr.7310089.

Please see attached reprints and uncorrected galley-proof/preprint in Appendix.

Task 2. **To over-express endometase in endometase-negative human prostate cells and characterize endometase positive cells (Months 5-25):**

a. Prepare endometase over-expression vectors.
b. Transfect pCIMMP-26 into normal human prostate cells, an androgen-dependent prostate cancer cell line LNCaP, and an androgen-independent prostate cancer cell line DU-145.
c. Identify endometase positive cells by immunological methods (ELISA and immunoblot).
d. Characterize endometase positive cells *in vitro* (colony formation on soft agar).

This task is completed. For more details please see Figures5-8 of the following paper published: Y.-G. Zhao, A. Xiao, R.G. Newcomer, H.I. Park, T. Kang, L.W.K. Chung, M.G. Swanson, H. E. Zhau, J. Kurhanewicz, and **Q.-X. Sang*** (2003) Activation of Pro-Gelatinase B by Endometase/Matrilysin-2 Promotes Invasion of Human Prostate Cancer Cells. *J. Biol. Chem.* **278**, 15056-15064. Please also see attached reprint and the following results (**Figure 1-3**) in the body of this report.

Task 3. **Analyze structure-function relationships of the endometase active site using synthetic matrix metalloproteinase (MMP) inhibitors and identify specific and potent endometase inhibitors for the cell invasion assays (Months 10-30):**

a. Optimize the synthetic fluorogenic peptide substrate cleavage assays using a Perkin-Elmer LS-50B luminescence spectrometer.
b. Measure the IC_{50} (inhibitor concentration at 50% enzyme activity) values and inhibition constants (k_I values) of new synthetic MMP inhibitors with endometase listed in *Table 1* and *Figure 2* of the proposal. Determine the inhibition kinetics and mechanisms.

This task has been accomplished. Multiple substrates have been tested and the assay has been optimized for the enzyme inhibition kinetic assays. The k_I values in nM of the following inhibitors against MMP-26 are: 2700 for MAG-148, 650 for MAG-174, 28 for YHJ-73, 160 for YHJ-72, 78 for YHJ-232, 103 for YHJ-233, 19 for YHJ-265, 6.7 for YHJ-96, 11 for YHJ-97, 8.0 for YHJ-3-44, 2.8 for YHJ-294-2, 450 for YHJ-294-1, 8.9 for YHJ-132, 86 for YHJ-133, 4.1 for YHJ-223, and 2.7 for YHJ-224. More IC50 values have been determined for other inhibitors. The inhibition mechanisms are competitive inhibition, reversible, slow-binding and tight binding for the most potent inhibitors.

Please see the attached reprints for more details about the structures of the newly developed synthetic MMP inhibitors and the enzyme inhibition kinetic data of those inhibitors against MMP-26/endometase and many other MMPs:

H.I. Park, B.E. Turk, F.E. Gerkema, L.C. Cantley, and **Q.-X. Sang*** (2002) Peptide substrate specificities and protein cleavage sites of human endometase/matrilysin-2/matrix metalloproteinase-26. *J. Biol. Chem.* **277,** 35168-35175.

H.I. Park, Y. Jin, D.R. Hurst, C.A. Monroe, S. Lee, M.A. Schwartz, and **Q.-X. Sang*** (2003) The intermediate S1' pocket of the endometase/matrilysin-2 active site revealed by enzyme inhibition kinetic studies, protein sequence analyses, and homology modeling. *J. Biol. Chem.*, 278:51646-51653.

D. R. Hurst, M.A. Schwartz, M.A. Ghaffari, Y. Jin, H. Tschesche, G.B. Fields, and **Q.-X. Sang*** (2004) Catalytic- and ecto-domains of membrane type 1-matrix metalloproteinase have similar inhibition profiles but distinct endopeptidase activities. *Biochem. J.* **377**, 775-779.

D.R. Hurst, M.A. Schwartz,Y. Jin, M.A. Ghaffari, P. Kozarekar, J. Cao, **Q.-X. Sang*** (2005) Inhibition of enzyme activity and cell-mediated substrate cleavage of membrane type 1-matrix metalloproteinase by newly developed mercaptosulfide inhibitors. *Biochem. J.* **392,** 527-536. Epub 2005 Jul 19.

Q.-X. Sang*, Y. Jin, R.G. Newcomer, S.C. Monroe, X. Fang, D.R. Hurst, S. Lee, Q. Cao, and M.A. Schwartz (2006) Matrix Metalloproteinase Inhibitors as Prospective Agents for the Prevention and Treatment of Cardiovascular and Neoplastic Diseases. *Current Topics in Medicinal Chemistry*. **6**, 289-316. Invited review.

Task 4. **Identify new substrates of endometase, compare the degrees of invasiveness of the human prostate cells selected from *Task 1* and *Task 2*, and test the efficacies of MMP inhibitors selected from *Task 3* in the prostate cell invasion (Months 20-36):**

a. Test intracelluar proteins, extracellular matrix proteins, and cell surface proteins of ARCaP and other cells to identify new endometase substrates, hence, understand its putative functions.

This task is completed. Please see the following published paper Figs. 6-8 and tables I and II for more details. H.I. Park, B.E. Turk, F.E. Gerkema, L.C. Cantley, and **Q.-X. Sang*** (2002) Peptide substrate specificities and protein cleavage sites of human endometase/matrilysin-2/matrix metalloproteinase-26. *J. Biol. Chem.* **277,** 35168-35175.

b. Test and compare the degrees of invasiveness of human normal, malignant, and endometase over-expression prostate cells, respectively, using modifed Boyden Chambers coated with extracellular matrix proteins or cell surface proteins.
c. Test and compare the efficacies of selective MMP inhibitors from *Task 3* in prostate cell invasion. Identify specific inhibitors that may lead to future cancer therapeutics.

This task is completed. For more details please see Figs. 4-8 of the following paper published. Y.-G. Zhao, A. Xiao, R.G. Newcomer, H.I. Park, T. Kang, L.W.K. Chung, M.G. Swanson, H. E. Zhau, J. Kurhanewicz, and **Q.-X. Sang*** (2003) Activation of Pro-Gelatinase B by Endometase/Matrilysin-2 Promotes Invasion of Human Prostate Cancer Cells. *J. Biol. Chem.* **278**, 15056-15064. Please see attached reprint and the following results presented in **Figures 2, 4-6** in the body of this report.

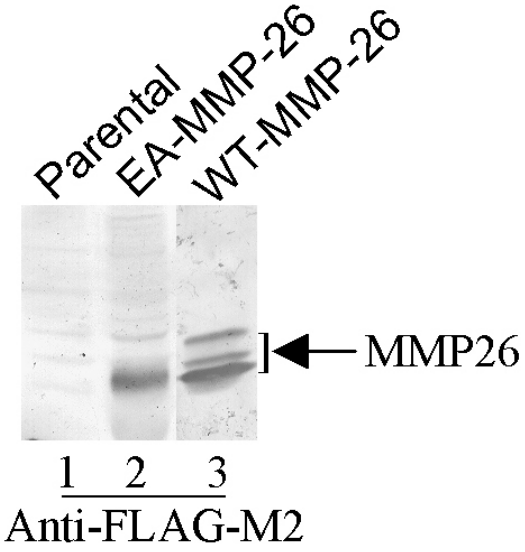


Fig. 1. Detection of MMP-26 in androgen-dependent LNCaP cells stably expressing MMP-26 wild type or an inactive form. LNCaP cells, a human prostate cancer cell line, were stably transfected with MMP-26 wild type (WT-MMP-26), or an inactive form (EA-MMP-26), both of which were tagged with FLAG tags at their C-termini. Cells were routinely cultured in DMEM supplemented with 10% fetal bovine serum and 400 μg/L of G418. For experiments, they were seeded into 24-well-plates until they reached 100% confluence. Cells were lysed with RIPA buffer, and cell lysates were analyzed by Western blotting using anti-FLAG-M2 antibody.

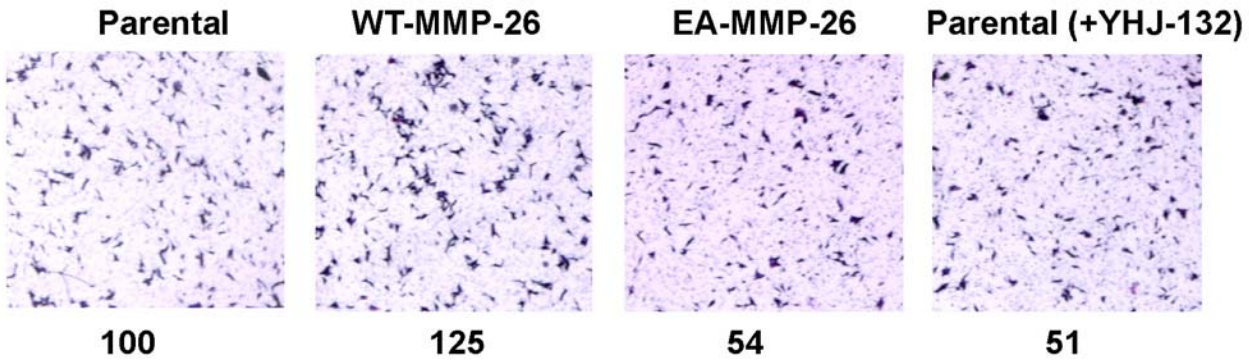
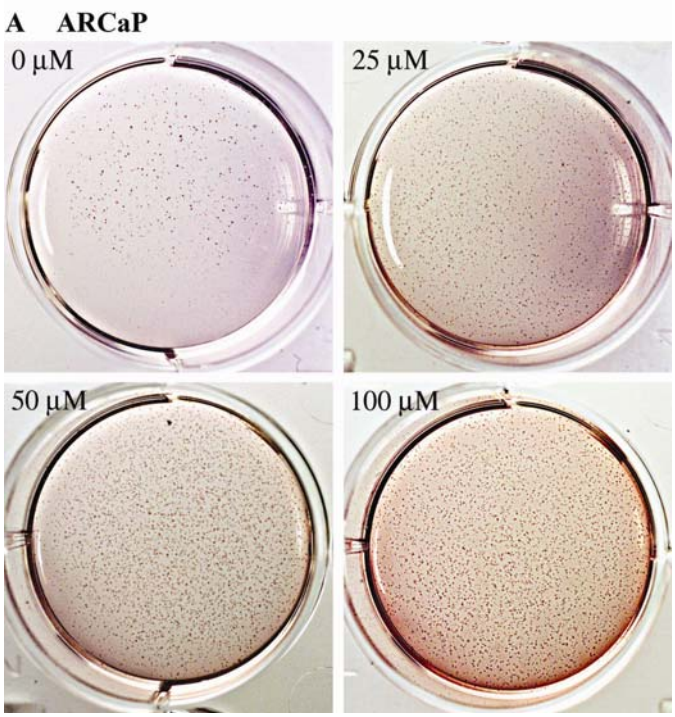


Fig. 2. MMP-26 plays a vital role in the invasion of androgen-dependent LNCaP cells. Parental LNCaP cells or its derivatives stably over-expressing MMP-26 wild type (WT-MMP-26) or inactive form (EA-MMP-26) were subjected to invasion assays utilizing a modified Boyden chamber coated with type IV collagen (25 μg/well). Following 48 hours incubation in serum-free medium alone or containing **10 μM YHJ-132, an MMP inhibitor synthesized by our group**, the inner chambers were fixed and the invading cells were photographed. Quantification of the invading cells was performed by integrated morphometry analysis (IMA); untreated parental LNCaP cells (Parental) were controls as 100% invasive. The WT-MMP-26 cells showed a 25% increase in invading cells, while the EA-MMP-26 or parental cells treated with YHJ-132 showed decreased invasion, at 54% and 51% of the parental cells, respectively.

A



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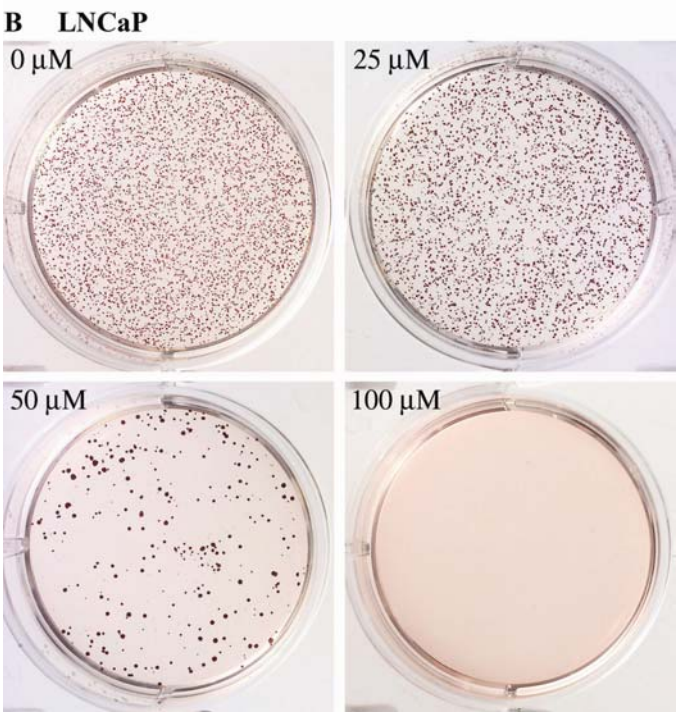

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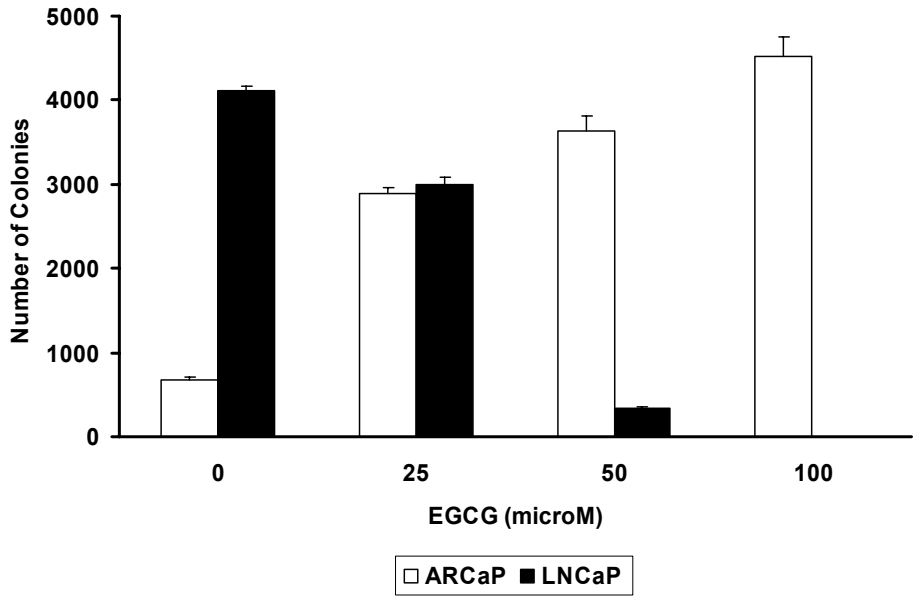

Fig. 3. Colony formation of endometase-positive ARCaP cells and endometase protein-negative LNCaP cells and their responses to a green tea polyphenol, Epigallocatechin-3 Gallate (EGCG). EGCG promotes colony formation in ARCaP cells but represses it in LNCaP cells. The anchorage-independent growth assay was performed in soft agar coated 6-well plates with $3x10^4$ cells in each well. Indicated doses of EGCG or same volume of water was added to both top and bottom agar and also to the media and the media was replaced every week with fresh EGCG. Cells were cultured for two weeks and pictures of (A) ARCaP cell grown wells and (B) those of LNCaP cells were taken. Pictures shown are representative of three independent experiments. The number of colonies in the whole well, but not in specific field, of the all three independent experiments was counted and the result is shown in (C). The data shown are mean value with standard error (SE). Analysis of variance (ANOVA) was used for statistical analysis of EGCG treatment of each cell line as a whole. The *P* values for EGCG treatment of ARCaP cells and LNCaP cells were 4.92E-7 and 5.02E-11, respectively. These extremely low probability values indicate that there are highly significant differences between treatments in both cell lines.

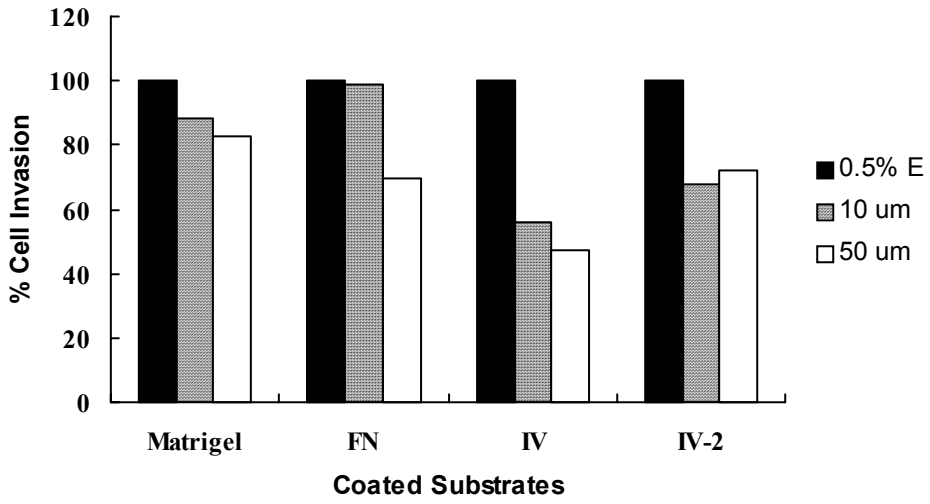

Fig. 4. Inhibition of ARCaP cell invasion by YHJ-132 using modified Boyden chambers coated with *Matrigel*, fibronectin (FN), and type IV collagen, respectively. The concentrations of YHJ-132 are 10 μM and 50 μM dissolved in 0.5% ethanol (E). The 0.5% ethanol was used as a control without inhibitors. YHJ-132 is stable up to for 24 hours in cell culture media. It is added to the cell culture media every 24 hours. Briefly, modified Boyden chambers containing polycarbonate filters with 8-μm pores (Becton Dickinson, Boston, MA) were coated with 100 μl (0.5 mg/ml) human plasma FN (Gibco, Carlsbad, California) or (0.5mg/ml) type IV collagen (Sigma) or *Matrigel*. IV-2 is a type IV collagen repeat experiment in which the YHJ-132 concentration was 25 μM instead of 10 μM. Three hundred μl of prepared cell suspension (1 x 10^6 cells/ml) in serum-free medium was added to each insert, and 500 μl of media containing 10% fetal bovine serum was added to the lower chamber. After 48 hours of incubation, invasive cells that had passed through the filters to the lower surface of the membrane were fixed in 4% paraformaldehyde (PFA) (Sigma, St. Louis, Missouri). The cells were then stained with 0.1% Crystal Violet solution and photographed with an Olympus DP10 digital camera (Melville, NY) under a Nikon FX microscope (Melville, NY). The cells were then counted by Integrated Morphometry Analysis (IMA).

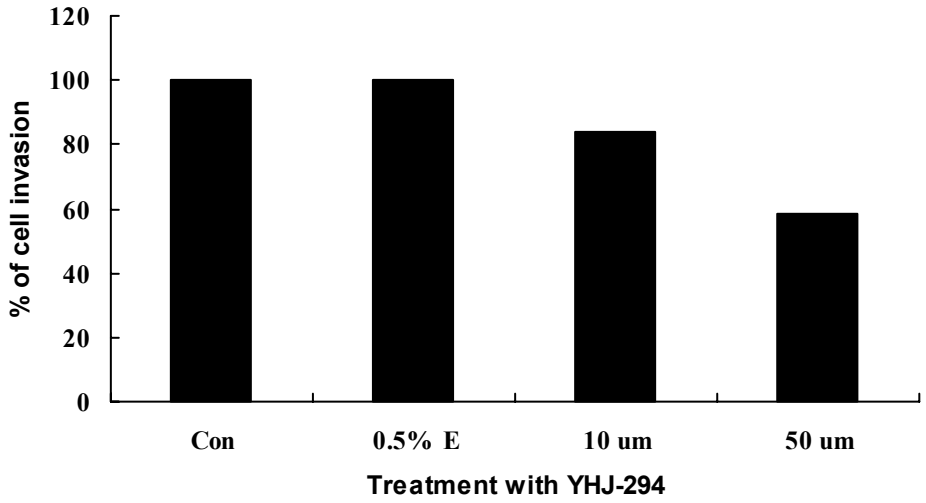

Fig. 5. Inhibition of ARCaP cell invasion by YHJ-294-2 using modified Boyden chambers coated with *Matrigel*. The concentrations of YHJ-294-2 are 10 μM and 50 μM dissolved in 0.5% ethanol (E). The 0.5% ethanol was used as a control without inhibitors. YHJ-294-2 is stable up to for 8 hours in cell culture media. It is added to the cell culture media every 8 hours. The experimental procedure is the same as described in Fig. 4.

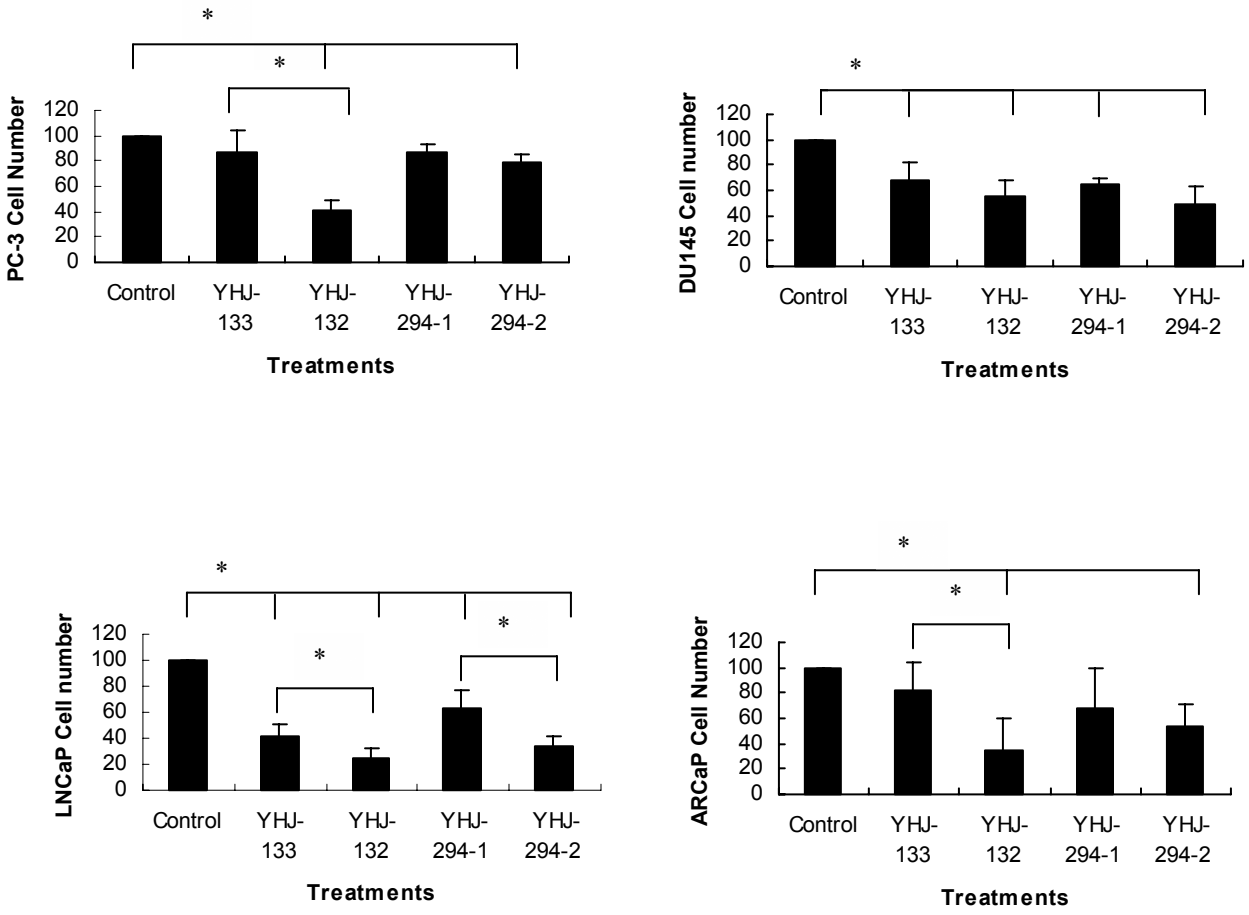

Fig. 6. Effects of 4 new synthetic MMP inhibitors on the invasiveness of 4 human prostate cancer cell lines. Polycarbonate filters with 8-micrometer pores were coated with type I collagen at a concentration of 25 micrograms/well, and 4 human prostate cancer cell lines, DU145, PC-3, LNCaP, and ARCaP, respectively, were cultured in inner champers of modified Boyden chambers at a concentration of 500,000 cells/well. Inhibitors, YHJ-133, YHJ-132, YHJ-294-1, or YHJ-294-2, was introduced into the serum free DMEM media at a concentration of 10 μM. 10% FBS DMEM media were added to outer champers, and cells were fixed and stained with 0.1% crystal violet solution after 6 hours (DU145) or 48 hours (PC-3, LNCaP, and ARCaP) incubation, then were counted by IMA and analyzed by one-way ANOVA with LSD correction. * stands for statistically significant comparisons ($p<0.05$).

Task 5. **Alternative approaches: Test and compare the rates of cell proliferation and apoptosis of the human prostate cells selected from *Task 1* and *Task 2* (Months 26-36):**

a. Test and compare the rates of cell proliferation of human normal, malignant, and endometase over-expression prostate cells, respectively, cultured on extracellular matrix proteins. Cell proliferation rates will be determined by assaying for 5-bromo-2'-deoxyuridine (BrdU) incorporation using the colorimetric ELISA assay kit from Boehringer Mannheim Co.

b. Test and compare the rates of cell apoptosis of human normal, malignant, and endometase over-expression prostate cells, respectively, cultured on extracellular matrix proteins. A quantitative Cell Death Detection ELISA^{PLUS} colorimetric assay kit (also from Boehringer Mannheim Co.) will be used. This assay is useful for the quantitation of apoptosis without cell labeling; it differentiates apoptosis from necrosis.

This task is completed.

Blockage of ARCaP cell invasion by MMP-26 antibodies is not due to the effects of the antibodies on cell attachment to extracellular matrix, cell proliferation, cytotoxicity, and apoptosis. Further investigation shows that the functional blocking antibodies do not affect ARCaP cell attachment to the substrates, do not inhibit ARCaP cell proliferation (**Fig. 7**), have no cytotoxicity, and do not promote the cell apoptosis, demonstrating that the diminished invasiveness of ARCaP cells is due to the functional neutralizing activity of the antibodies against MMP-26 and is not due to reduced cell numbers and is not due to reduced cell attachment to the extracellular matrix substrates.

Cells attachment experiments. 2.25×10^5 ARCap/LNCap/DU145/ PC-3 cells in serum free DMEM media containing different concentrations of one of the pre-immune-IgGs or anti-MMP-26 IgGs were cultured in fibronectin, and matrigel coated 24-well plates. The growing cells were stopped at 3 hours, 6 hours, 12 hours, 24 hours, 48 hours, and 60 hours by rinsing with PBS and fixing in 4% PFA/PBS solution. Then the cells were stained with 0.1% Crystal Violet (Sigma, USA) solution. The attached cells number was counted under in 10 high power fields (400x) of each of the duplicate samples under a microscope.

Results: The ARCaP cells number attached on the FN and Matrigel coated 24-well plate had no significant difference among the untreated wells, pre-immune IgGs treated wells and the anti-MMP-26 antibodies treated the wells ($p>0.05$) at the time points of 3 hours, 6 hours, 12 hours, 24 hours, 48 hours, and 60 hours (data not shown). These results indicate that the inhibition of ARCaP cell invasion by anti-MMP-26 antibodies is not due to the effect on the cells attachment to extracellular matrix components.

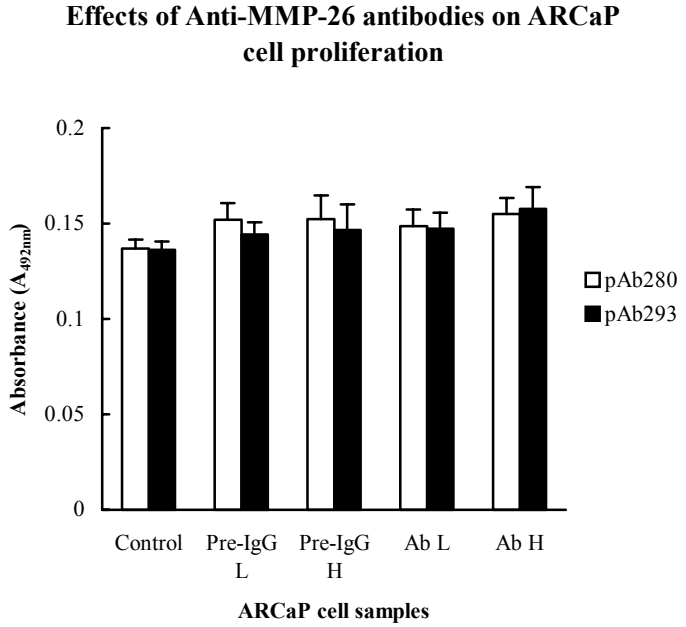

Fig. 7. The anti-MMP-26 polyclonal Abs have no effect on the proliferation of ARCaP cells. The ARCaP cells proliferation was determined using the BrdU Labeling Cell Proliferation ELISA (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. **Pre-IgG L**, preimmune IgG low concentration (10 μg/ml); **Pre-IgG H,** preimmune IgG high concentration (50 μg/ml); **Ab L**, antibody low concentration (10 μg/ml); and **Ab H,** antibody high concentration (50 μg/ml).

Cells proliferation assays. ARCaP cell proliferation was determined using the Cell Proliferation ELISA from Roche Molecular Biochemicals (Indianapolis, IN) following the manufacturer's instructions. In brief, the ARCap cells were plated in a fibronectin, and matrigel pre-coated 96-well tissue culture plate (Falcon, Becton Dickinson Labware, Lincoln Park, New Jersey) at a density of 1 x 10^4 cells/well in DMEM medium containing 10% FBS for 24 hours. Then the cells were cultured in serum free DMEM medium in the presence of pre-immune IgG of 98R3, pre-immune IgG of 98R12, pAb280 and pAb293 (10μg/ml, 50μg/ml, respectively) in duplicate wells for another 24 hours. After adding BrdU (100μM) labeling solution for 12 hours in 37° C, 5%CO_2, the cells were fixed by adding FixDenat solution for 30mins at room temperature. Followed by adding anti-BrdU POD and incubated for 90 min at room temperature. Finally, added substrate and incubate for 30 mins and the values of absorbance were read at 492 nm using an Automatic Microplate Reader (Titertek Multiskan MC-340, Flow Laboratories, Virginia). Standard errors were calculated and were presented as error bars in the Figures.

Key Research Accomplishments

1. This research project has completed the tasks listed in the "Statement of Work", generated three papers published in "The Journal of Biological Chemistry", one "Cancer Research" paper, two "Biochemical Journal paper", and many other publications. The grant also partially supported training of research students and postdoctoral associates.

2. This work has verified a putative biochemical mechanism by which endometase/matrilysin-2/matrix metalloproteinase-26 (MMP-26) may promote human prostate cancer cell invasion.

3. We showed that the levels of MMP-26 protein in human prostate carcinomas from multiple patients were significantly higher than those in prostatitis, benign prostate hyperplasia, and normal prostate glandular tissues. The highest expression level of MMP-26 is in the preinvasive human prostate tumor, HGPIN. Statistical analyses have been performed.

4. MMP-26 was capable of activating pro-MMP-9 by cleavage at the Ala^{93}-Met^{94} site of the prepro-enzyme. This activation proceeded in a time- and dose-dependent manner, facilitating the efficient cleavage of fibronectin by MMP-9. The activated MMP-9 products generated by MMP-26 appeared more stable than those cleaved by MMP-7 under the conditions tested.

5. To investigate the contribution of MMP-26 to cancer cell invasion via the activation of MMP-9, highly invasive and metastatic human prostate carcinoma cells, androgen-repressed prostate cancer (ARCaP) cells, were selected as a working model. ARCaP cells express both MMP-26 and MMP-9. Specific anti-MMP-26 and anti-MMP-9 functional blocking antibodies both reduced the invasiveness of ARCaP cells across fibronectin or type IV collagen.

6. The introduction of MMP-26 antisense cDNA into ARCaP cells reduced the MMP-26 protein level in these cells and strongly suppressed the invasiveness of ARCaP cells.

7. Double immunofluorescence staining and confocal laser scanning microscopic images revealed that MMP-26 and MMP-9 were co-localized in parental and MMP-26 sense-transfected ARCaP cells. Moreover, MMP-26 and MMP-9 proteins were both expressed in the same human prostate carcinoma tissue samples examined. Furthermore, MMP-26 and its most potent tissue inhibitor of metalloproteinases TIMP-4 are both expressed in the same human prostate cancer tissues.

8. These results indicate that MMP-26 may be a physiological and pathological activator of pro-MMP-9, and the activation of pro-MMP-9 by MMP-26 may be an important mechanism contributing to the invasive capabilities of prostate carcinomas.

9. Peptide libraries were used to profile the substrate specificity of MMP-26 from the P4 to P4′ sites. The optimal cleavage motifs for MMP-26 were Lys-Pro-Ile/Leu-Ser (P1)-Leu/Met (P1′)-Ile/Thr-Ser/Ala-Ser.

10. The strongest preference was observed at the P1′ and P2 sites where hydrophobic residues were favored. Proline was preferred at P3 and Serine at P1. The overall specificity was similar to that of other MMPs except that more flexibility was observed at P1, P2′, and P3′.

11. Synthetic inhibitors of gelatinases and collagenases inhibited MMP-26 with similar efficacy. A pair of stereoisomers had a only 40-fold difference in K_i^{app} values against MMP-26 compared to a 250-fold difference against neutrophil collagenase, indicating that MMP-26 is less stereo-selective for its inhibitors.

12. MMP-26 auto-digested itself during the folding process; two of the major autolytic sites were Leu^{49}-Thr^{50} and Ala^{75}–Leu^{76}, which still left the cysteine switch sequence (PH**C**^{82}GVPD) intact. This suggests that Cys^{82} may not play a role in the latency of the zymogen.

13. MMP-26 cleaved Phe^{352}–Leu^{353} and Pro^{357}–Met^{358} in the reactive loop of alpha 1 proteinase inhibitor and His^{140}–Val^{141} in insulin-like growth factor binding protein-1, likely rendering these substrates inactive.

14. Among the fluorescent peptide substrates analyzed, Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2 displayed the highest specificity constant (30000 / Molar second) with MMP-26.

15. The intermediate S1' pocket of the endometase active site has been revealed by enzyme inhibition kinetic studies using our novel MMP inhibitors, protein sequence analyses, and molecular modeling/computational biochemistry.

16. Potent and selective inhibitors of endometase have been identified and tested in biochemical experiments, and some preliminary data were also obtained for the inhibition of human prostate cancer cell invasion by one of the potent inhibitors.

17. Transgenic human prostate cancer cell lines (both androgen-repressed and androgen-dependent) have been made and MMP-26 over-expression cells, knock out cells, and dominant-negative catalytically inactive mutants have been generated and tested. The degree of invasiveness of these human prostate cancer cell lines were correlated with the expression levels of MMP-26 protein. These results verified our hypotheses.

18. Human breast cancer tissues were examined as controls and comparisons for prostate cancer studies. Breast carcinoma *in situ* also expressed high levels of MMP-26 and MMP-26 may play a role in the initiation of invasion of human breast cancers.

19. Tissue inhibitors of metalloproteinases TIMP-2 and TIMP-4 are potent inhibitors of MMP-26 and they have inhibited the activation of pro-MMP-9 by MMP-26.

20. MMP-26 gene is expressed by human breast carcinoma/epithelial cancer cells as examined by *in situ* hybridization, confirming MMP-26 is expressed by epithelial cells and its epithelial origin. These data have been reported in the "Cancer Research" paper. Please see attached reprints.

21. This report proposes a working model for the future studies of proMMP-26 activation, the design of inhibitors, and the identification of optimal physiological and pathological substrates of MMP-26 *in vivo*.

22. This report showed the relative stability and efficacy of some of the novel inhibitors in inhibiting human prostate cancer cell invasion. These inhibitors blocked the activities of matrix metalloproteinases under cell culture conditions.

23. A new generation of novel MMP inhibitors have been rationally designed, synthesized, tested, and characterized biochemically and cellularly. They are more water-soluble, stable, and more potent and selective. They are able to inhibit human prostate cancer cell invasion through type IV collagen, *Matrigel*, fibronectin, and other extracellular matrix substrates. Most promising inhibitors are YHJ-132 and YHJ-294-2.

24. The effects of 4 newly synthesized MMP inhibitors on the invasiveness of ARCaP, LNCaP, DU145, and PC3 human prostate cells have been tested and YHJ-132 is the most potent inhibitor tested and it can inhibit more than 50% invasion at 10 μM concentration.

25. Colony formation on soft agar of MMP-26 positive ARCaP and MMP-26 negative LNCaP human prostate cancer cells was examined. They respond differently upon EGCG treatment.

26. Tests showed that blockage of ARCaP cell invasion by MMP-26 functional blocking antibody is not due to the effects on cell attachment to extracellular matrix, cell proliferation, cytotoxicity, and apoptosis.

27. All the tasks have been accomplished and more promising data are generated.

28. This report demonstrates that the PI's laboratory has been very productive and produced exceptional results beyond what have been described in the Statement of Work.

29. Sixteen scientific research papers related to this project have been published, one in press, and additional manuscripts are in the process of getting published or under preparation.

30. Thirty-two posters/abstracts were presented at conferences. We have acknowledged the partial support of this funded grant to those publications and presentations.

Reportable Outcomes

A. Sixteen published papers and one preprint in press:

1. H.I. Park, B.E. Turk, F.E. Gerkema, L.C. Cantley, and **Q.-X. Sang*** (2002) Peptide substrate specificities and protein cleavage sites of human endometase/matrilysin-2/matrix metalloproteinase-26. *J. Biol. Chem.* **277,** 35168-35175.

2. T.-B. Kang, Y.-G. Zhao, D. Pei, J.F. Sucic, and **Q.-X. Sang*** (2002) Intracellular activation of human adamalysin 19/disintegrin and metalloproteinase 19 by furin occurs via one of the two consecutive recognition sites. *J. Biol. Chem.* **277,** 25583-25591.

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4. Y.-G. Zhao, A. Xiao, R.G. Newcomer, H.I. Park, T. Kang, L.W.K. Chung, M.G. Swanson, H. E. Zhau, J. Kurhanewicz, and **Q.-X. Sang*** (2003) Activation of Pro-Gelatinase B by Endometase/Matrilysin-2 Promotes Invasion of Human Prostate Cancer Cells. *J. Biol. Chem.* **278,** 15056-15064.

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8. Y.-G. Zhao, A.-Z. Xiao, H.I. Park, R.G. Newcomer, M. Yan, Y.-G. Man, S.C. Heffelfinger, and **Q.-X. Sang*** (2004) Endometase/matrilysin-2 in human breast ductal carcinoma *in situ* and its inhibition by tissue inhibitors of metalloproteinases-2 and -4: a putative role in the initiation of breast cancer invasion. *Cancer Res.* **64,** 590-598.

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10. Y.-G. Man* and **Q.-X. Sang*** (2004) The Significance of Focal Myoepithelial Cell Layer Disruptions in Human Breast Tumor Invasion: a Paradigm Shift from the "Protease-centered" Hypothesis. *Exp. Cell Res.* **301,** 103-118.

11. W. Qiu, S.-X. Bai, M.-R. Zhao, X.-Q. Wu, Y.-G. Zhao, **Q.-X. Sang**, Y.-L. Wang (2005) Spatio-

temporal expression of matrix metalloproteinase-26 in human placental trophoblasts and fetal red cells during normal placentation. *Biol. Reprod.* **72,** 954-959 [Epub ahead of print, 2004 Dec 15].

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13. D.R. Hurst, M.A. Schwartz,Y. Jin, M.A. Ghaffari, P. Kozarekar, J. Cao, **Q.-X. Sang*** (2005) Inhibition of enzyme activity and cell-mediated substrate cleavage of membrane type 1-matrix metalloproteinase by newly developed mercaptosulfide inhibitors. *Biochem. J.* **392,** 527-536. Epub 2005 Jul 19.

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16. **Q.-X. Sang***, Y. Jin, R.G. Newcomer, S.C. Monroe, X. Fang, D.R. Hurst, S. Lee, Q. Cao, and M.A. Schwartz (2006) Matrix Metalloproteinase Inhibitors as Prospective Agents for the Prevention and Treatment of Cardiovascular and Neoplastic Diseases. *Current Topics in Medicinal Chemistry*. **6**, 289-316. Invited review.

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3. Y.-G. Zhao, H.I. Park, M.A. Schwartz, M.-C. Jia, and Q.-X. Sang. A New Metalloproteinase in Cancer Progression and Angiogenesis. *Pathobiology of Cancer Workshop.* American Association for Cancer Research. Keystone, Colorado. July 14-21, 2002.

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C. In addition to the Principal Investigator, Dr. Sang, the following students and research associates at Professor Sang's laboratory were partially funded by this grant.

Douglas R. Hurst, Graduate Student Research Assistant
Robert G. Newcomer, Graduate Student Assistant
Mark Dru Roycik, Graduate Student Assistant
Megan E. Moruski, Undergraduate Student Assistant
Jennifer Walker, Undergraduate Student Assistant
Kevin K. Desai, Undergraduate Student Assistant
Dr. Hyun I. Park, Postdoctoral Research Associate
Dr. Yunge Zhao, Postdoctoral Research Associate
Margaret Mary Coryn, Student Assistant
Sara C. Monroe, Research Assistant
Dr. Tiebang Kang, Postdoctoral Research Associate
Dr. Aizhen Xiao, Postdoctoral Research Associate
Dr. Ziad J. Sahab, Postdoctoral Research Associate
Seakwoo Lee, Graduate Student Assistant
Daniel Trueblood, Graduate Student Assistant

Conclusions

The spread of prostate cancer cells to other parts of the body is the leading cause of patient death. In 2000, we reported the discovery, cloning, and characterization of human matrix metalloproteinase-26 (MMP-26), **endometase**. We have tested three specific hypotheses: 1) The expression levels of MMP-26 is correlated with the metastatic potentials and the degrees of malignancy of human prostate cells; 2) MMP-26 has unique structure and enzymatic function; 3) MMP-26 enhances prostate cancer invasion by digesting extracellular matrix proteins and inactivating serine proteinase inhibitors, and specific inhibitors of MMP-26 block prostate cancer invasion. We have showed that the levels of MMP-26 protein in human prostate carcinomas from multiple patients were significantly higher than those in prostatitis, benign prostate hyperplasia, and normal prostate glandular tissues. Most importantly, the highest expression levels of MMP-26 and its most potent tissue inhibitor of metalloproteinases TIMP-4 are in human high-grade intraepithelial neoplasia (HGPIN), indicating that these two proteins may serve as biomarkers for the early diagnosis of a subset of patients with HGPIN possibly before there is invasion and metastasis. Appropriate treatment strategies may applied to those patients to save their lives before life-threatening process, metastasis, begins. Highest levels of MMP-26 and TIMP-4 are also expressed by human breast ductal carcinoma *in situ* (DCIS), which is equivalent to HGPIN in human prostate.

Both MMP-26 gene and protein are expressed by the carcinoma cells confirming its epithelial origin. MMP-26 promoted prostate cancer invasion via activation of pro-gelatinase B/MMP-9. Functional blocking antibodies against either MMP-26 or MMP-9 blocked human prostate cancer cell invasion. Furthermore, antisense cDNA of MMP-26 and catalytically inactive mutant MMP-26 also inhibited prostate cancer cell invasion. The over-expression of MMP-26 cDNA in both androgen-dependent and independent cells lines enhanced the invasiveness of these cells. Tissue inhibitors of metalloproteinases TIMP-2 and TIMP-4 are also potent inhibitors of MMP-26. The endometase active site structure and function have been investigated and revealed to have an intermediate S1' pocket using synthetic metalloproteinase inhibitors, protein sequence analyses and homology modeling studies. These findings may aid in rational inhibitor/potential drug design for the development of more potent and selective inhibitors of MMP-26. Optimal peptide substrate specificity and protein cleavage sites have been identified in both peptide libraries and potentially physiologically relevant proteins. These results suggest that endometase/MMP-26 may promote human prostate and breast cancer cell invasion and it is specifically expressed in human prostate and breast cancer tissues.

Multiple novel MMP inhibitors have been designed, synthesized, tested, and found to be more potent, water-soluble, and stable under cell culture conditions. Some of these inhibitors have been tested for their stability in cell culture media and ability in invasion through extracellular matrix proteins using modified Boyden invasion chamber model. Three additional human prostate cancer cell lines have been tested in addition to the originally proposed ARCaP cell line. Those inhibitors are relatively stable up to 1-2 days and effective in inhibiting human prostate cancer cell invasion. This project may have identified a novel marker for prostate cancer early detection and a new target for prostate cancer treatment. The tasks listed in the original Statement of Work have been accomplished and more promising data are generated. This report demonstrates that the PI's laboratory has been very productive and produced exceptional results beyond what have been described in the Statement of Work. Fifteen scientific research papers related to this project have been published and additional manuscripts are in press or under preparation. This project was extended for one year to complete all the tasks under a Federal Demonstration, No-cost Extension agreement. This current report is the final scientific report for this funded project/grant.

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Please see references cited in the following 16 papers and Appendix:

1. H.I. Park, B.E. Turk, F.E. Gerkema, L.C. Cantley, and **Q.-X. Sang*** (2002) Peptide substrate specificities and protein cleavage sites of human endometase/matrilysin-2/matrix metalloproteinase-26. *J. Biol. Chem.* **277,** 35168-35175.

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8. Y.-G. Zhao, A.-Z. Xiao, H.I. Park, R.G. Newcomer, M. Yan, Y.-G. Man, S.C. Heffelfinger, and **Q.-X. Sang*** (2004) Endometase/matrilysin-2 in human breast ductal carcinoma *in situ* and its inhibition by tissue inhibitors of metalloproteinases-2 and -4: a putative role in the initiation of breast cancer invasion. *Cancer Res.* **64,** 590-598.

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Appendix

Fifteen published papers/reprints and one uncorrected galley proof/preprint are attached.

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Peptide Substrate Specificities and Protein Cleavage Sites of Human Endometase/Matrilysin-2/Matrix Metalloproteinase-26*

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Human endometase/matrilysin-2/matrix metalloproteinase-26 (MMP-26) is a novel epithelial and cancer-specific metalloproteinase. Peptide libraries were used to profile the substrate specificity of MMP-26 from the P4–P4′ sites. The optimal cleavage motifs for MMP-26 were Lys-Pro-Ile/Leu-Ser(P1)-Leu/Met(P1′)-Ile/Thr-Ser/Ala-Ser. The strongest preference was observed at the P1′ and P2 sites where hydrophobic residues were favored. Proline was preferred at P3, and Serine was preferred at P1. The overall specificity was similar to that of other MMPs with the exception that more flexibility was observed at P1, P2′, and P3′. Accordingly, synthetic inhibitors of gelatinases and collagenases inhibited MMP-26 with similar efficacy. A pair of stereoisomers had only a 40-fold difference in K_i^{app} values against MMP-26 compared with a 250-fold difference against neutrophil collagenase, indicating that MMP-26 is less stereoselective for its inhibitors. MMP-26 autodigested itself during the folding process. Two of the major autolytic sites were Leu^{49}–Thr^{50} and Ala^{75}–Leu^{76}, which still left the cysteine switch sequence (PHC^{82}GVPD) intact. This suggests that Cys^{82} may not play a role in the latency of the zymogen. Interestingly, inhibitor titration studies revealed that only ~5% of the total MMP-26 molecules was catalytically active, indicating that the thiol groups of Cys^{82} in the active molecules may be dissociated or removed from the active site zinc ions. MMP-26 cleaved Phe^{352}–Leu^{353} and Pro^{357}–Met^{358} in the reactive loop of α_1-proteinase inhibitor and His^{140}–Val^{141} in insulin-like growth factor-binding protein-1, probably rendering these substrates inactive. Among the fluorescent peptide substrates analyzed, Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH$_2$ displayed the highest specificity constant (30,000/molar second) with MMP-26. This report proposes a working model for the future studies of pro-MMP-26 activation, the design of inhibitors, and the identification of optimal physiological and pathological substrates of MMP-26 *in vivo*.

Matrix metalloproteinases (MMPs)[1] share a conservative metal binding sequence of H*E*X*G*H*XX*G*XX*HS and a turn containing methionine (1). Evidence suggests that MMPs may play important roles in extracellular matrix (ECM) remodeling in physiological processes (2, 3). Excessive breakdown of the ECM by MMPs is observed in pathological conditions including periodontitis, rheumatoid arthritis, and osteoarthritis. MMPs also participate in tumor cell invasion and metastasis by degrading the basement membrane and other ECM components and allowing the cancer cells to gain access to blood and lymphatic vessels (4). Analyses of a large number of peptide and protein substrates and more recent work with phage display and synthetic peptide libraries have led to the identification of consensus cleavage site motifs for a number of different MMPs (5–13). The substrate specificities of MMPs are quite similar to each other, showing strong preferences for hydrophobic residues at P1′. Although distinct MMPs often prefer the same type of amino acid residues at corresponding positions surrounding the cleavage site, differences in the orders of preference for specific residues at each position may more precisely determine MMP specificity for substrates.

Endometase (matrilysin-2/MMP-26) is the smallest member of the MMP family, with a molecular mass of 28 kDa (14–17). Sequence homology calculations identified metalloelastase (MMP-12) and stromelysin-1 (MMP-3) as the closest relatives. Nevertheless, the specificity constant profile of peptide substrates with MMP-26 was quite different from that with MMP-12 and MMP-3 (14). According to protein substrate studies *in vitro*, MMP-26 might process matrix proteins such as fibronectin, vitronectin, fibrinogen, type IV collagen, gelatinase B (MMP-9), and gelatin (14–17).

MMP-26 has been found to be highly expressed in several cancer cell lines. A significant level of expression in normal tissues was found only in the uterus and placenta. The limited occurrence of MMP-26 in normal tissues suggests that the production of this enzyme may be strictly regulated during specific events, such as implantation, and that MMP-26 could be a target enzyme for the treatment of cancer and other pathological conditions.

The biological function and substrate specificity of MMP-26 are not yet fully understood. According to the protein substrate

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[1] The abbreviations used are: MMP, matrix metalloproteinase; α_1-PI, α_1-protease inhibitor; Brij-35, polyoxyethylene lauryl ether; IGFBP-1, insulin-like growth factor binding protein-1; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; ECM, extracellular matrix; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; Dnp, 2,4-dinitrophenyl; Dpa, *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Mca, (7-methoxycoumarin-4-yl)acetyl; Nva, non-valine.

studies *in vitro*, it may participate in ECM degradation. In this study, we take a step forward toward understanding the biochemical properties and functions of MMP-26 by identifying the cleavage sites of protein and peptide substrates, characterizing the substrate specificities of MMP-26 and measuring the potencies of synthetic inhibitors.

EXPERIMENTAL PROCEDURES

Materials—Dnp-Pro-Leu-Gly-Met-Trp-Ser-Arg-OH, Dnp-Pro-Leu-Ala-Tyr-Trp-Ala-Arg-OH, Mca-Pro-β-cyclohexylalanyl-Gly-Nva-His-Ala-Dpa-NH_2, Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2, insulin-like growth factor binding protein-1 (IGFBP-1), and MMP-specific synthetic inhibitors were purchased from Calbiochem, and Dnp-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-NH_2 and Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2 were purchased from Bachem. Hydroxamic acid derivatives of amino acids, buffers, cysteine, α_1-protease inhibitor (α_1-PI), and 1,10-phenanthroline were purchased from Sigma. Metal salts, Brij-35, sodium dodecyl sulfate, dithioerythreitol, and 2-mercaptoethanol were purchased from Fisher. Peptide libraries were synthesized at the Tufts University Core Facility (Boston, MA) as described previously (12).

Preparation of Partially Active MMP-26—MMP-26 was expressed in the form of inclusion bodies from transformed *E. coli* cells as described previously (14). The inclusion bodies were isolated and purified using B-PER™ bacterial protein extraction reagent according to the manufacturer's instructions. The insoluble protein was dissolved in 8 M urea to ~5 mg/ml. The protein solution was diluted to ~100 μg/ml in 8 M urea and 10 mM dithiothreitol for 1 h, dialyzed in 4 M urea, 1 mM dithiothreitol, 50 mM HEPES, or Tricine, pH 7.5, for at least 1 h and then folded by dialysis in buffer containing 50 mM HEPES or Tricine, 0.2 M NaCl, 10 mM $CaCl_2$, 20 μM $ZnSO_4$, 0.01% Brij-35, pH 7.5, for 16 h. To enhance the activity of MMP-26, the folded enzyme was dialyzed twice for 24 h at 4 °C in the folding buffer without Zn^{2+} ion. The total enzyme concentration was measured by UV absorption using $\epsilon_{280} = 57130\ M^{-1}\ cm^{-1}$, which was calculated by Genetics Computer Group software.

Peptide Library Methods—The methods were performed as described previously (12). To determine the specificity for the primed positions (18), an amino-terminally acetylated dodecamer peptide mixture (1 mM) consisting of a roughly equimolar mixture of the 19 naturally occurring L-amino acids excluding cysteine at each site was incubated with MMP-26 in 50 mM HEPES, pH 7.4, 200 mM NaCl, 5 mM $CaCl_2$ at 37 °C until 5–10% of the peptides were digested. An aliquot (10 μl) of the mixture was subjected to automated amino-terminal peptide sequencing. The data in each sequencing cycle were normalized to the total molar amount of amino acids in that cycle so that a value of 1 indicated the average value. Undigested peptides and the amino-terminal fragments of digested peptides are amino-terminally blocked and therefore do not contribute to the sequenced pool.

The specificity of the unprimed side was determined by libraries with the sequence MAXXXXXLRGAARE(K-biotin) for the P3 site and MAXXPXXLRGGGEE(K-biotin) for other sites, where X represents a degenerate position, K-biotin is ε-(biotinamidohexanoyl)lysine, and the amino terminus is unblocked. Libraries were partially digested with MMP-26 as described above, quenched with EDTA (10 mM), and treated in batch with 400 μl of avidin-agarose resin (Sigma). The mixture was transferred to a column, which was washed with 25 mM ammonium bicarbonate. The unbound fraction was evaporated to dryness under reduced pressure, suspended in water, and sequenced. Data were normalized as described above.

Kinetic Assays—Assays of fluorescent peptide substrates were performed by following the procedures reported in the literature (14, 29). For substrates containing the tryptophan residue, the fluorescence was observed at an excitation wavelength of 280 nm and emission wavelength of 360 nm, and for substrates containing 3-methoxycoumarin, fluorescence was measured at an excitation wavelength of 328 nm and emission wavelength of 393 nm. All of the kinetic experiments were conducted in 50 mM HEPES buffer containing 10 mM $CaCl_2$, 0.2 M NaCl, and 0.01% Brij-35. To assess inhibition potency for tight binding inhibitors, the apparent inhibitor dissociation constants (K_i^{app} values) were calculated by fitting the data to Morrison's equation (19). The inhibitor dissociation constants (K_i values) were determined by Dixon's plot (20) for less potent inhibitors. The inhibition assays were performed with a peptide substrate (1 μM), Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, and 5–10 different inhibitor concentrations. The substrate stock solutions were prepared in Me_2SO and then further diluted to 50% Me_2SO in water. The final Me_2SO concentration in the assays was 1%. The inhibitors were dissolved in Me_2SO to 5 or 2 mM and diluted with methanol with the exception of inhibitor IV (Calbiochem catalogue number: 444250), which was dissolved in assay buffer. The final methanol concentration in the inhibition assays was 5% (v/v). The specificity constants (k_{cat}/K_m values) were determined by the equation $V = (k_{cat}/K_m)[E][S]$, which is modified from the Michaelis-Menten equation when $[S] \ll K_m$.

The enzyme became a mixture of several states after partial activation by dialysis. The total concentration of 400 nM MMP-26 was measured by absorption at 280 nm and calculated using a molar extinction coefficient of 57,130 $M^{-1}\ cm^{-1}$. The enzyme was titrated with MMP inhibitor I (GM-6001) to determine the concentration of catalytically active MMP-26. The titration analysis revealed the concentration of active MMP-26 to be 21 nM, which was ~5% of the total protein concentration after dialysis. For an accurate titration, the concentration of an enzyme is required to be at least 100-fold more than the inhibition constant of the titrant (21). To avoid the depletion of substrate by a high MMP-26 concentration, a less specific substrate, Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-NH_2, designed for MMP-3 (22), was used for detection of the initial rate. The cleavage of this substrate by MMP-26 was the slowest among peptide substrates studied in our laboratory (14).

IGFBP-1 and α_1-PI Digestion by MMP-26—IGFBP-1, α_1-PI, and MMP-26 solutions were diluted or dissolved in 50 mM HEPES buffer at pH 7.5 containing 10 mM $CaCl_2$, 0.2 M NaCl, and 0.01% Brij-35. IGFBP-1 (4 μg) and MMP-26 (0.63 μg) in a total volume of 50 μl were incubated for 2 days at room temperature. Each day, 10 μl of reaction mixture was taken, and the reaction was stopped by boiling for 5 min after 2× SDS-PAGE sample buffer containing 2% SDS, 100 mM dithiothreitol, and 50 mM EDTA was added. The cleaved products were separated by a 12% acrylamide gel and detected by silver staining. For cleavage of α_1-PI, 90 μg of α_1-PI were incubated with 1.3 μg of MMP-26 in a total volume of 100 μl. The samples were collected after 1 h, 1day, and 2 days. The cleaved products were separated by a 15% SDS-PAGE and detected by silver staining.

Determination of Cleavage Products by Matrix-assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS)—The cleavage sites of fluorogenic peptide substrates and α_1-PI were determined by measuring the mass of the cleavage products with a Bruker protein time-of-flight mass spectrometer. The reaction mixture was mixed with an equivalent volume of α-cyano-4-hydroxycinamic acid (4.5 mg/ml in 50% CH_3CN, 0.05% trifluoroacetic acid) matrix solution containing synthetic peptide calibrants. Because the high salt concentration increased the noise in the mass spectra, the digestion reaction was performed with 10 mM HEPES buffer containing 5 mM $CaCl_2$ overnight at room temperature. For fluorogenic substrates, MMP-9 was used as a positive control.

RESULTS

Substrate Specificities of MMP-26—The substrate specificity of MMP-26 was investigated using a recently described peptide library method (12). Data are shown in Fig. 1. The residues preferred at each site from P4–P4′ are summarized in Table I. The strongest selectivity was seen at the P1′ site where large hydrophobic residues were preferred. Small residues, alanine and serine, were preferred at the P3′ site. Although P2′ and P4′ displayed indistinct specificity compared with the P1′ site, a lack of a preference for a basic residue (Arg or Lys) at the P2′ site was unique to MMP-26 (Table I). Among the unprimed positions, the P3 site showed the highest selectivity preferring proline and valine. The P1 site was not as selective as the P3 site, although small residues such as serine were preferred. The preference of MMP-26 for proline at P3, hydrophobic residues at P2 and P1′ sites, and serine at P1 is similar to that of other MMPs (5–13).

Inhibition of MMP-26 by Synthetic Inhibitors—Inhibition constants for several inhibitors designed for collagenases and gelatinases were measured with MMP-26, and these values are shown in Fig. 2. Among the four inhibitors tested, inhibitor I (23) was the most potent for MMP-26 with a K_i^{app} of 0.36 nM. Inhibitor II inhibited MMP-26 with a K_i^{app} of 1.5 nM, which is similar to the inhibition constant with neutrophil collagenase MMP-8 (4 nM) (24). Inhibitor III is a less potent stereoisomer of inhibitor II, and MMP-8 discriminates between the two with a 250-fold difference in their inhibition constants (1000 *versus* 4

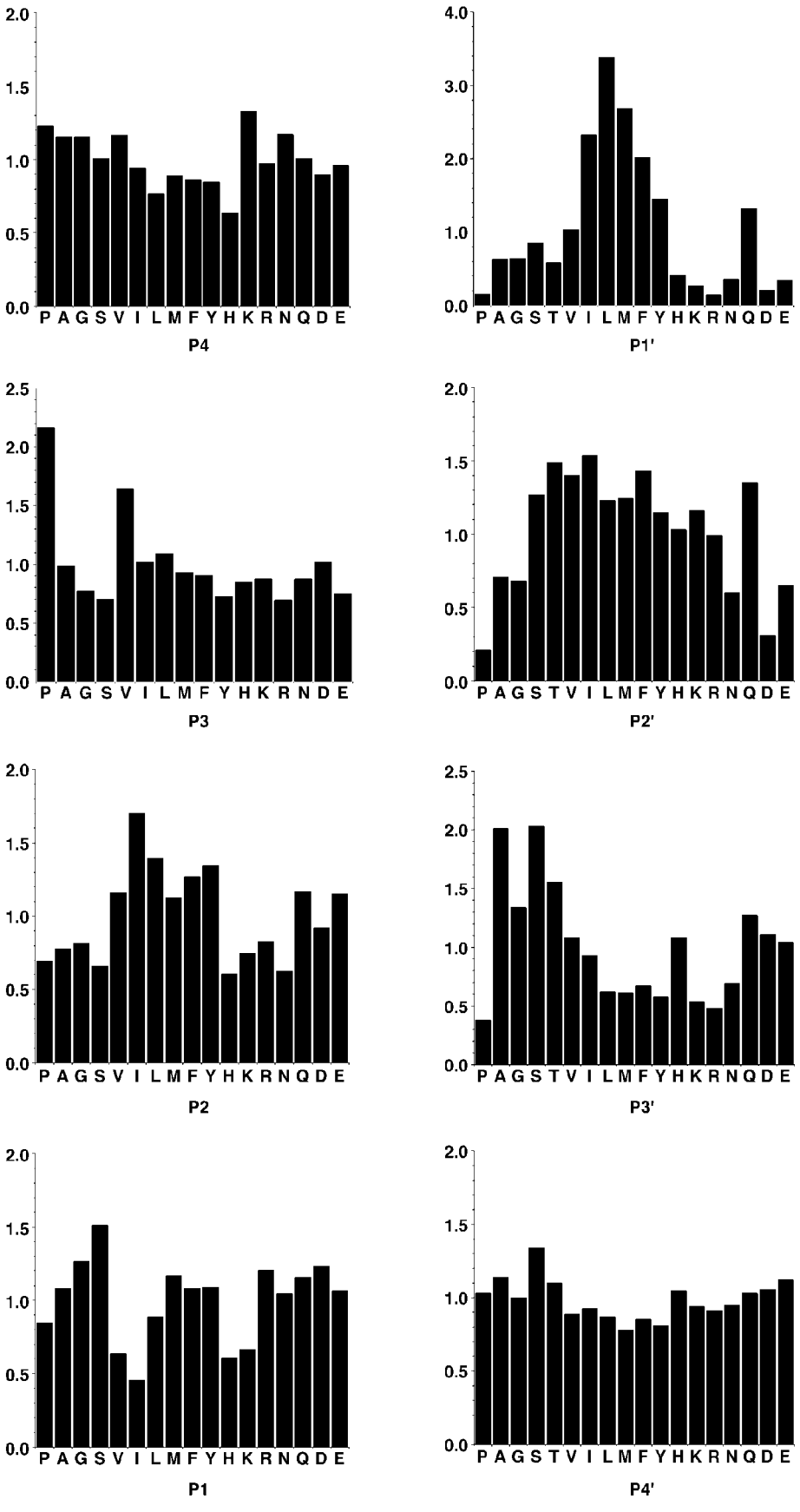

FIG. 1. **Cleavage site specificity of MMP-26 (endometase).** The *figures* on the *right* represent the relative distribution of amino acid residues at positions COOH terminus (P1′–P4′) to the MMP-26 cleavage site determined by sequencing the cleavage fragments of a random dodecamer (Ac-*XXXXXXXXXXXX*). Data are normalized so that a value of 1 corresponds to the average quantity per amino acid in a given sequencing cycle and would indicate no selectivity. Tryptophan was not included in the analysis because of poor yield during sequencing. The *figures* on the *left* represent specificity of positions amino terminus to the MMP-26 cleavage site. For the P3 position, data shown were obtained using the library MA*XXXXX*LRGAARE(K-biotin). For all other positions, the P3 proline library MG*XX*P*XX*LRGGGEE(K-biotin) was used. Glutamine and threonine were omitted in some cycles because of high background on the sequencer. Data were normalized as for the primed sites.

nM). There was a 40-fold difference between the K_i^{app} values of the pair of stereoisomers with MMP-26 (60 *versus* 1.5 nM). Inhibitor IV inhibited MMP-26 with a K_i^{app} of 2.9 μM and an IC_{50} value of 3.4 μM. This IC_{50} value is similar to the IC_{50} values with interstitial collagenases MMP-1 and MMP-8 (both are 1 μM) (25).

TABLE I
Cleavage site motifs for MMP-26[a] compared with those of six other MMPs[b]

Enzyme	Cleavage position							
	P4	P3	P2	P1	P1′	P2′	P3′	P4′
MMP-26	Lup (1.3)	Pro (2.2) Val (1.6)	Ile (1.7) Leu (1.4) Tyr (1.3)	Ser (1.5)	Leu (3.4) Met (2.7) Ile (2.3) Phe (2.0) Tyr (1.5) Gln (1.3)	Ile (1.5) Iwe (1.5) Phe (1.4) Gln (1.4)	Ser (2.0) Ada (2.0) Thr (1.6) Gly (1.3)	Ser (1.3)
MMP-1	Val	Pro	Met	Ser	Met	Met	Ala	
MMP-2	Ile	Pro	Val	Ser	Leu	Arg	Ser	
MMP-3	Lys	Pro	Phe	Ser	Met	Met	Met	
MMP-7	Val	Pro	Leu	Ser	Leu	Val	Met	
MMP-9	Val	Pro	Leu	Ser	Leu	Arg	Ser	
MMP-14	Ile	Pro	Glu	Ser	Leu	Arg	Met	
MMP	Val	Pro	Leu	Ser	Leu	Arg	Ala	
Consensus[c]		Val	Tyr		Met Ile	Ile		

[a] Quantities were determined from sequencing data as described for Fig. 1, and values ≥1.3 are listed. All primed sites were obtained using the library Ac-*XXXXXXXXXXXX*. MA*XXXXX*LRGAARE(K-biotin) and MG*XXPXX*LRGGGEE(K-biotin) were used to produce the data at the unprimed sites.
[b] Data from Turk *et al.* (12). A series of consensus peptides/optimal cleavage site motifs were selected and listed for each MMP.
[c] Data summarized from Turk *et al.* (12). These listed residues were selected among amino acids that appeared at least in 5 of the 6 MMPs with values ≥1.3.

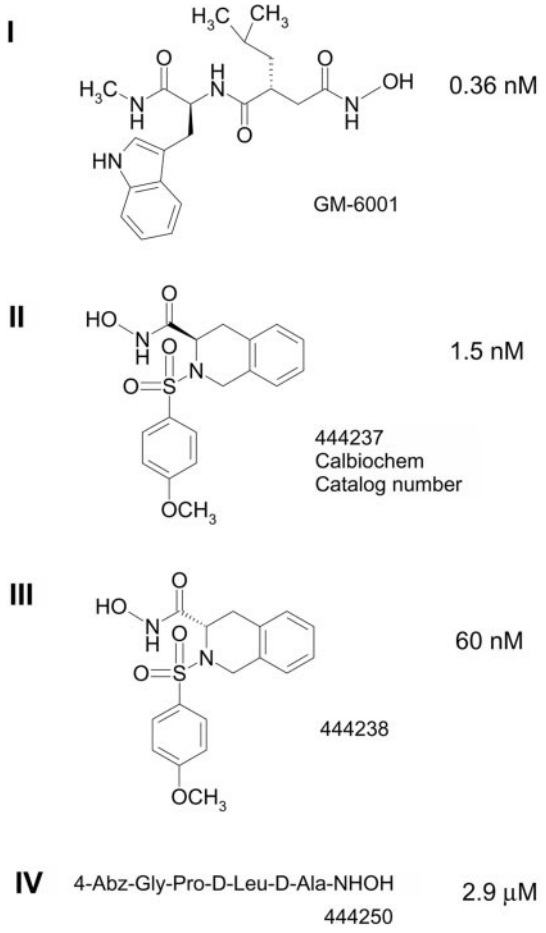

FIG. 2. **The structures of MMP inhibitors and their inhibitor dissociation constants with MMP-26.** The apparent inhibition constants (K_i^{app} values) were determined by Morrison's equation for tight binding inhibitors (compounds *I*, *II*, and *III*) (19), and the inhibition constant (K_i value) was determined by Dixon's plot for a less potent inhibitor (compound *IV*) (20). The values were 0.36, 1.5, 60, and 2900 nM for compounds I, II, III, and IV, respectively.

Autocleavage Sites of Recombinant MMP-26—Dialysis of the folded pro-form of MMP-26 results in an increase in activity because of autolysis of the prodomain. MMP-26 was collected after two 24-h dialyses with fresh buffer at 4 °C (further dialysis or incubation gradually reduced the activity). Partially activated MMP-26 was compared with the zymogen form on a silver-stained polyacrylamide gel (Fig. 3). The band near 30 kDa was confirmed to be pro-MMP-26 by amino-terminal sequencing (Fig. 3, *lane 2*) (14). Several bands below 30 kDa appeared after the dialysis, three of which were located between 20 and 25 kDa (Fig. 3, *lane 3*). One or more of the three cleavage products may be active forms of MMP-26 and was analyzed by amino-terminal sequencing. Only the top two bands were successfully sequenced. The top band resulted from cleavage of a peptide bond between Leu^{49} and Thr^{50}, and the band below it was a product of cleavage between Ala^{75} and Leu^{76} (sequence based on Ref. 14). The cleavage at either site does not remove the cysteine switch sequence PH**C^{82}**GVPDGSD.

Cleavage of Fluorogenic Substrates by MMP-26—Initial screening of a number of fluorogenic peptide substrates revealed that gelatinase and collagenase peptide substrates were most efficiently cleaved by MMP-26 (14, 17). Therefore, we chose peptide substrates designed for gelatinases or collagenases for further study, three of which contained Trp and two of which contained 7-methoxy coumarin as the fluorogenic group, respectively (26–30). The active MMP-26 concentration was determined by active site titration with inhibitor I (Fig. 4) using the least efficient substrate tested as described under "Experimental Procedures." The titration analysis revealed the concentration of active MMP-26 to be ~5% of the total enzyme concentration (21 of 400 nM). The cleavage sites of the six fluorogenic peptide substrates were determined by identifying the mass of the products by mass spectrometry. Mass spectra of the cleavage products revealed that the cleavage sites of the substrates by MMP-26 and MMP-9 were identical as shown in the example of peptide III (Fig. 5). The specificity constants (k_{cat}/K_m) of these six peptide substrates with MMP-26 were measured and calculated as shown in Table II. MMP-26 hydrolyzed peptide V with the highest specificity constant (3.0×10^4 M^{-1} s^{-1}), which is still 10-fold lower than the specificity constant with MMP-2 (3.97×10^5 M^{-1} s^{-1}) (26).

Cleavage Site of α_1-PI and IGFBP-1—MMP-26 cleaved α_1-PI near the COOH terminus to produce a COOH-terminal fragment of approximately 5 kDa (Fig. 6, *lanes 6* and *7*). This fragment was detected by silver staining of a 15% SDS-PAGE gel run under optimized conditions to identify proteins of molecular masses <10 kDa as described previously (31). A 24-h

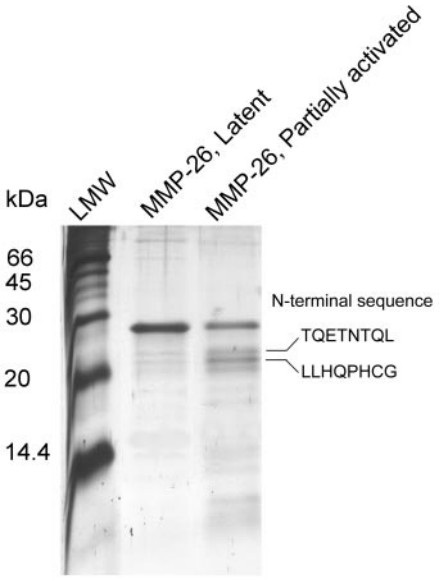

FIG. 3. **Autolysis of MMP-26 during dialysis.** *Lanes 1–3* were low molecular weight markers and the folded MMP-26 before and after dialysis at 4 °C for 24 h, respectively. The cleavage sites of MMP-26 that formed the two major bands around 20 kDa were revealed to be The^{51}–Gln^{52} and Ala^{75}–Leu^{76} by amino-terminal sequencing.

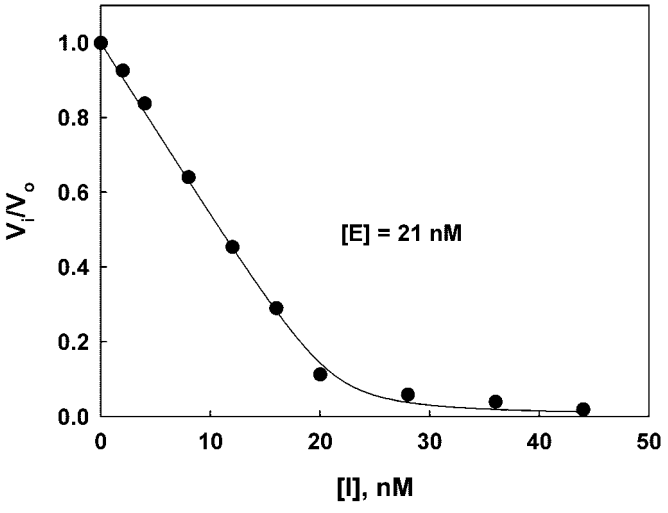

FIG. 4. **Determination of the active MMP-26 concentration by titration of MMP-26 with inhibitor I.** Total MMP-26 concentration was estimated to be 400 nM by molar absorptivity. The estimated active concentration was 21 nM by fitting the titration data into Morrison's equation (19). The assays were performed as described under "Experimental Procedures" with 1 μM of the substrate.

incubation of α_1-PI with MMP-26 at room temperature led to the formation of a fragment below 14.4 kDa (*lane 6*), which was not cleaved any further after 2 days of incubation (*lane 7*). The mass spectrum of the α_1-PI and MMP-26 mixture (Fig. 7*B*) exhibited two new peaks located at 4260 and 4774, which were not observed in the spectrum of α_1-PI alone (Fig. 7*A*). Based on molecular mass analysis, the cleavage sites resulting in these fragments should be Phe^{352}–Leu^{353} (~4774 Da) and Pro^{357}–Met^{358} (~4260 Da) near the COOH terminus of α_1-PI.

A comparison of *lanes 2* and *7* in Fig. 8 indicated that there was no detectable proteolysis of IGFBP-1 without MMP-26. The dark band around 30 kDa (IGFBP-1) disappeared, and a band below 14.4 kDa appeared when IGFBP-1 was incubated with MMP-26 for 1 or 2 days (*lanes 4* and *5*, respectively). The amino-terminal sequence of this band was determined to be Val-The-Asn-Ile-Lys-Lys-Trp-Lys, demonstrating that it arises

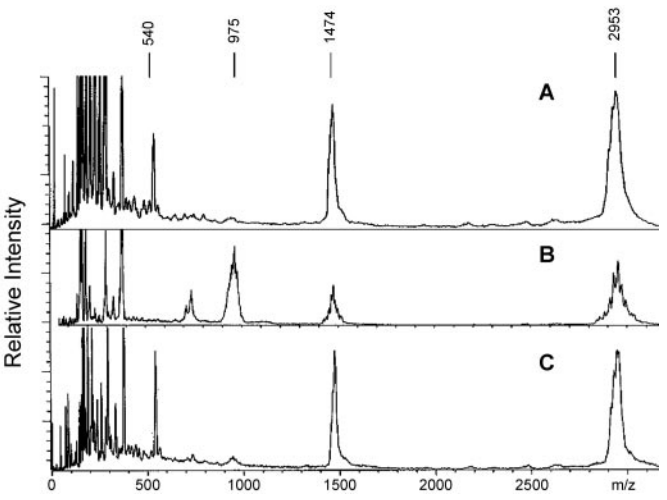

FIG. 5. **An example of the determination of fluorogenic peptide cleavage sites by MALDI TOF mass spectrometry.** 80 μM peptide substrate III (Table II), Dnp-Pro-Leu-Gly-Leu-Trp-Ala-(D)-Arg-OH) was incubated overnight with 5 nM MMP-9 (human neutrophil gelatinase) (*A*), alone (*B*), and with 20 nM endometase (*C*), pH 7.5, and 10 mM HEPES containing 5 mM $CaCl_2$ at room temperature. The two peaks observed at *m/z* 1474 and 2953 were internal synthetic peptide mass calibrants. The peaks at *m/z* 975 and 542 were the substrate and the cleaved peptide fragment, Leu-Trp-Ala-(D)-Arg-OH, produced by cleavage of the Gly–Leu peptide bond by MMP-9 and endometase, respectively.

TABLE II
Peptide substrates of MMP-26[a]

Fluorogenic substrate cleavage sites[b]	k_{cat}/K_m
	$s^{-1}\,M^{-1}$
P3 P2 P1 P1′ P2′ P3′ P4′	
Dnp-Pro-Leu-Gly–Met-Trp-Ser-Arg-OH (I)	9.4×10^3
Dnp-Pro-Leu-Ala–Tyr-Trp-Ala-Arg-OH (II)	3.5×10^3
Dnp-Pro-Leu-Gly-Leu-Trp-Ala-(D)Arg-OH (III)	4.9×10^3
Mca-Pro-Cha-Gly–Nva-His-Ala-Dpa-NH_2 (IV)	1.7×10^4
Mca-Pro-Leu-Ala–Nva-Dpa-Ala-Arg-NH_2 (V)	3.0×10^4
Mca-Pro-Leu-Gly–Leu-Dpa-Ala-Arg-NH_2 (VI)	2.2×10^4

[a] All of the assays were performed in pH 7.5 buffer containing 50 mM HEPES, 0.2 M NaCl, 0.01 M CaCl2, 0.01% Brij-35 at 25°C. The range of substrate concentrations used were 1μM, and the active MMP-26 concentration used was 2 nM for the substrates containing the Mca group and 10 nM for the substrates containing the Trp residue.

[b] The cleavage sites of the substrates were determined by mass spectrometry as described under "Experimental procedures" and Fig. 5.

from cleavage at the same site (His^{140}–Val^{141}) as stromelysin-3 (MMP-11), which produces an inactive 9-kDa fragment (32).

DISCUSSION

The results obtained from peptide library studies indicate that MMP-26 substrate specificities are similar to those of other MMPs where hydrophobic residues are preferred at P1′and P2, proline is preferred at P3, and serine is preferred at P1. The optimal cleavage motifs/consensus peptide sequences for MMP-26 were Lys-Pro-Ile/Leu-Ser(P1)-Leu/Met(P1′)-Ile/Thr-Ser/Ala-Ser (Table I), which are not identical to those of MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, and MMP-14 (12). Based on this sequence specificity knowledge, new fluorescence resonance energy transfer substrates more specific for MMP-26 will be designed and developed. These data may provide critical information applicable to the design of new MMP-26-specific inhibitors and to the identification of novel physiological and pathological substrates of MMP-26 *in vivo*.

The inhibition constants of four synthetic inhibitors with MMP-26 were comparable to those with gelatinases and collagenases, the enzymes for which the inhibitors were designed. This corroborates the findings that the substrate specificity of

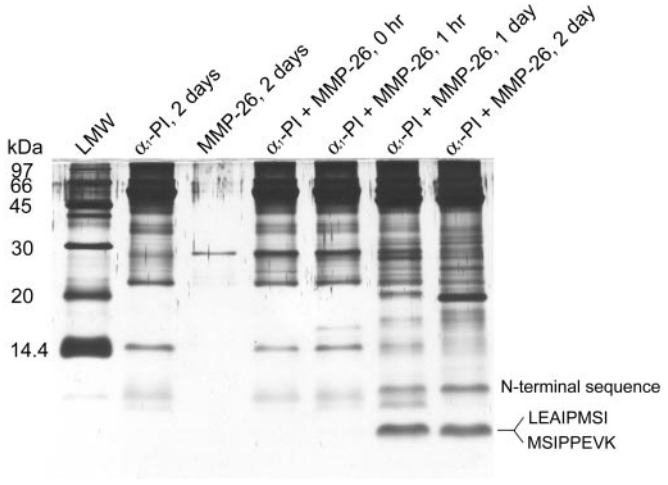

FIG. 6. **Cleavage of human α_1-PI by MMP-26.** After incubation of an α_1-PI (900 μg/ml) and MMP-26 (13 μg/ml) mixture for 1 day (*lane 6*) and 2 days (*lane 7*) at room temperature, the COOH-terminal cleavage products were detected by silver staining a 15% SDS-PAGE gel. Samples containing α_1-PI were overloaded to detect the bands of around 4.5 kDa in *lanes 6* and *7*, which might be 4.8- and 4.2-kDa fragments produced by MMP-26 proteolysis of α_1-PI. The two amino-terminal sequences were deduced from the mass spectrometry results shown in Fig. 6 compared with the primary structure of human α_1-PI.

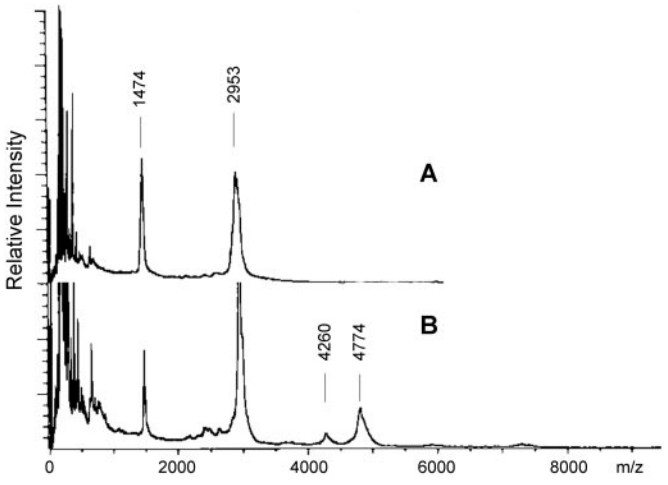

FIG. 7. **Cleavage sites of α_1-PI by MMP-26 determined by MALDI TOF mass spectrometry.** α_1-PI alone (*A*) and with MMP-26 (*B*) were incubated for 1 day in 10 mM HEPES buffer at pH 7.5 containing 5 mM $CaCl_2$. The peaks at *m/z* 1474 and 2953 were two internal calibrants. The two peaks observed at *m/z* 4260 and 4774 were produced from α_1-PI cleavage by MMP-26 at the sites Pro^{357}–Met^{358} and Phe^{352}–Leu^{353}.

MMP-26 is quite close to that of other MMPs. Inhibitor I/GM6001 was the most potent inhibitor of MMP-26 tested with a K_i^{app} of 0.36 nM. GM6001 also potently inhibits MMP-2 (K_i = 0.5 nM) and MMP-8 (K_i = 0.1 nM) but is less effective against MMP-3 (K_i = 27 nM) (23). Inhibitor III is a less potent stereoisomer of inhibitor II, and MMP-8 discriminates between the two with a 250-fold difference in their inhibition constants. There was only 40-fold difference between the K_i^{app} values of the stereoisomers with MMP-26, indicating that MMP-26 is less stereoselective for its inhibitors. Inhibitor IV was more selective for MMP-1 and MMP-8 (IC_{50} = 1 μM against both enzymes) than MMP-9 (IC_{50} = 30 μM) and MMP-3 (IC_{50} = 150 μM) (25). This inhibitor has an IC_{50} value of 3.4 μM with MMP-26, similar as that with MMP-1 and MMP-8.

A survey of known protein cleavage sites determined *in vitro* for MMP-26 is summarized in Table III. The survey indicates that hydrophobic residues are preferred at P1′ and appear in almost all of the substrates. Residues occurring at other positions that agree with the consensus from the peptide libraries include proline (3 times) at P3, hydrophobic residues (6 times) at P2, and Ser, Ala, and Thr (4 times) at P3′. Residues at the other positions seem random and do not coincide with residue predictions by the peptide libraries, although the libraries do indicate less stringent selectivity at these positions. Accordingly, no individual protein cleavage site precisely matches the consensus motif determined by the peptide library studies, suggesting that the cleavage sites in these protein substrates are probably suboptimal for cleavage by MMP-26. The folding topology of the protein may be a contributing factor to the enzyme-substrate interactions. Although the protein cleavage site may not be the optimal sequence, the peptide chain might assume a conformation that is easily accessible to a protease active site; for example, an exposed loop is found in the bait region of α_2-macroglobulin (33), and the reactive loop is found in the bait region of α_1-PI (34). Alternatively, the cleavage of a suboptimal site may be promoted by recruitment to the enzyme via a substrate-binding exosite. In addition, the presence of unfavorable residues around the cleavage site may slow down the rate of digestion by a protease, regulating the degradation process.

MMP-26 has been shown to digest several components of the extracellular matrix, such as fibronectin, collagens, fibrinogen, and vitronectin, but not any of several plasma proteins tested with the exception of α_1-PI (14, 17). It has been reported that the cleavage of the reactive loop residues around 350–365 in α_1-PI by MMP-1 and MMP-3 inactivates the inhibitor (34–36). The digestion of α_1-PI by MMP-26 generates two major peaks

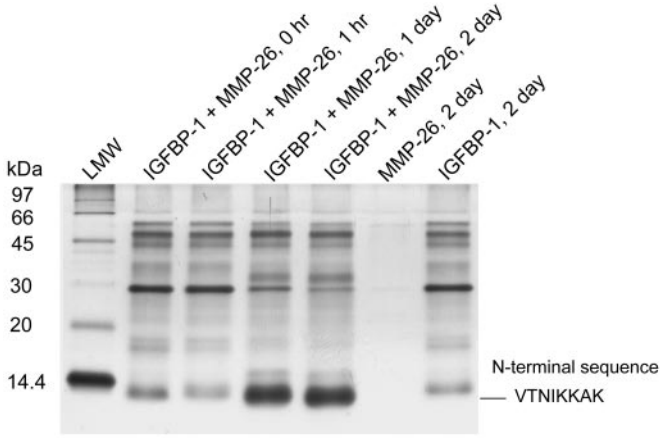

FIG. 8. **Cleavage of IGFBP-1 by MMP-26.** IGFBP-1 (80 μg/ml) was incubated with MMP-26 (13 μg/ml) for 0 h (*lane 2*), 1 h (*lane 3*), 1 day (*lane 4*), and 2 days (*lane 5*). The dense band below 14.4 kDa observed after 1 day (*lane 4*) was the product of IGFBP-1 cleavage by MMP-26 at the His^{140}–Val^{141} site.

TABLE III
Protein sequences hydrolyzed by MMP-26

Proteins	Cleavage sites[a]
α_1-PI[a]	GAMF-LEAI
	EAIP-MSIP
MMP-26 (autolysis)[b]	QMHA-LLHQ
	SPLL-TQET
MMP-26 (autolysis)[c]	QLLQ-QFHR
IGFBP-1[b]	KALH-VTNI
Fibronectin[d]	SPVA-VSQS
Vitronectin[d]	KPEG-IDSR
Fibrinogen[d]	SKPN-MIDA
	HTEK-LVTS
	GDKE-LRTG

[a] A line is inserted in the cleavage site.
[b] Data from this study.
[c] Data from Marchenko *et al.* (41).
[d] Data from Marchenko *et al.* (17).

that originate from the cleavage at two sites near the COOH-terminal region, Phe^{352}–Leu^{353} (~4774 Da) and Pro^{357}–Met^{358} (~4260 Da). These are the same cleavage sites for MMP-1 (35). In addition, MMP-3 cleaves the Pro^{357}–Met^{358} bond (34). MMP-11 cleaves the Ala^{350}–Met^{351} bond (36), a site distinct from those of MMP-26 and MMP-1. Interestingly, direct evidence showed that α_1-PI was a critical substrate for MMP-9 *in vivo* in a mouse model of the autoimmune disease *bullous pemphigoid* (37). Thus, MMP-26 may inactivate α_1-PI like the other MMPs to promote serine proteinase activity, enhancing extracellular matrix degradation in cancers or other pathological processes.

The insulin-like growth factors, IGFBPs, and IGFBP proteases are involved in the regulation of somatic growth and cellular proliferation. The level of free insulin-like growth factor in a system is modulated by rates of insulin-like growth factor production and clearance and the degree of binding to IGFBPs (38). IGFBP-1 inhibits IGF-I-induced proliferation of the MCF-7 human breast adenocarcinoma (32). Through their inactivation of IGFBP-1, MMPs were able to promote cell growth and survival by the increase of the effective insulin-like growth factor concentration in the surrounding medium (32). MMP-26 cleaves the His^{140}–Val^{141} bond in IGFBP-1 as does MMP-11. Therefore, the cleavage of IGFBP-1 by MMP-26 to produce the 9-kDa inactive form may sustain the survival of cancer cells, increasing the chance of metastasis.

The cleavage sites in the fluorogenic substrates seem in good agreement with the motifs determined by the peptide library approach. Although the six commercial fluorogenic peptide substrates tested were not designed for the specificity of MMP-26, some of them resemble closely to the consensus sequences of peptide substrates for MMP-26 determined by the peptide library studies, proline at P3, a hydrophobic residue at P2, P1′, and P2′, and small residues at P3′, with the exception that serine is preferred at P1 and P4′, Lys is preferred at P4, but a basic residue is not preferred at P2′. The best substrate tested for MMP-26 was peptide V, Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2. This peptide appears to be very close to optimal sequences determined by the peptide library studies where there is a selected residue at essentially every position (see Fig. 1 and Table I) with the exception that the peptide libraries do not have Nva at P1′.

The cleavage sites in the protein substrates tested do not match exactly the optimal motifs identified by the peptide library approach; however, upon close examination of the protein cleavage site data presented in Table III, it seems that the amino acid residues at P1 and P4′ are less selective. This is in good agreement with the peptide library data. Furthermore, P1′ is more selective, and Leu, Met, and Ile are preferred at P1′ (Fig. 1). This finding is consistent with the protein cleavage site data shown in Table III in which 7 of the 11 residues (64%) at P1′ are these residues. Moreover, two Lys residues are found at the P4, and two Ser residues are found at P4′ of the protein cleavage sites, which is also unique to MMP-26 according to the library data.

The relative rates of cleavage in the six fluorogenic substrates also correspond to the peptide library data relatively well. The best substrate is peptide V with a specificity constant of 3.0×10^4 M^{-1} s^{-1}. In addition to peptide V, peptides IV and VI are also relatively good substrates for MMP-26 with specificity constants of 1.7×10^4 M^{-1} s^{-1} and 2.2×10^4 M^{-1} s^{-1}, respectively (Table II). The worst substrate of MMP-26 in Table II is peptide II with a specificity constant ~10 times slower than peptide V. Neither Ala at P1 nor Tyr at P1′ in the peptide II is preferred. On the other hand, the rate of cleavage of peptide V, the best peptide of MMP-26 in Table II, is 10 times slower than the rate of substrate cleavage by MMP-2 (3.97×10^5 M^{-1} s^{-1}) (26). The slower rate of peptide and protein digestion by MMP-26 suggests that this enzyme is not the most powerful MMP catalytically or the optimal substrates for MMP-26 have not been identified.

It is also possible that a manageable rate of MMP-26 catalysis may be required in biological processes such as normal implantation where tight control of substrate degradation is highly desirable. In the latter scenario, the function of MMP-26 may not be limited to the direct degradation of ECM. MMP-26 may play a more critical role in controlling the activities of growth factors or proteases that mediate such processes. Consequently, biologically significant substrates of MMP-26 may be growth factor-binding proteins, receptors, zymogens, and enzyme inhibitors.

MMP-26 is not only unique in terms of its tissue and cell-specific expression as reported by us and others (14–17) but also because of its unique cysteine switch sequence (P**H**^{81}CGVPDGSD) and thus its unique pathway of proenzyme activation. Many members of the MMP family follow the classic cysteine-switch activation model (39, 40). The inactivity of a pro-MMP is generally attributable to a complex between the sulfhydryl group of a cysteine residue in the cysteine switch sequence (P**R**CGVPDV) of the prodomain and the active site zinc atom in the catalytic domain. The activation of a pro-MMP can be achieved proteolytically by hydrolysis of the propeptide on the carboxyl-terminal side of the cysteine switch residue near the border between the propeptide and catalytic domains. This proteolytic step may be catalyzed by another proteinase or it may be an autolytic step (39, 40). However, Marchenko *et al.* (41) have challenged the cysteine-switch model. Their report showed that the activating cleavage site of pro-MMP-26 occurs at Gln^{59}–Gln^{60}, leaving the putative cysteine switch sequence intact. It was suggested that the Arg to His substitution existing in the unique P**H**^{81}CGVPDGSD cysteine-switch motif of pro-MMP-26 abolishes the ability of Cys^{82} to interact with the zinc ion of the catalytic domain (41).

We have identified two of the major autolytic sites in MMP-26 to be Leu^{49}–Thr^{50} and Ala^{75}–Leu^{76}. Although different from the Gln^{59}–Gln^{60} site, the cleavage at these two sites also does not remove the cysteine switch sequence (PH**C**^{82}GVPD) from the enzyme, suggesting that Cys^{82} may not play a role in the latency of the zymogen, which is consistent with the hypothesis proposed by Marchenko *et al.* (41). Alternatively, the thiol group of Cys^{82} could be transiently dissociated from the zinc ion at the active site, allowing a water molecule to bind to the zinc ion and the enzyme to exhibit catalytic activity. Our inhibitor titration data demonstrated that ~5% of the total enzyme molecules was active. This observation may support the concept that the thiol groups of Cys^{82} in the active enzyme molecules are dissociated or removed from the active site zinc ions and the thiol groups of the Cys^{82} in remaining 95% of the total enzyme molecules are still coordinated with the zinc ions at the active sites, forming a steady-state equilibrium between the active enzyme molecules and the zymogen molecules. However, this hypothesis and the detailed activation mechanisms of pro-MMP-26 remain to be thoroughly investigated (42). In summary, this work provides new knowledge on the MMP-26 substrate specificity to build a working model for the future design of MMP-26 inhibitors, studies of pro-MMP-26 activation, and identification of optimal physiological and pathological substrates of MMP-26 *in vivo*.

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Intracellular Activation of Human Adamalysin 19/Disintegrin and Metalloproteinase 19 by Furin Occurs via One of the Two Consecutive Recognition Sites*

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Adamalysin 19 (a disintegrin and metalloproteinase 19, ADAM19, or meltrin β) is a plasma membrane metalloproteinase. Human ADAM19 zymogen contains two potential furin recognition sites (R*X*(K/R)R), ^{196}KRPR^{200}R and ^{199}RRMK^{203}R, between its pro- and catalytic domains. Protein N-terminal sequencing revealed that the cellular mature forms of hADAM19 started at ^{204}EDLNSMK, demonstrating that the preferred furin cleavage site was the ^{200}RMK^{203}R ↓ ^{204}EDLN. Those mature forms were catalytically active. Both Pittsburgh mutant of α_1-proteinase inhibitor and dec-Arg-Val-Lys-Arg-chloromethyl ketone, two specific furin inhibitors, blocked the activation of hADAM19. Activation of hADAM19 was also blocked by brefeldin A, which inhibits protein trafficking from the endoplasmic reticulum to the Golgi, or A23187, a calcium ionophore known to inhibit the autoactivation of furin. When ^{202}KR were mutated to AA, the proenzyme was also activated, suggesting that ^{197}RPRR is an alternative activation site. Furthermore, only pro-forms of hADAM19 were detected in the ^{199}RR to AA mutant, which abolished both furin recognition sites. Moreover, the zymogens were not converted into their active forms in two furin-deficient mammalian cell lines; co-expression of hADAM19 and furin in these two cell lines restored zymogen activation. Finally, co-localization between furin and hADAM19 was identified in the endoplasmic reticulum-Golgi complex and/or the trans-Golgi network. This report is the first thorough investigation of the intracellular activation of adamalysin 19, demonstrating that furin activated pro-hADAM19 in the secretory pathway via one of the two consecutive furin recognition sites.

The adamalysin, ADAM[1] (for a disintegrin and metalloprotease), or metalloprotease/disintegrin/cysteine-rich family includes proteins containing disintegrin- and metalloprotease-like domains. These proteinases are involved in diverse processes, such as development, cell-cell interaction, and protein ectodomain shedding (1–5). For example, ADAM10/kuzbanian (KUZ) and ADAM17/tumor necrosis factor-α convertase play key roles in the processing of both Notch1 receptor, which is critical in development, and amyloid precursor protein, which is related to the pathogenesis of Alzheimer's disease (3, 4, 6). Six different ADAMs, ADAM2, -9, -12, -15, -23, and -28, are able to interact with integrins such as $\alpha_6\beta_1$, $\alpha_v\beta_3$, $\alpha_9\beta_1$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, and $\alpha_4\beta_1$, regulating cell-cell interactions in normal and pathological processes (7, 8). In the prodomain of ADAMs, there is a cysteine switch sequence similar to the motif found in matrix metalloproteases (MMPs) (9, 10), keeping ADAMs in latent forms (2, 3, 11, 12). There are one or more furin cleavage sites between the pro- and metalloprotease domains of almost all members of the ADAM family discovered (2, 13–15), but only several ADAM precursors, including ADAM1, -9, -12, -15, and -17 and ADAMTS1, -4, and -12, have been shown to be activated by furin or furin-like proprotein convertases (16–23).

Adamalysin 19/ADAM19, a type 1 membrane protein containing an intact zinc-binding site in its metalloprotease domain, was cloned from mice (24, 25) and humans (26, 27). Human adamalysin 19 was recently demonstrated to be an active metalloproteinase through its cleavage of α_2-macroglobulin (α_2-M) *in vitro* (27). The endopeptidase activity of adamalysin 19 was blocked by specific antibodies against its catalytic and disintegrin domain peptides (28). Mouse ADAM19 cleaved intracellular neuregulin, a member of the epidermal growth factor (EGF) family *in vivo* (29). Human ADAM19 and its mouse homolog are highly similar, sharing 80.6% identity in nucleotide sequences and 84.1% identity in amino acid sequences (24, 27). Among its many roles, hADAM19 may be important in osteoblast differentiation (24), as a marker for the differentiation and characterization of dendritic cells, in the distinction between macrophages and dendritic cells (26), and in the intracellular processing of neuregulin (29). ADAM19 is synthesized as a zymogen, and its mechanism of activation has not been thoroughly investigated.

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[1] The abbreviations used are: ADAM, a disintegrin and metalloproteinase; ADAMTS, ADAM with thrombospondin-like motifs; α_2-M, α_2-macroglobulin; BACE, β-amyloid-converting enzyme; BFA, brefeldin A; decRVKR-CMK, dec-Arg-Val-Lys-Arg-chloromethyl ketone; DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; ER, endoplasmic reticulum; FBS, fetal bovine serum; MDCK, Madin-Darby canine kidney; MMP, matrix metalloproteinase; MT-MMP, membrane-type MMP; pAT, α_1-antitrypsin or α_1-proteinase inhibitor; α_1-PI, α_1-proteinase inhibitor; pATp, Pittsburgh mutant of α_1-antitrypsin/proteinase inhibitor; PACE4, paired basic amino acid-converting enzyme 4; PBS, phosphate-buffered saline; PC, proprotein convertase; 7.P15 cell, furin-deficient monkey kidney COS-7 strain cell; RIPA, radioimmune precipitation buffer; RPE.40 cell, furin-deficient Chinese hamster ovary-K1 strain cell; TGN, trans-Golgi network.

The proprotein convertases (PCs) are a large family of serine proteinases that recognize dibasic or R*X*(K/R)R motifs and cleave the peptide bond on the carboxyl side (30–32). As a major proprotein convertase, furin is concentrated in the trans-Golgi network (TGN) and cycles between this compartment and the cell surface through the endocytic pathway. The autoactivation and intracellular trafficking of furin are well characterized. Numerous studies have shown that furin activates a large number of proproteins in multiple compartments (30–32). For instance, furin has been demonstrated to mediate the activation of proenzymes, such as β-amyloid-converting enzyme (BACE), some matrix metalloproteinases (MMPs), including MMP-11, -14, -16, and -24, and some ADAMs, including ADAM1, -9, -12, -15, and -17 and ADAMTS1, -4, and -12 (16–23, 30, 31, 33–40). However, the molecular mechanism and pathway by which the cells regulate the potentially important interactions between these proenzymes and the proprotein convertase in cells are not fully understood. In this report, we present evidence that furin is responsible for the activation of hADAM19 and this activation can occur via one of the two consecutive recognition sites and that furin is co-localized with the substrate in the ER-Golgi complex and/or TGN.

EXPERIMENTAL PROCEDURES

Chemicals, Cell Lines, Cell Culture, and Immunological Reagents—All common laboratory chemicals, proteinase inhibitors, brefeldin A (BFA), anti-FLAG-M2 monoclonal antibody, and its agarose conjugates were purchased from Sigma. Anti-furin antibodies were from Affinity Bioreagents, Inc.(Golden, CO). Protein A/G PLUS agarose was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The CMK-based furin inhibitor dec-Arg-Val-Lys-Arg-chloromethyl ketone (decRVKR-CMK); a calcium ionophore, A23187; and a matrix metalloproteinase inhibitor, ilomastat (GM6001), were from BACHEM (Philadelphia, PA). Restriction enzymes were from Promega or Invitrogen. COS1, Madin-Darby canine kidney (MDCK), and derivative cells were maintained as described (40–43). The furin-deficient Chinese hamster ovary-K1 strain RPE.40 and furin-deficient COS-7 cell strain 7.P15 were cultured as described (44). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin G, and streptomycin were from Invitrogen. α_2-M was from Roche Molecular Biochemicals. Goat anti-mouse conjugated with fluorescein isothiocyanate and goat anti-rabbit-conjugated rhodamine red were from Jackson Immunoresearch Laboratory, Inc. (West Grove, PA). Rabbit polyclonal hADAM19 antibodies pAb361 (anti-metalloproteinase domain) and pAb362 (anti-disintegrin domain) were generated by our laboratory as reported (28).

PCR Primers, Mutagenesis, and Expression Constructs—pCR3.1uni-ADAM19 wild type and mutants with or without the FLAG tag were generated by high fidelity polymerase chain reaction with *Pfu* polymerase (Stratagene) as described (40–43). The primer sequences for wild type ADAM19 were 5′-ACC ATG CCA GGG GGC GCA GGC GCC-3′ (forward primer) and 5′-GAT TTT CGA GCT AAT CAT CCC TCC-3′ (reverse primer). For deletion from the transmembrane domain to the cytoplasmic domain, sequences were 5′-ACC ATG CCA GGG GGC GCA GGC GCC-3′ (forward primer) and 5′-AGG ACC CAC ACT CTC AGG GGG-3′ (reverse primer). For ^{196}KR to AA mutant, sequences were 5′-CAG ACC AAG **GCG GCA** CCT CGC AGG-3′ (forward primer) and 5′-CCT GCG AGG **TGC CGC** CTT GGT CTG-3′ (reverse primer). For ^{199}RR to AA mutant, sequences were 5′-G AAG CGA CCT **GCC GCG** ATG AAA AGG-3′ (forward primer) and 5′-CCT TTT CAT **CGC GGC** AGG TCG CTT C-3′ (reverse primer). For ^{202}KR to AA mutant, sequences were 5′-CGC AGG ATG **GCA GCG** GAA GAT TTA AAC-3′ (forward primer) and 5′-GTT TAA ATC TTC **CGC TGC** CAT CCT GCG-3′ (reverse primer). Expression constructs for the wild type full-length form and the truncation form were named F46 and D52, respectively. The ^{196}KR to AA, ^{199}RR to AA, and ^{202}KR to AA mutants of full-length and truncation forms were called ^{196}RA-F, ^{196}RA-D, ^{199}RA-F, ^{199}RA-D, ^{202}RA-F, ^{202}RA-D, respectively. All constructs were confirmed by DNA sequencing. The expression vectors for furin and its soluble form, paired basic amino acid-converting enzyme 4 (PACE4), α_1-PI (pAT), and Pittsburgh mutant of α_1-PI (pATp) were constructed as previously described (37).

DNA Transfection and Generation of Stable hADAM19 Expression Cell Lines—LipofectAMINE 2000-mediated DNA transfections into MDCK cells were performed following the instructions provided by Invitrogen. Stable lines were selected in the presence of G418 (400 μg/ml) and screened by Western blotting as described (40–42).

Western Blotting—The experiments were carried out as described previously (40, 41). Briefly, cells were grown to 80% confluence and were treated as indicated. After centrifugation at 14,000 × *g* for 15 min at 4 °C to clear any debris, the serum-free media were prepared for SDS-PAGE. The cells were lysed with RIPA (50 mM Tris, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2.5 μM GM6001, 10 μg/ml aprotinin, 10 μg/ml E64, and 10 μg/ml pepstatin A) for 15 min in ice. The supernatant was collected after centrifugation at 14,000 × *g* for 20 min at 4 °C. After electrophoresis, the proteins were transferred onto nitrocellulose membranes and probed with anti-FLAG-M2 or anti-hADAM19 and developed as described (40, 41).

Purification of Soluble hADAM19 and Protein N-terminal Sequencing—All proteins were purified on anti-FLAG-M2 affinity columns as described (42, 43). Briefly, cells stably expressing wild type soluble hADAM19 (D52-5) or ^{199}RR to AA mutant (^{199}RA-D-6) were grown to 100% confluence, then washed with PBS twice and incubated for 48 h in serum-free medium containing GM6001 (to prevent the degradation of hADAM19). The conditioned media were collected, centrifuged to clear debris, and loaded onto an anti-M2 immuno-affinity column (1 ml of resuspended agarose) prewashed with Tris-buffered saline. The bound materials were extensively washed with Tris-buffered saline, eluted with FLAG peptides, and collected in 200-μl fractions. The fractions were analyzed by Western blot using anti-hADAM19 antibodies or anti-FLAG-M2. The fraction containing the highest hADAM19 protein concentration was prepared for protein N-terminal sequencing. After separation by SDS-PAGE, the samples were transferred to a polyvinylidene difluoride membrane and stained with Coomassie Blue R-250. After destaining, the hADAM19 bands were excised and sent to the Bioanalytical Core Facility at the Florida State University for N-terminal amino acid sequencing.

α_2-M Trapping Assay to Determine Endopeptidase Activity of hADAM19 Species—The detailed experimental procedure was previously reported (27, 28). Briefly, 10 μl of the fraction containing purified soluble hADAM19 was mixed with 24 μl of α_2-M (0.2 unit/ml), adjusted to a total volume of 100 μl by adding HEPES buffer (50 mM HEPES, pH 7.5, 200 mM NaCl, 10 mM $CaCl_2$, 25 μM $ZnCl_2$, 0.05% Brij-35), and incubated at 37 °C for 1–5 days. A 20-μl aliquot of the mixture was removed daily, put into 2× SDS-PAGE sample buffer, and boiled. Following SDS-PAGE, the protein bands in the gels were visualized by silver staining.

Transient Transfection into COS1, RPE.40, or 7.P15 Cells—COS1, RPE.40 or 7.P15 cells were seeded in 24- or 6-well plates for 16–24 h at 80% confluence prior to transfection. The cells were then transfected with the indicated plasmids using LipofectAMINE 2000. After 6–10 h, serum-free or 5% FBS DMEM containing 2.5 μM GM6001, with or without CMK, BFA, or A23187 at indicated concentrations, were added for another 24 h. The conditioned media and cell lysates were analyzed by Western blotting. For co-transfection experiments in these cells, the indicated plasmid was transfected alone as a control and then co-transfected with expression plasmids directing the production of furin, PACE4, pAT, pATp, furin and pAT, or furin and pATp. After 6–10 h, serum-free or 5% FBS DMEM containing GM6001 (2.5 μM) was added to the transfected cells for 16–24 h. Then, the conditioned media and cell lysates were analyzed by Western blotting as described above.

Glycosylation Analysis—N-Glycosylation *in vitro* was investigated by endoglycosidase F treatment as described previously (40, 41). Briefly, transfected cells were grown to 80% confluence and incubated in serum-free medium for 24 h. The conditioned media were then collected, and the cells were lysed with RIPA. After centrifugation, the conditioned media or the supernatant from RIPA were treated with glycosidase F (5 units, Roche Molecular Biochemicals) for 20 h at 37 °C and analyzed by Western blotting.

Confocal Microscopy—The procedures have been described in detail previously (38, 40). Briefly, MDCK cells expressing hADAM19 wild type or ^{199}RA mutant were grown on coverslips in six-well plates with or without treatment with CMK, BFA, or A23187. After fixing with Lina's fixation buffer for 30 min, the cells were permeabilized with buffer A (0.3% Triton X-100, 1% neutral detergent solution, 1% bovine serum albumin, and 0.01% NaN_3 in PBS) for 1 h and incubated for 3 h with anti-furin and anti-FLAG-M2 (1:100 dilution in buffer A) for double staining. After washing with PBS three times, secondary antibodies conjugated with either fluorescein isothiocyanate or rhodamine red were added to the cells for 1 h, followed by four washes with PBS. Confocal microscopy experiments were performed at the Biological Science Imaging Resource Facility at Florida State University.

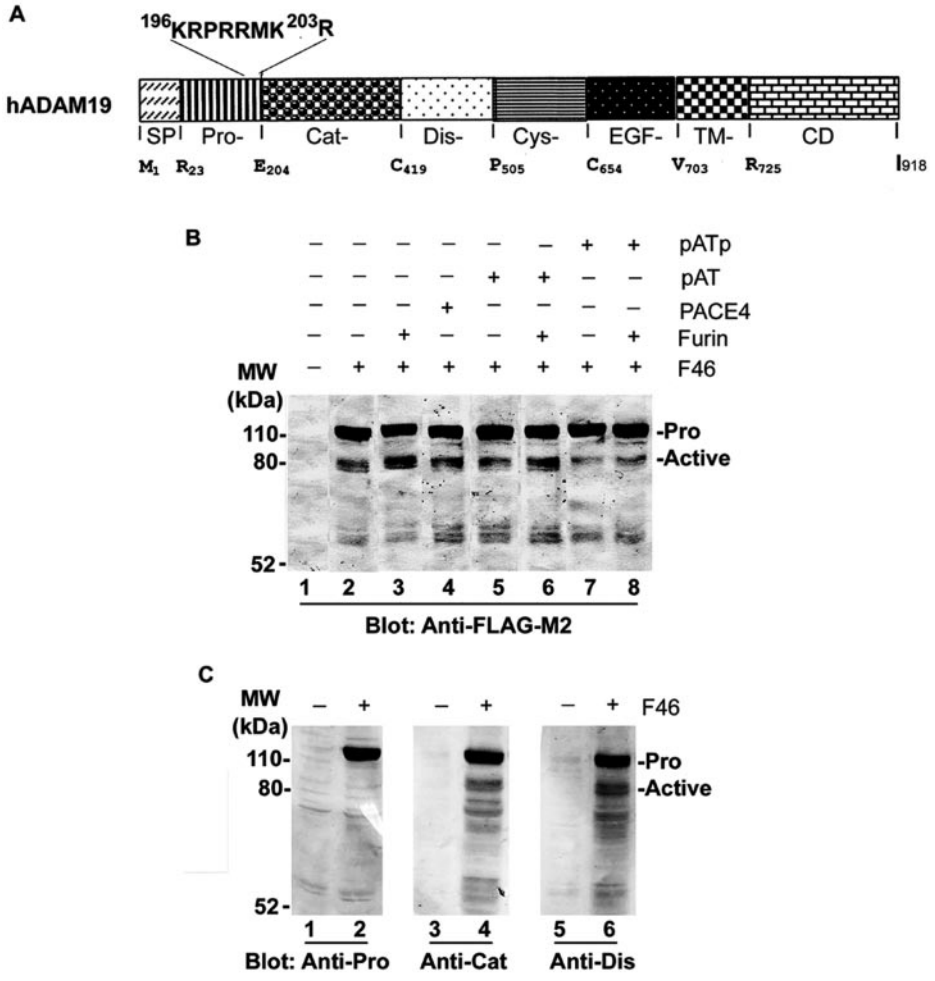

FIG. 1. **The prodomain removal of hADAM19 by furin.** *A*, a schematic diagram of the hADAM19 domain structure. The furin recognition sequence is shown *above* the border between the pro- and catalytic domains. *SP*, signal peptide; *Pro-*, prodomain; *Cat-*, catalytic domain; *Dis-*, disintegrin domain; *Cys-*, cysteine-rich domain; *EGF-*, EGF-like domain; *TM*, transmembrane domain; *CD*, cytoplasmic domain. *B*, detection of hADAM19 by Western blotting with anti-FLAG-M2 monoclonal antibody. Cell lysates from COS1 cells transfected with the blank vector (*lane 1*), pCR3.1-hADAM19 (F46) alone (*lane 2*), or co-transfected with plasmid encoding furin (*lane 3*), PACE4 (*lane 4*), pAT (*lane 5*), pAT and furin (*lane 6*), pATp (*lane 7*), or pATp and furin (*lane 8*). The pro- and active forms of hADAM19 are indicated. *C*, characterization of hADAM19 using polyclonal antibodies against different domains of hADAM19. Cell lysates from *lanes 1* and *2* in *B* were analyzed by Western blotting with the following hADAM19 polyclonal antibodies: anti-prodomain (*Pro*) (*lanes 1* and *2*), anti-catalytic domain (*Cat*) (*lanes 3* and *4*), or anti-disintegrin domain (*Dis*) (*lanes 5* and *6*).

RESULTS

Removal of the hADAM19 Prodomain Is Dependent on Furin Activity—The sequence of hADAM19 contains two potential furin recognition sites (R*X*(K/R)R), ^{196}KRPRRMK^{203}R, between its pro- and catalytic domains (Fig. 1*A*). To ascertain the role of furin in the cleaving of the hADAM19 prodomain, wild type hADAM19 (F46) with a C-terminal FLAG tag was transfected into COS1 cells alone or co-transfected with furin, PACE4, α_1-proteinase inhibitor (pAT), both furin and pAT, Pittsburgh mutant of α_1-proteinase inhibitor (pATp, a specific inhibitor of furin) (37, 45), or both furin and pATp. As shown in Fig. 1*B*, active hADAM19 forms were increased by the introduction of furin, but not PACE4. Furthermore, pATp blocked the processing of hADAM19; furin could not restore this processing when cells were co-transfected with furin and pATp (*lane 8*). pAT had little effect on endogenous or furin-induced processing of hADAM19 (*lanes 2*, *3*, *5*, and *6*). Because pATp did not completely block the hADAM19 processing, the low levels of endogenous processing may also be meditated by other proprotein convertases in addition to furin (*lanes 7* and *8*).

Interestingly, both the pro- and active forms of hADAM19 were doublets. These doublets may be differentially glycosylated forms. According to protein sequence analyses, hADAM19 has five potential glycosylation sites (27). Indeed, endoglycosidase F converted the doublets into a single pro- or active form, respectively (data not shown). To verify that the active hADAM19 lacks a prodomain, hADAM19 antibodies against the pro-, catalytic, or disintegrin domains (28) were used to probe the proteins in the cell lysates. The results in Fig. 1*C* clearly showed that the processed 80-kDa hADAM19 came from the removal of its prodomain because it was not recognized by the antibody against the prodomain peptide; however, it was detected with antibodies against its catalytic and disintegrin domains, respectively. These data showed that furin activity played a major role for the intracellular removal of hADAM19 prodomain.

To further demonstrate a direct role for furin in the activation of the hADAM19 zymogen, COS1 cells transfected with wild type hADAM19 (F46) were incubated with dec-Arg-Val-Lys-Arg-CMK (decRVKR-CMK), a widely used inhibitor of furin (17, 38, 40, 46, 47). As shown in Fig. 2*A*, decRVKR-CMK blocked the activation of hADAM19 in a dose-dependent manner. Because furin is mainly localized in TGN and the autoactivation of furin is calcium-dependent (48), we investigated whether hADAM19 activation occurred in the trans-Golgi network and required calcium. COS1 cells transfected with wild type hADAM19 were treated with BFA, which blocks protein trafficking from the ER to the Golgi apparatus (48, 49), or A23187, a calcium ionophore known to inhibit the maturation of furin (48). As shown in Fig. 2*B*, only the pro-forms of hADAM19 were detected upon treatment with either BFA or A23187. These results are consistent with furin-mediated activation of hADAM19.

Deletion of the Transmembrane Domain and the Cytoplasmic Tail of hADAM19 Does Not Alter the Processing of the Prodomain by Furin—To isolate soluble hADAM19 protein for enzyme activity assays, a construct encoding the extracellular domain (ectodomain) of hADAM19 containing a C-terminal FLAG tag was generated; this construct was called D52 and lacked the transmembrane domain and cytoplasmic domain

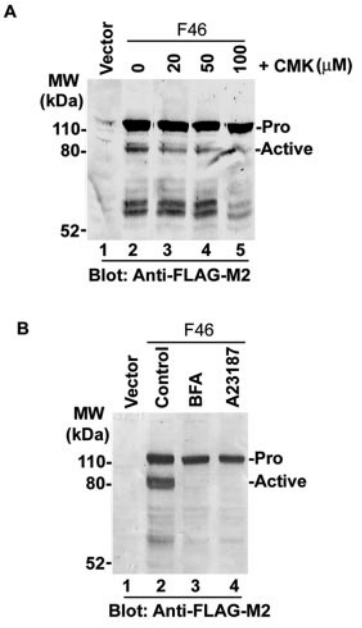

FIG. 2. **Blocking hADAM19 processing with CMK, BFA, or A23187.** *A*, dose-dependent inhibition of hADAM19 processing by decRVKR-CMK. COS1 cells transfected with the blank vector (*lane 1*) or pCR3.1hADAM19 (F46, *lanes 2–5*) were grown in 24-well plates with 0.1% methanol (v/v) (*lanes 1* and *2*) or CMK at 20 μM (*lane 3*), 50 μM (*lane 4*), or 100 μM (*lane 5*) for 24 h. The cell lysates were analyzed by Western blotting with anti-FLAG-M2. *B*, prevention of the hADAM19 prodomain removal by BFA and A23187. COS1 cells were transfected with the blank vector (*lane 1*) or F46 (*lanes 2–4*). Cells were grown in 24-well plates without (*lanes 1* and *2*) or with either 10 μg/ml BFA (*lane 3*) or 0.5 μM A23187 (*lane 4*) for 24 h. The cell lysates were analyzed as in *A*.

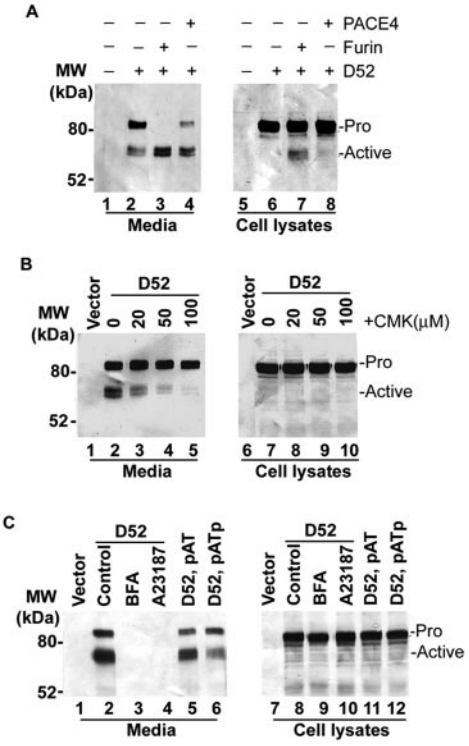

FIG. 3. **Activation of the ectodomain of hADAM19.** *A*, enhancement of the processing of soluble hADAM19 by furin. COS1 cells were transfected with the blank vector (*lanes 1* and *5*), or vector expressing soluble hADAM19 (D52) alone (*lanes 2* and *6*), or co-expressing with furin (*lanes 3* and *7*), or with PACE4 (*lanes 4* and *8*). Both conditioned media and cell lysates (*lanes 5–8*) were analyzed by Western blotting with anti-FLAG-M2. *B*, dose-dependent inhibition of the processing of soluble hADAM19 by CMK. The conditioned media (*lanes 1–5*) and cell lysates (*lanes 6–10*) were from COS1 cells transfected with the blank vector (*lanes 1* and *6*) or D52 (*lanes 2–5* and *7–10*). Cells were grown in 24-well plates with 0.1% methanol (v/v) (*lanes 1*, *2*, *6*, and *7*) or CMK at 20 μM (*lanes 3* and *8*), 50 μM (*lanes 4* and *9*), and 100 μM (*lanes 5* and *10*) for 12–16 h followed by incubation in serum-free media for 24 h. Samples were analyzed by Western blotting as in *A*. *C*, processing of soluble hADAM19 in the secretory pathway. COS1 cells were grown in 24-well plates overnight and then transfected with the blank vector (*lanes 1* and *7*) or D52 alone (*lanes 2–4* and *8–10*), or co-transfected with plasmids encoding either pAT (*lanes 5* and *11*) or pATp (*lanes 6* and *12*). The next day, cells were treated with either 10 μg/ml BFA (*lanes 3* and *9*) or 0.5 μM A23187 (*lanes 4* and *10*) in serum-free medium for 24 h. The conditioned media (*lanes 1–6*) or cell lysates (*lanes 7–12*) were analyzed as in *A*.

(Fig. 4*A*). This hADAM19 ectodomain construct was transfected into COS1 cells. As shown in Fig. 3*A*, only the active forms of soluble hADAM19 were detected in the media from COS1 cells co-expressing the hADAM19 ectodomain and furin. Both pro- and active forms were detected in the media from the cells co-transfected with PACE4 or transfected with the ectodomain construct alone. Additionally, the active forms were detected in the cell lysates only when cells were co-transfected with furin (Fig. 3*A*, *lanes 5–8*).

A dose-dependent inhibition of soluble hADAM19 activation by decRVKR-CMK was observed (Fig. 3*B*). However, there was no significant effect of decRVKR-CMK on the intracellular levels of hADAM19. Furthermore, there was no secretion of soluble hADAM19 in the transfected cells treated with either BFA or A23187 (Fig. 3*C*). Also, pATp dramatically decreased the amount of active forms in the medium when it was expressed in COS1 cells, but pAT failed to do so (Fig. 3*C*). Once again, no significant differences were seen in response to these treatments in the cell lysates (Fig. 3*C*). These results show that the soluble forms of hADAM19 were processed in the same manner as the full-length form and are consistent with furin-mediated activation of hADAM19.

There Are Two Alternative Furin Recognition Sites between the Pro- and Catalytic Domain of hADAM19—Upon the examination of the hADAM19 protein sequence, two consecutive furin recognition sites (R*X*(K/R)R), ^{196}KRPR^{200}R and ^{199}RRMK^{203}R, were found (Fig. 1*A*). We hypothesized that these two furin recognition sites are alternatively used for the intracellular activation of pro-ADAM19 by furin. To test this hypothesis, three mutants were generated in full-length and ectodomain hADAM19, which converted the ^{196}KR, ^{199}RR, and ^{202}KR into AA, respectively. These were named as ^{196}RA-F, ^{196}RA-D, ^{199}RA-F, ^{199}RA-D, ^{202}RA-F, and ^{202}RA-D, respectively, indicating that one (^{196}RA-F, ^{196}RA-D, ^{202}RA-F, or ^{202}RA-D) or no (^{199}RA) furin recognition site existed in the hADAM19 mutants (Fig. 4*A*).

All of the plasmids were transfected into COS1 cells to compare the levels of activated hADAM19 for the wild type and RA mutants in both the full-length (RA-F) and ectodomain forms (RA-D). As shown in Fig. 4 (*B* and *C*), no active forms of ^{199}RA mutants were detected as a result of the absence of a furin cleavage motif, whereas almost equivalent amounts of the active forms of the ^{196}RA and ^{202}RA mutants were detected. The wild type, full-length hADAM19 (Fig. 4*B*) and ectodomain form (Fig. 4*C*) were studied in parallel with the mutants. In addition, the protein levels of hADAM19 were almost equal among the cell lysates from these transfectants (Fig. 4, *B* and *C*). These results strongly supported the hypothesis that processing of the prodomain of hADAM19 was dependent on the presence of either one of the two consecutive furin recognition sites between the pro- and catalytic domains.

To further confirm that furin processed hADAM19 via one of the two alternative furin recognition sites, samples of the media from the four cell lines in Fig. 4*C* were incubated with the medium containing soluble furin, which was obtained from the

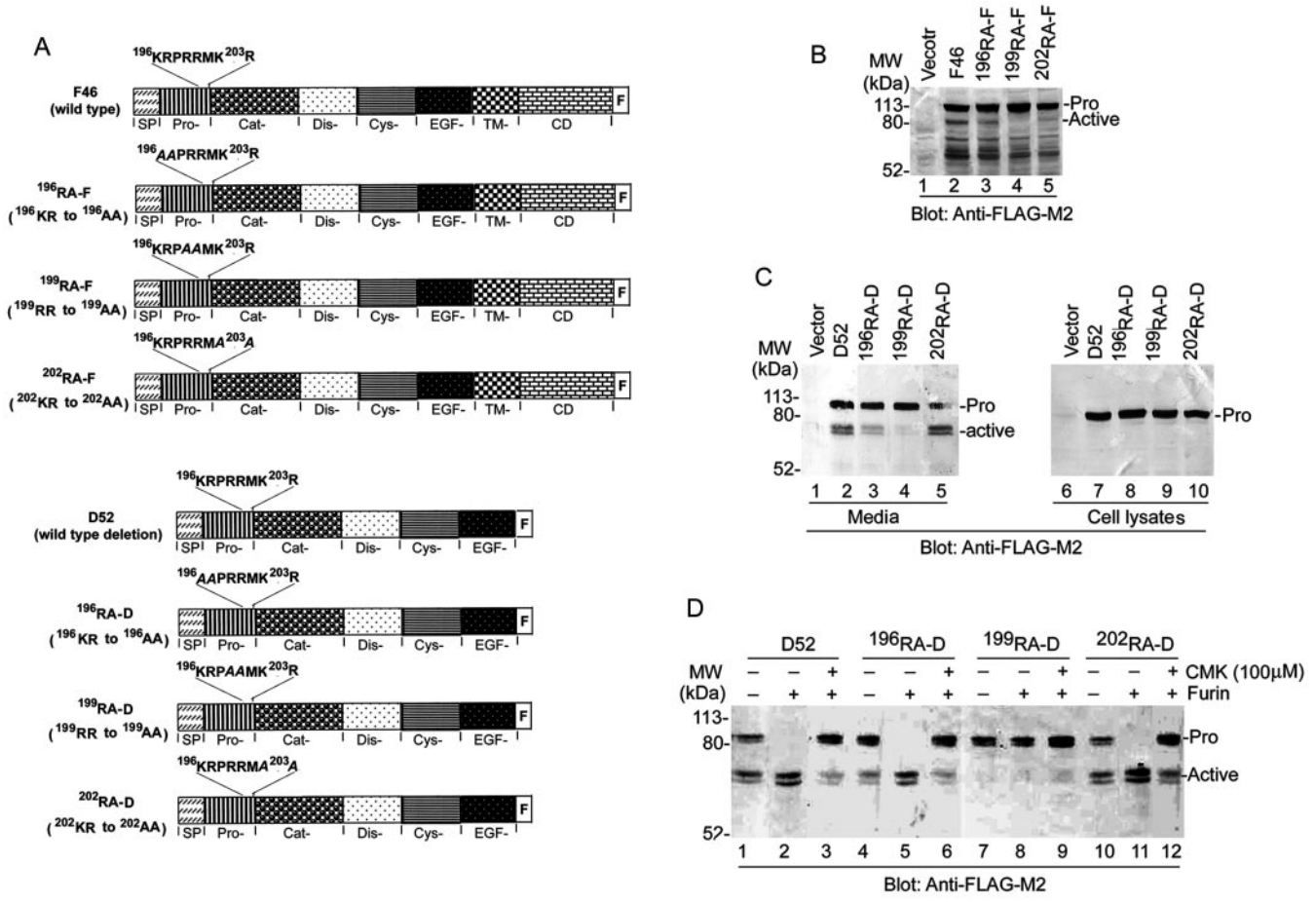

FIG. 4. **Requirement of furin motifs between the pro- and catalytic domains for the activation of hADAM19.** *A*, a schematic illustration for the wild type expression vector pCR3.1hADAM19 and its mutant constructs. All the constructs have a C-terminal FLAG tag. *SP*, signal peptide; *Pro-*, prodomain; *Cat-*, catalytic domain; *Dis-*, disintegrin domain; *Cys-*, cysteine-rich domain; *EGF-*, EGF-like domain; *TM*, transmembrane domain; *CD*, cytoplasmic domain; *F*, FLAG tag. *B*, processing of hADAM19 in the ^{199}RR to AA mutant is abolished. COS1 cells were transfected with the blank vector (*lane 1*), F46 (*lane 2*), ^{196}RA-F (*lane 3*), ^{199}RA-F (*lane 4*), or ^{202}RA-F (*lane 5*), and grown in 24-well plates for 24–36 h. The cells were lysed with RIPA followed by SDS-PAGE and Western blotting with anti-FLAG-M2. *C*, inhibition of the processing of the hADAM19 ectodomain in the ^{199}RR to AA mutant. The conditioned media (*lanes 1–5*) and cell lysates (*lanes 6–10*) were from COS1 cells transfected with the blank vector (*lanes 1* and *6*), D52 (*lanes 2* and *7*), ^{196}RA-D (*lanes 3* and *8*), ^{199}RA-D (*lanes 4* and *9*), or ^{202}RA-D (*lanes 5* and *10*) overnight, followed by incubation in serum-free medium for 24 h. The samples were analyzed by Western blotting with anti-FLAG-M2. *D*, activation of the soluble hADAM19 by exogenous soluble furin. The condition media from *lanes 2–4* in *C* were mixed with equal volumes of conditioned serum-free media from COS1 cells transfected with the blank vector (*lanes 1*, *4*, *7*, and *10*) or soluble furin (*lanes 2*, *3*, *5*, *6*, *8*, *9*, *11*, and *12*). After being incubated with (*lanes 3*, *6*, *9*, and *12*) or without 50 μM CMK (*lanes 1*, *2*, *4*, *5*, *7*, *8*, *10*, and *11*) at 37 °C for 24 h, the samples were analyzed by Western blotting as in *C*.

furin-transfected COS1 cell culture. The medium from COS1 cells transfected with a blank vector was used as a negative control. As shown in Fig. 4*D*, soluble furin did not process the ^{199}RA mutant of the soluble hADAM19. However, the wild type and the mutant soluble proteins containing a furin recognition motif were cleaved by furin. This furin-mediated processing was sensitive to decRVKR-CMK inhibition, consistent with the intracellular processing results obtained earlier (Figs. 3*A* and 4*C*). These results demonstrated that furin could activate hADAM19 at both furin cleavage sites between the prodomain and the catalytic domain of the zymogen.

Removal of the Prodomain Was Required for hADAM19 to Exert Its Proteolytic Activity—In our previous reports, an *in vitro* assay was established using α_2-M to test the activity of hADAM19 (27, 28). To assess the importance of zymogen activation to the proteolytic activity of hADAM19, stable lines of wild type hADAM19 and its ^{199}RA mutants were generated in MDCK cells, in which the endogenous furin activity is high (38, 40, 50). One stable line was chosen from each group as a representative to be treated with CMK, BFA, or A23187 and to examine whether hADAM19 would display the same processing as it did in COS1 cells. As predicted, CMK, BFA, or A23187 blocked the activation of wild type hADAM19 in the stably transfected MDCK cells called F46-4 (Fig. 5*A*). MDCK cells stably expressing the full-length ^{199}RA (^{199}RA-F-9) showed no conversion of the pro-hADAM19 to its active form (Fig. 5*A*). Furthermore, as shown in Fig. 5*B*, the active forms were only detected in the medium from MDCK cells stably expressing soluble hADAM19 (D52-5). When D52-5 cells were treated with decRVKR-CMK for 24 h, the pro-forms of soluble hADAM19 were predominantly detected from the cell culture medium. There were no active forms detected in the medium from the ^{199}RA-D6 mutant cells, which were MDCK cells stably expressing soluble hADAM19 with the ^{199}RA mutation (Fig. 5*B*).

Soluble hADAM19 proteins were purified from conditioned media of D52-5 and ^{199}RA-D6 cells. The endopeptidase activity of the purified metalloproteinases was tested using an α_2-M trapping and cleaving assay. As shown in Fig. 5*C*, only the wild type proteins could complex with α_2-M and generate two cleaved products. This activity was completely blocked by EDTA. The ^{199}RA mutant proteins were inactive, likely because the prodomain containing the cysteine switch residue

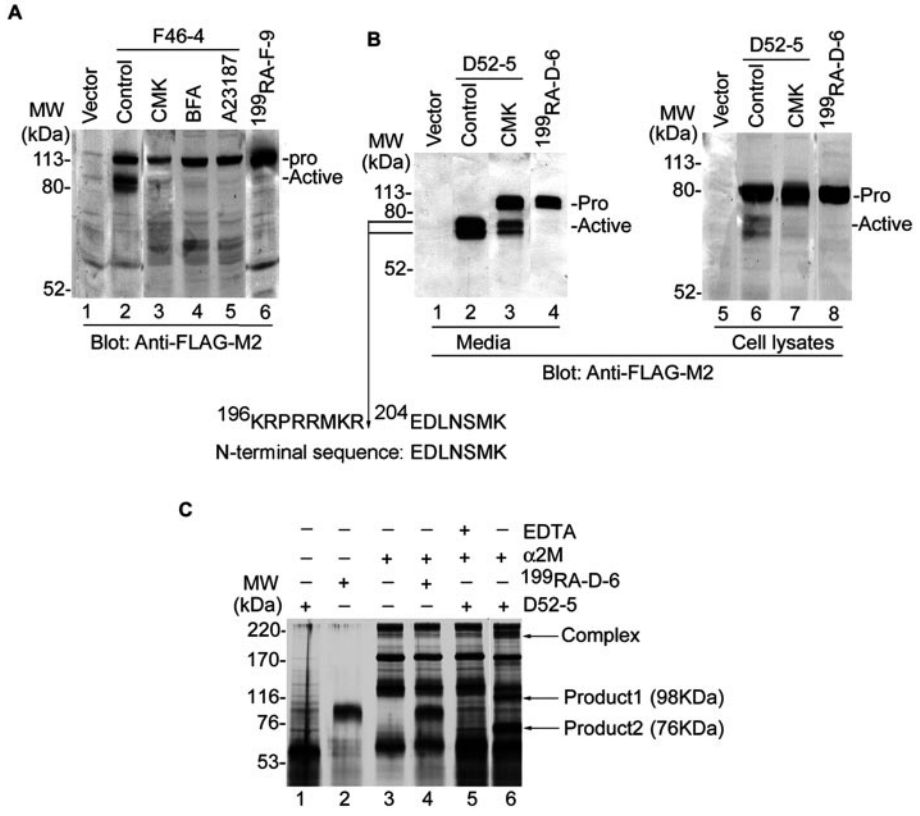

FIG. 5. **Requirement of the zymogen activation for the proteolytic activity of hADAM19.** *A*, processing of hADAM19 in stably transfected MDCK cells. MDCK cells stably expressing wild type hADAM19 (F46-4; *lanes 2–5*) or ^{199}RA mutant (^{199}RA-F-9; *lane 6*) were seeded in six-well plates at 50% confluence. The next day, cells were treated with CMK (100 μM, *lane 3*), BFA (10 μg/ml, *lane 4*), or A23187 (0.5 μM, *lane 5*). *Lanes 2* and *6* represent untreated controls. The cells were lysed with RIPA followed by analysis of Western blotting using anti-FLAG-M2. MDCK cells stably transfected with the blank vector were an additional control (*lane 1*). *B*, the requirement of furin in the processing of soluble hADAM19 in stably transfected MDCK cells. MDCK cells stably expressing soluble hADAM19 (D52-5) (*lanes 2*, *3*, *6*, and *7*) or soluble ^{199}RA mutant (^{199}RA-D-6) (*lanes 4* and *8*) were grown in 24-well plates to 100% confluence. Cells were incubated in serum-free media without (*lanes 1*, *2*, *4*, *5*, *6*, and *8*) or with CMK (100 μM, *lanes 3* and *7*) for 24 h. Both conditioned media (*lanes 1–4*) and lysates (*lanes 5–8*) were analyzed by Western blotting as in *A*. MDCK cells stably transfected with the blank vector is a control (*lane 1*). *C*, the proteolytic activity of soluble mature hADAM19. Purified soluble hADAM19 from D52-5 and ^{199}RA-D6 were incubated in reaction buffer alone (*lanes 1* and *2*) or with α_2-M in the absence (*lanes 4* and *5*) or presence of EDTA (*lane 5*) for 48 h. α_2-M in reaction buffer alone (48 h) was a control (*lane 3*). The α_2-M·hADAM19 complex and the cleavage products of α_2-M by hADAM19 are labeled on the *right*. Note that pro-soluble hADAM19 (^{199}RA-D-6) did not form the complex with α_2-M.

was not removed by furin (Fig. 5*B*). These results suggest that hADAM19 activation also obeys the cysteine-switch mechanism for zymogen latency and activation. Furthermore, N-terminal sequences of the purified hADAM19 proteins from the media revealed that the processed doublets (Fig. 5*B*, *lane 2*) had the identical N-terminal sequences of ^{204}EDLNSMK, suggesting that furin prefers to cleave hADAM19 using the recognition site of ^{200}RMK^{203}R rather than ^{197}RPR^{200}R. The doublets have different glycosylation patterns as verified by endoglycosidase F treatment experiments (data not shown). These results confirmed the prediction that hADAM19 was activated by furin through cleavage of the ^{203}R-^{204}E peptide bond at the sequence ^{199}RRMKR ↓ ^{204}EDLNSMK.

Two Furin-deficient Cell Strains (RPE.40 and 7.P15) Do Not Activate Pro-hADAM19, and the Introduction of Furin into These Cells Restores Zymogen Activation—To further confirm that furin activity was required for the intracellular activation of pro-hADAM19, the wild type of full-length and soluble forms of hADAM19 (F46 and D52) were transfected into RPE.40 cells and 7.P15 cells, two furin-deficient cell strains (44, 51). Processing of hADAM19 to its mature forms was negligible in these cell lines. However, the active forms were clearly detectable when cells co-expressed furin (data not shown). As shown in Fig. 6, there were barely detectable levels of the active forms of soluble hADAM19 in the media of the two D52-transfected cell lines. High levels of active forms were only detected in the media when the cells co-expressed furin, although PACE4 also increased the amount of active forms when it was co-expressed with D52 (Fig. 6, *A* and *B*). Curiously, the active forms were only detected in the lysates of cells co-expressing D52 and furin. These data further confirm that furin was responsible for the intracellular activation of hADAM19.

Furin Was Co-localized with hADAM19 in the ER-Golgi Complex and/or TGN—To verify that hADAM19 was a physiologically relevant substrate of furin, the cellular localization of furin and hADAM19 was examined by confocal microscopy using MDCK cells stably expressing hADAM19 (Fig. 7). Untreated cells are marked as F46-4 (control) (Fig. 7, *top panels*). Co-localization of hADAM19 and furin was clearly observed, and was consistent with ER-Golgi complex and/or TGN localizations (*top*, *middle panel*). Furthermore, hADAM19 was also seen at the edges of the plasma membrane (*right lane*, *top panel*) where furin was rare (*left lane*, *top panel*). Interestingly, a similar pattern of co-localization between the ^{199}RA mutant and furin was also observed (Fig. 7, *bottom panels*), suggesting that the co-localization was independent of the recognition sites for furin in hADAM19. To test whether the agents that block the activation of hADAM19 could prevent co-localization between furin and hADAM19 (Fig. 5*A*), the cells were treated with CMK, BFA, or A23187. None of these treatments interfered with the co-localization pattern of furin and hADAM19 (Fig. 7), suggesting that furin may be co-localized with

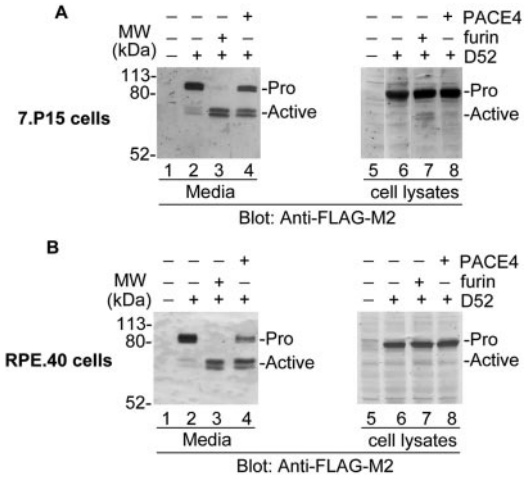

FIG. 6. **Processing the hADAM19 prodomain in furin-deficient mammalian cell lines.** *A*, hADAM19 in 7.P15 cells. 7.P15 cells were transfected with the blank vector (*lanes 1* and *5*) or D52 alone (*lanes 2* and *6*) or co-transfected with D52 and plasmids encoding furin (*lanes 3* and *7*) or PACE4 (*lanes 4* and *8*). Cells were grown in 24-well plates for 12–16 h followed by incubation in serum-free medium for 24 h. Conditioned media (*lanes 1–4*) and cell lysates (*lanes 5–8*) were analyzed by Western blotting with anti-FLAG-M2. *B*, hADAM19 in RPE.40 cells. RPE.40 cells were transfected with the blank vector (*lanes 1* and *5*) or D52 alone (*lanes 2* and *6*) or were co-transfected either D52 and plasmids encoding furin (*lanes 3* and *7*) or PACE4 (*lanes 4* and *8*). Cells were grown in 24-well plates overnight followed by incubation in serum-free media for 24 h. Conditioned media (*lanes 1–4*) and cell lysates (*lanes 5–8*) were analyzed by Western blotting as in *A*.

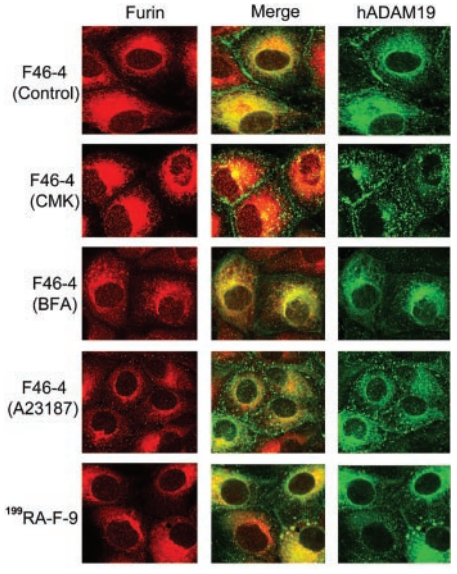

FIG. 7. **Co-localization of hADAM19 and furin.** MDCK cells stably expressing wild type hADAM19 (F46-4) or ^{199}RA mutant (^{199}RA-F-9) grown on coverslips in six-well plates were treated with nothing (control), 100 μM CMK, 10 μg/ml BFA, or 0.5 μM A23187 for 24 h. The fixed slides were stained for both hADAM19 with anti-FLAG-M2 (*right panels*) and furin with anti-furin (*left panels*). The merged pictures for both hADAM19 and furin are presented in the *middle column*. Note that hADAM19 is co-localized with furin, independent on furin motif, with neither CMK, BFA, nor A23187 altering the staining pattern.

hADAM19 in perinuclear ER-Golgi complex and/or TGN independent of the furin catalytic activity.

DISCUSSION

Proteolysis of the extracellular matrix and cell surface proteins mediated by metalloproteases, including MMPs and ADAMs, is of vital importance for tissue-remodeling processes during normal and pathological conditions, such as tissue morphogenesis, wound healing, inflammation, and tumor cell invasion and metastasis (3–7, 52, 53). Metalloproteases are synthesized as inactive proenzymes or zymogens, and their latency is maintained by a cysteine-switch residue in the propeptide domain in which the thiol group is coordinated to the active site zinc (II) (2, 9–12). To display any proteolytic activities, the prodomain located N-terminal to the catalytic domain must be removed from the zymogen in most cases. Recently, PCs, such as furin and or furin-like serine peptidases, have been recognized as very important enzymes for the zymogen activation, although various mechanisms have been proposed for the activation of pro-MMPs and pro-ADAMs. Furin or furin-like PCs mediate zymogen activation by recognizing a conserved R*X*(K/R)R motif in the boundary between pro- and catalytic domains. This motif is present in almost all ADAMs and nine MMPs (2, 13, 53). By analyzing the intracellular activation of hADAM19, we have demonstrated that both furin activity and one of the two consecutive sites in ^{197}RPRRMK^{203}R in ADAM19 are required for activation, which is dependent on calcium and proper secretory pathway trafficking. Furthermore, we have provided direct evidence that furin is co-localized with hADAM19 in ER-Golgi complex and/or TGN. This colocalization between furin and hADAM19 is independent of the furin recognition site and is resistant to a variety of treatments, such as CMK, BFA, and A23187, that inhibit furin activity, vesicular trafficking, and calcium signal, respectively. These findings are consistent with the report published recently showing that furin was co-localized with MMP16 independent of their apparent enzyme-substrate relationship (40).

Latency and Activation of ADAMs—The classic cysteine switch mechanism for pro-MMP latency and activation was originally proposed for MMPs (10) and may be applied for many MMPs discovered with the exception of MMP-3, MMP-23, and MMP-26 (41, 54–58). The activation of pro-MMP-3 by a mercurial compound was triggered by a perturbation of the conformation of the precursor rather than a direct disruption of the Cys-zinc interaction (54). A salt bridge in pro-MMP-3 might also contribute to the latency of the proenzyme (55). Organomercurial treatment failed to activate pro-MMP-26 with a unique cysteine-switch motif, P**H**^{81}CG*XX*D, and when the conserved cysteine-switch sequence, P**R**^{81}CG*XX*D, in the prodomain of pro-MMP-26 was restored by mutagenesis, the cysteine-switch activation mechanism was not induced (58).

Regarding the ADAM family members, the active ADAMs, such as ADAM1, -9, -10, -12, -15, -17, -19, -28, and ADAMTS1, -4, and -12, contain a catalytic site consensus sequence (HE*XX*H) in their metalloprotease domains (2, 11–14, 16–21, 27–29, 53, 59–61). They may also have a putative cysteine-switch residue in their prodomain to keep them inactive (9). For example, the investigation by Leochel *et al.* (11) demonstrated that the latency and activation mechanism of ADAM12 was similar to the cysteine switch model proposed for MMPs. ADAM9, -15, and -17 showed catalytic activity against their substrates only after their prodomains were removed (12, 21, 22). However, for many ADAMs, including ADAM19, no direct evidence has been provided to support the hypothesis that the Cys-zinc coordination is required for latency. For ADAM17/tumor necrosis factor-α convertase, the prodomain was not only an inhibitor of the catalytic domain, but also appeared to act like a chaperone, facilitating secretion, folding, or both of the ADAM protein (12). In this report, we have demonstrated that, after the removal of the prodomain of hADAM19 by furin, the enzyme has endopeptidase activity against α_2-M. However, furin is unable to cleave the prodomain of the ^{199}RR to AA hADAM19 mutant lacking a furin recognition site in the boundary of the pro- and catalytic domains. This mutant has no

proteolytic activity using an α_2-M trapping assay. These results demonstrate that at least one of the furin recognition sites is required for the removal of the propeptide domain by furin to activate pro-hADAM19. The detailed mechanism of pro-ADAM19 latency and activation and the role of the cysteine-switch sequence remain to be further investigated.

For the activation of ADAM zymogens, two mechanisms have been reported. One is the removal of the prodomain by autolysis, but it was shown only in ADAM28 (60). The predominant mechanism for the activation of ADAMs is mediated by furin or furin-like PCs in the secretory pathway. This mechanism has been shown in many ADAMs, including ADAM1, -9, -12, -15, -17, and -19, and ADAMTS1, -4, and -12, using N-terminal sequencing, specific inhibitors of furin, blockers of protein trafficking from ER to Golgi, exogenous soluble furin *in vitro*, furin-deficient cell lines, and mutagenesis at the furin recognition site(s) (R*X*(K/R)R) between the pro- and catalytic domain (Refs. 16–23; this report). In the present report, we provide a thorough investigation of ADAM zymogen activation mediated by furin (Figs. 1–6) and evidence that furin is co-localized with ADAMs in the ER-Golgi complex and/or TGN (Fig. 7), showing that ADAMs are similar to MMPs in these respects (38, 40).

There Are Two Consecutive Furin Recognition Sites in the Boundary of the Pro- and Catalytic Domains of hADAM19—The minimal furin recognition sequence requires basic residues at P_1 and P_4 (R*XX*R) and in some cases, at the P_1 position, an amino acid with a hydrophobic aliphatic side chain is not suitable (31). Typically, there is only one furin recognition site between the pro- and catalytic domain of the substrates of furin as found in most members of the ADAM family, seven MMPs, pro-BACE, and Notch1 receptor (2, 13–15, 30, 31, 33–40, 53, 62). In this report, we present evidence for the first time that there are two consecutive furin recognition sites, ^{197}RPR^{200}R and ^{200}RMK^{203}R, between the pro- and catalytic domain in hADAM19, which adhere to the rules for efficient cleavage by furin (31). Only pro-forms were detectable in the ^{199}RA mutant, which lacked a furin recognition site between its pro- and catalytic domain, whereas the mutants of both ^{196}RA and ^{202}RA, which possessed recognition sites, were converted into the active forms. Thus, the Arg residue at the P_4 site is required for the intracellular hADAM19 maturation mediated by furin (Figs. 4 and 5*B*). Interestingly, N-terminal sequencing of wild type mature forms (Fig. 5) confirmed that the preferred intracellular cleavage site for hADAM19 activation is the one nearer to the catalytic domain, ^{200}RMK^{203}R, as predicted before (26, 27). This motif is conserved in mice as ^{201}RMK^{204}R (24). The distal motif, ^{197}RPR^{200}R in humans, however, is replaced with ^{198}QPR^{201}R in mice, which is not efficiently cleaved by furin.

A notion that pro-hADAM19 activation by furin may be sequential, *i.e.* ^{200}R^{201}M is cleaved first followed by ^{203}R^{204}E, seems to be consistent with the partially activated soluble species seen for ^{196}RA-D compared with ^{202}RA-D data in Fig. 4*C*; however, it does not agree with the data shown in Fig. 4*B*, where it is seen that the presence of furin with the full-length ^{196}RA-F leads to more activated species than ^{202}RA-F. The delicate changes in the interactions between furin and the different mutants that have subtle structural and conformational differences might be partially responsible for the different activation levels observed. Moreover, among all the protein N-terminal sequence data of wild type hADAM19 activated species, only ^{204}EDLNSMK was found; the alternative cleavage site product of ^{201}MKRED was not detected. Most importantly, the minimal furin recognition sequence requires basic residues at P_1 and P_4 (R*XX*R) and the Arg residue at the P_4 site is required for the intracellular hADAM19 maturation mediated by furin (Figs. 4 and 5*B*). It may not be possible for furin, an endopeptidase, to effectively cleave the product of the ^{200}R-^{201}M cleavage because the ^{201}MK^{203}R-^{204}ED sequence lacks the required Arg at the P_4 site. Thus, our data suggest that the ^{203}R-^{204}E site is the predominant cleavage site and ^{200}R-^{201}M is an alternative cleavage site by furin when the predominant site is missing. This is consistent with the model proposed for wild type MT1-MMP, in which the pro-MT1-MMP is processed primarily at the ^{108}RRKR site to generate the active proteinase and the secondary site within ^{86}K*XX*RR*X*R is cleaved only when the primary ^{108}RRKR motif was mutated (39).

Notably, there are two potential consecutive furin recognition sites in other metalloproteinase zymogens, including ADAM11 (AB009675, ^{292}RLRRK^{297}R), ADAM22 (AF155382, ^{219}RPKRSK^{225}R), ADAMTS4 (AF148213, ^{206}RPRRAK^{212}R), MT2-MMP (NM_002428, ^{126}RRRRK^{131}R), and MT5-MMP (AJ010262, ^{118}RRRRNK^{224}R). The ones nearer to the catalytic domains are conserved in different species, whereas the distal ones might be acquired later during evolution. Although the significance of the two alternative recognition sites in these precursors remains poorly understood, we may speculate that the processing of these zymogens are crucial for some biological events; the zymogens may be activated by furin at a different cleavage site even if the primary site is abolished by mutation.

Significance of Furin and Its Related PC Pathways in the Processing of Precursors—Furin and its related PCs have been demonstrated as the major enzymes responsible for the maturation of many precursors, such as some ADAMs and MMPs (Refs. 16–23 and 37–40; this report). Furthermore, zymogens of BACE, a major enzyme related to Alzheimer's disease, and some growth factors and cell surface receptors, such as transforming growth factor β, insulin-like growth factor, hepatocyte growth factor receptor, and Notch1 receptor, are converted into their active forms by these PC pathways (30, 31, 33–36, 62). Thus, this activation mechanism by a PC may play key roles in many physiological and pathological events. In fact, furin knockout mice are embryonic lethal (63), and inhibition of furin results in absent or decreased invasion and tumorigenicity of human cancer cells (64, 65). The inability to activate many types of proproteins, including some pro-ADAMs and pro-MMPs, in furin null mice may contribute to the abnormal phenotypes during early development and morphogenesis in those mice. On the other hand, the design and synthesis of furin specific inhibitors may lead to a new strategy in the treatment of cancer and other diseases, such as Alzheimer's disease, in the human adult.

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Autolytic Processing at Glu^{586}-Ser^{587} within the Cysteine-rich Domain of Human Adamalysin 19/Disintegrin-Metalloproteinase 19 Is Necessary for Its Proteolytic Activity*

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We investigated the regulation of the proteolytic activity of human adamalysin 19 (a disintegrin and metalloproteinase 19, hADAM19). It was processed at Glu^{586}(P1)-Ser^{587}(P1′) site in the cysteine-rich domain as shown by protein N-terminal sequencing. This truncation was autolytic as illustrated by its R199A/R200A or E346A mutation that prevented the zymogen activation by furin or abolished the catalytic activity. Reagents that block furin-mediated activation of pro-hADAM19, decRVKR-CMK, A23187, and brefeldin A abrogated this processing. The sizes of the side chains of the P1 and P1′ residues are critical for the processing of hADAM19. The amount of processing product in the E586Q or S587A mutant with a side chain almost the same size as that in the wild type was almost equal. Conversely, very little processing was observed when the size of the side chain was changed significantly, such as in the E586A, E586G, or S587F mutants. Two mutants with presumably subtle structural distinctions from wild type hADAM19, E586D and S587T, displayed rare or little processing and had very low capacities to cleave α2-macroglobulin and a peptide substrate. Therefore, this processing is necessary for hADAM19 to exert its proteolytic activities. Moreover, a new peptide substrate, Ac-RPLE-SNAV, which is identical to the processing site sequence, was cleaved at the E-S bond by soluble hADAM19 containing the catalytic and disintegrin domains. This enzyme cleaved the substrate with K_m, k_{cat}, and k_{cat}/K_m of 2.0 mM, 2.4/min, and 1200 M^{-1} min^{-1}, respectively, using a fluorescamine assay. Preliminary studies showed that a protein kinase C activator, phorbol 12-myristate 13-acetate, promoted the cellular processing of hADAM19; however, three calmodulin antagonists, trifluoperazine, W7, and calmidazolium, impaired this cleavage, indicating complex signal pathways may be involved in the processing.

Ectodomain shedding is a process in which a wide variety of transmembrane proteins, such as growth factors and growth factor receptors, cytokines and their receptors, amyloid precursor protein (APP),[1] adhesion molecules, and enzymes, proteolytically release their extracellular domains. It is believed to play key roles in normal development, arthritis, inflammation, and tumorigenesis (1–5). Although the signal pathways regulating ectodomain shedding remain poorly understood, numerous studies have shown that structurally different proteins share common pathways (5–8). For example, phorbol 12 myristate 13-acetate (PMA), a protein kinase C (PKC) activator, is generally a potent inducer of ectodomain shedding. Other signals, such as calcium, calmodulin (CaM), tyrosine kinase, mitogen-activated protein kinase (MAPK), and phosphatase, also play roles in certain shedding processes (9–23). On the other hand, the shedding process, in most cases, is hindered by hydroxamate-based inhibitors of metalloproteinases, such as BB94, GM6001, and tumor necrosis factor-α proteinase inhibitor (TAPI) (5–8, 19, 24–26).

Inhibitor studies have shown that TIMP-3, not TIMP-1 or TIMP-2, impairs many shedding processes, indicating that the proteins comprising the a disintegrin and metalloprotease (ADAM)/adamalysin/metalloprotease, disintegrin, cysteine-rich (MDC) family, rather than the matrix metalloproteinase (MMP) family, are the predominant sheddases (27–30). Indeed, five ADAMs have been implicated in shedding processes so far. ADAM17/TACE, a major sheddase, has a role in the shedding of tumor necrosis factor-α (TNF-α), transforming growth factor-α (TGF-α), L-selectin, both TNF receptors, interleukin-1 receptor II, HER4, Notch, and TNF-related activation-induced cytokine (TRANCE). It also acts as a PMA-induced APP α-secretase (1–6, 31). ADAM10/Kuzbanian, another major sheddase, is required for Notch signaling. It can cleave the Notch ligand Delta, heparin-binding epidermal growth factor (HB-EGF), TNF-α, L1 adhesion molecule, and ephrin A2 and is

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[1] The abbreviations used are: APP, amyloid precursor protein; Ab, antibody; ADAM, a disintegrin and metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin-like motifs; α2-M, α2-macroglobulin; CaM, calmodulin; decRVKR-CMK, decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MDC, metalloprotease/disintegrin/cysteine-rich; MDCK, Madin-Darby canine kidney; MMPs, matrix metalloproteinases; MT-MMPs, membrane-type MMPs; NRG, neuregulin; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TACE, tumor necrosis factor α convertase; TAPI, tumor necrosis factor-α proteinase inhibitor; TIMPs, tissue inhibitors of metalloproteinases; TGF-α, transforming growth factor-α; TNF-α, tumor necrosis factor- α; TRANCE, TNF-related activation-induced cytokine; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

an APP α-secretase (1–5, 32, 33). ADAM9 is believed to participate in the PMA-stimulated shedding of HB-EGF (34) and can function as an APP α-secretase (35). ADAM19/MDC β has been linked to shedding of the epidermal growth factor receptor-ligand neuregulin-β1 (36). ADAM12/MDC α has recently been shown to be responsible for endogenous shedding of HB-EGF in the heart (37). In addition, MMP7 has a functionally relevant role in shedding of TNF-α and FasL (38, 39), and MT1-MMP can be autolytically shed and is an enzyme capable of releasing both CD44 and TRANCE (21, 40, 41). However, not many proteinases responsible for ectodomain shedding of proteins have been identified.

Adamalysin 19/ADAM19/MDC β, cloned from mice (42, 43) and humans (44, 45), is a type I membrane-bound protein containing the basic domains of ADAMs, such as the prodomain, metalloprotease and disintegrin domain, cysteine-rich domain, transmembrane domain, and cytoplasmic domain (3). In addition to the processing of neuregulin (NRG) (36), human adamalysin 19 (hADAM19) is believed to play a role in osteoblast differentiation, in the distinction between macrophages and dendritic cells, and as a marker for the differentiation and characterization of dendritic cells (44). Recently, we demonstrated that hADAM19 is activated by furin in the secretory pathway and that the intracellular removal of the prodomain is required for its proteolytic activity, which was assessed with an α2-macroglobulin (α2-M) trapping assay *in vitro* (46). Emerging evidence indicates that metalloproteinase activity is regulated by the process of shedding or truncation, as in the cases of MT1-MMP, MT5-MMP, ADAM13, and ADAMTS4 (40, 47–49). Here we show that autolytic processing at $Glu^{586} \downarrow Ser^{587}$ of hADAM19 within its cysteine-rich domain is required for its endopeptidase activity and that efficient processing of hADAM19 is mainly dependent on the sizes of both Glu^{586} and Ser^{587}. We also show that PKC, CaM, and calcium signals may regulate the hADAM19 processing, which is not sensitive to GM6001 or TIMP-3. Moreover, we present a peptide substrate that mimics the processing site and may be used to determine the activity of soluble hADAM19 by a fluorescamine assay.

MATERIALS AND METHODS

Chemicals, Cell Lines, Cell Culture, and Immunological Reagents—All common laboratory chemicals, proteinase inhibitors, PMA, trifluoperazine, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), calmidazolium, PD98059, wortmannin, LY290042, pervanadate, and anti-FLAG-M2 monoclonal antibody (mAb) and its agarose conjugates were purchased from Sigma Chemical Co. (St. Louis, MO). The CMK-based furin inhibitor, dec-Arg-Val-Lys-Arg-chloromethyl ketone (decRVKR-CMK), and a matrix metalloproteinase inhibitor, ilomastat (GM6001), were purchased from Bachem (Philadelphia, PA). TIMP-3 was purchased from R & D Systems (Minneapolis, MN). Restriction enzymes were purchased from Promega (Madison, WI) or Invitrogen (Gaithersburg, MD). COS1 and Madin-Darby canine kidney (MDCK) cells and its derivatives were maintained as described (45). Dulbecco's modified Eagle's medium was purchased from Invitrogen (Gaithersburg, MD). Fetal bovine serum, penicillin G, and streptomycin were purchased from Invitrogen (Rockville, MD). α2-M was purchased from Roche Molecular Biochemicals (Indianapolis, IN). Rabbit polyclonal hADAM19 antibodies pAb361 (anti-metalloproteinase domain, anti-Cat) and pAb362 (anti-disintegrin domain, anti-Dis) were generated by our laboratory as reported previously (50).

PCR Primers, Mutagenesis, and Expression Constructs—All inserts tagged with FLAG at their C terminus were cloned into pCR3.1uni, including wild type hADAM19 (F46), soluble hADAM19 (D52), ^{199}RA-D (described in Ref. 46), and all mutants used in this study. The primer sequences for full-length (E346A-F) and soluble (E346A-D) $Glu^{346} \rightarrow$ Ala mutants were: forward primer, 5′-C ATG GCC CAC **GCG** ATG GGC CAC-3′; reverse primer, 5′-GTG GCC CAT **CGC** GTG GGC CAT G-3′. For deletion from the cysteine-rich domain to the end of the C terminus (D-CR): forward primer, 5′-ACC ATG CCA GGG GGC GCA GGC GCC-3′; reverse primer, 5′-GGT ACC ATC CAT CTG GTA GAA G-3′. For the soluble $Glu^{586} \rightarrow$ Ala mutant (E586A-D): forward primer, 5′-CGG CCC CTG **GCG** TCC AAC GCG-3′; reverse primer, 5′-CGC GTT GGA **CGC** CAG GGG CCG-3′. For the soluble $Glu^{586} \rightarrow$ Gly mutant (E586G-D): forward primer, 5′-CGG CCC CTG **GGG** TCC AAC GCG-3′; reverse primer, 5′-CGC GTT GGA **CCC** CAG GGG CCG-3′. For the soluble $Glu^{586} \rightarrow$ Asp mutant (E586D-D): forward primer, 5′-CGG CCC CTG **GAC** TCC AAC GCG-3′; reverse primer, 5′-CGC GTT GGA **GTC** CAG GGG CCG-3′. For the soluble $Glu^{586} \rightarrow$ Gln mutant (E586Q-D): forward primer, 5′-CGG CCC CTG **CAG** TCC AAC GCG-3′; reverse primer, 5′-CGC GTT GGA **CTG** CAG GGG CCG-3′. For the soluble $Ser^{587} \rightarrow$ Ala mutant (S587A-D): forward primer, 5′-CCC CTG GAG **GCC** AAC GCG GTG-3′; reverse primer, 5′-CAC CGC GTT **GGC** CTC CAG GGG-3′. For the soluble $Ser^{587} \rightarrow$ Thr mutant (S587T-D): forward primer, 5′-CCC CTG GAG **ACC** AAC GCG GTG-3′; reverse primer, 5′-CAC CGC GTT **GGT** CTC CAG GGG-3′. For the soluble $Ser^{587} \rightarrow$ Phe mutant (S587F-D): forward primer, 5′-CCC CTG GAG **TTC** AAC GCG GTG-3′; reverse primer, 5′-CAC CGC GTT **GAA** CTC CAG GGG-3′. All constructs were confirmed by DNA sequencing.

DNA Transfection and Generation of Stable Cell Lines—COS1 cells were seeded into 24-well plates for 16–24 h at 80% confluence and transfected with the indicated plasmids using LipofectAMINE2000 according to the instructions provided by Invitrogen (Gaithersburg, MD). After 6–10 h, serum-free Dulbecco's modified Eagle's medium and the indicated reagents were added, and the mixture was incubated for another 24 h. The conditioned media and cell lysates were then analyzed by Western blotting (46). The same transfection procedure was performed to generate stable MDCK cell lines, and the selection for hADAM19 was begun in the presence of G418 (400 μg/ml) after transfection for 24 h. The conditioned media and/or cell lysates of the clones were subjected to Western blotting to confirm the expression of hADAM19 (46).

Western Blotting—The experiments were carried out as described previously (46). Briefly, cells were grown to 80% confluence and were treated as indicated. After centrifugation for 15 min at 14,000 $\times$ *g* and 4 °C to clear any debris, the serum-free media were collected and prepared for SDS-PAGE. The cells were lysed with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 2.5 μM GM6001, 10 μg/ml aprotinin, 10 μg/ml E64, and 10 μg/ml pepstatin A) for 15 min on ice. The supernatant was collected after centrifugation for 20 min at 14,000 $\times$ *g* and 4 °C. After electrophoresis, the proteins were transferred onto nitrocellulose membranes, probed with anti-FLAG-M2 or anti-hADAM19, and developed as before (46).

Purification of Soluble hADAM19 and N-terminal Sequencing—All proteins were purified on anti-FLAG-M2 affinity columns as described previously (46); however, HEPES buffer (50 mM HEPES, pH 7.5, 200 mM NaCl, 10 mM $CaCl_2$, 25 μM $ZnCl_2$, 0.05% Brij-35) was used instead of TBS buffer for the purpose of determining the activity of hADAM19 by a fluorescamine assay. Briefly, cells from stable lines expressing soluble hADAM19, D52-5, E586D-D, S587T-D, and D-CR, were grown to 100% confluence, washed twice with phosphate-buffered saline, and incubated for 48 h. in serum-free media. The conditioned media were collected, centrifuged to clear any debris, and loaded onto an anti-M2 immunoaffinity column (1 ml of resuspended agarose) that had been prewashed with HEPES buffer. The bound materials were extensively washed with HEPES buffer, eluted with FLAG peptides, and collected in 500-μl fractions. The fractions were analyzed by Western blot using anti-hADAM19 antibodies or anti-FLAG-M2, and the protein was quantified by its UV absorbance at 280 nm. The fractions containing the most wild type or mutant hADAM19 proteins were used for the α2-M trapping assay and fluorescamine assay. In the case of D52-5, the most concentrated fraction was also prepared for protein N-terminal sequencing to determine the shedding site. After separation by SDS-PAGE, the samples were transferred to a PVDF membrane and stained with Coomassie Blue R-250. After destaining, the hADAM19 bands were excised and sent to Margaret Seavy at the Bioanalytical Core Facility at the Florida State University for N-terminal amino acid sequencing.

α2-M Trapping Assay—The detailed experimental procedure was previously reported (46, 50). Briefly, equal amounts of purified wild type and mutated soluble hADAM19 were mixed with 24 μl of α2-M (0.2 unit/ml), respectively, adjusted to a total volume of 100 μl with HEPES buffer, and incubated at 37 °C for the indicated times. A 20-μl aliquot of the mixture was removed at the indicated times, put in 2× SDS-PAGE sample buffer, and boiled. Following SDS-PAGE, the protein bands in the gels were visualized by silver staining.

Determination of Kinetic Parameters of hADAM19 Using a New Peptide Assay—The N-terminal acetylated peptide (Ac-RPLESNAV) was synthesized by Dr. Umesh Goli at the Biochemical Analysis, Synthesis,

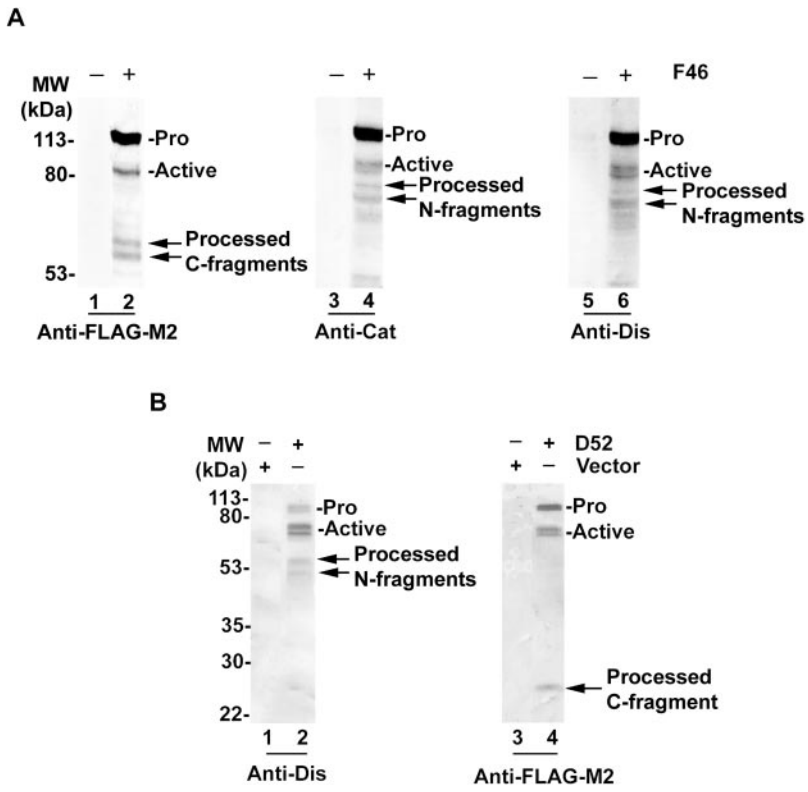

FIG. 1. **hADAM19 is processed within the cysteine-rich domain in COS1-transfected cells.** *A*, detection of hADAM19 by Western blotting with anti-FLAG-M2 mAb (*lanes 1* and *2*), anti-catalytic domain pAb (Anti-Cat) (*lanes 3* and *4*), or anti-disintegrin domain polyclonal antibody (Anti-Dis) (*lanes 5* and *6*). Cell lysates with radioimmune precipitation assay buffer from COS1 cells transfected with the blank vector (*lanes 1*, *3*, and *5*) or pCR3.1hADAM19 (F46) (*lanes 2*, *4*, and *6*). The pro, active, and processed forms of hADAM19 are indicated. *B*, characterization of soluble hADAM19. COS1 cells were transfected with the blank vector (*lanes 1* and *3*) or soluble hADAM19 (D52) (*lanes 2* and *4*). The conditioned media were analyzed by Western blotting using anti-disintegrin domain Ab (Anti-Dis) (*lanes 1* and *2*) and anti-FLAG-M2 mAb (*lanes 3* and *4*).

and Sequence Service Laboratory at Florida State University. The stock solutions of the peptide substrates were prepared in 0.05 M HEPES, pH 7.5, 0.2 M NaCl, 0.01 M $CaCl_2$, and 0.01% Brij-35. The peptide concentrations ranged from 0.2 to 4.0 mM, and the enzyme concentration was 160 nM. N-terminal sequencing for this released peptide was performed after overnight hydrolysis by the enzyme. Hydrolysis of the peptide by the enzyme was monitored by measuring fluorescence intensity (51). After incubation for the indicated times, the hydrolytic reaction was quenched by adding 20 μl of 100 mM 1,10-phenanthroline into 100 μl of reaction mixture. For the control sets, the 1,10-phenanthroline solution was added at the start of incubation. After the reaction was quenched, 100 μl of 0.1% fluorescamine in 10% Me_2SO/90% assay buffer was added into each reaction mixture. Relative fluorescence of the product coupled with fluorescamine was determined on a PerkinElmer Life Sciences LS-50B spectrofluorometer using an excitation wavelength of 386 nm and an emission wavelength of 477 nm. Excitation and emission slit widths were both 10 nm. Relative fluorescence was converted to nanomoles of product using a standard curve obtained with arginine. The final solution was diluted further with the assay buffer, if it was necessary. The values of k_{cat} and K_m were determined by fitting the data to the Michaelis-Menten equation. A molar extinction coefficient of 33,120 M^{-1} cm^{-1}, which was calculated by using Genetic Computer Group (GCG) software, was used to determine the concentration of soluble hADAM19.

RESULTS

hADAM19 Is Processed within the Cysteine-rich Domain—Shedding or truncation is increasingly being recognized as a key regulator of the activity of some metalloproteinases (40, 47–49). It was intriguing to investigate the possibility of shedding or truncation in hADAM19. As shown in Fig. 1*A*, in addition to the pro and active forms of hADAM19, we detected a doublet of protein of about 58 kDa by anti-FLAG-M2 in the lysate of the transfected COS1 cells expressing full-length hADAM19 with C-terminal FLAG tags (F46). We did not detect the doublet using antibodies against the catalytic and disintegrin domains of hADAM19, indicating that the 58-kDa protein is the processed C-terminal fragment lacking the metalloproteinase and disintegrin domains of hADAM19. On the other hand, another doublet of around 72 kDa was detected in the same lysate by these two hADAM19 antibodies but not anti-FLAG-M2 (Fig. 1*A*), suggesting that the 72-kDa protein is the processed N-terminal fragment containing the metalloproteinase and disintegrin domains of hADAM19. Taken together, a cellular processing occurs in hADAM19 after its disintegrin domain in the COS1-transfected cells. Notably, the doublets result from different forms of glycosylation as verified by glycosidase F treatment (Ref. 46 and data not shown). To probe the domain in which the processing occurs, we made two deletion forms of hADAM19 with C-terminal FLAG tags, soluble hADAM19 (D52), which lacked the transmembrane domain and cytoplasmic domain, and a form that lacked the cysteine-rich domain through the cytoplasmic domain (D-CR) (see Fig. 4). When D52 and D-CR were transfected into COS1 cells, we were able to detect several proteins in the conditioned media using both anti-FLAG-M2 and the antibody against the disintegrin domain, including the soluble pro and active forms of hADAM19. However, a 26-kDa protein in the conditioned medium from D52-transfected cells was clearly detectable using anti-FLAG-M2, but not the disintegrin domain antibody, suggesting that the 26-kDa protein is the processed C-terminal fragment of soluble hADAM19. In contrast, a doublet at 60–65 kDa was detected in the medium by the anti-disintegrin domain antibody, but not anti-FLAG-M2, indicating that the 60- to 65-kDa protein is the processed N-terminal fragment of soluble hADAM19 (Fig. 1*B*). There were no additional proteins detectable in the conditioned medium from the cells transfected with D-CR (data not shown); therefore, the processing of hADAM19 might be accounted for by shedding within its cysteine-rich domain.

PMA Up-regulates Processing of Full-length but Not Soluble hADAM19—PMA is a potent inducer of ectodomain shedding of many transmembrane proteins (5, 8–12, 14, 16, 19, 20, 34). To examine the effect of PMA on the processing of hADAM19, the COS1 cells transfected with F46 were treated with PMA overnight. As shown in Fig. 2*A*, PMA obviously enhanced hADAM19 processing in the COS1-transfected cells as detected under reducing conditions. Interestingly, we failed to detect the

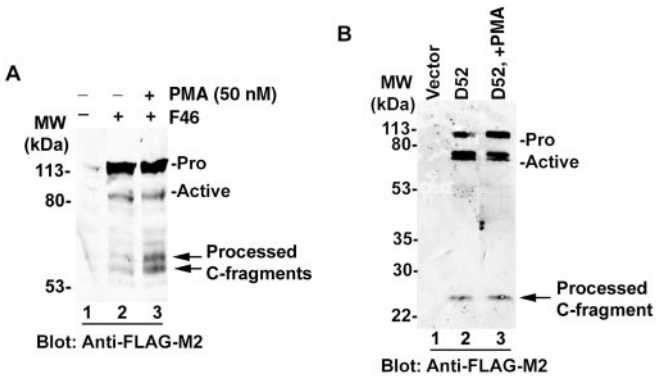

FIG. 2. **PMA enhances the processing at cysteine-rich domain of full-length, but not soluble, hADAM19.** *A*, enhancement of the processing at cysteine-rich domain of full-length hADAM19 by PMA. COS1 cells were transfected with the blank vector (*lane 1*) or F46 (*lanes 2* and *3*). After treated without (*lanes 1* and *2*) or with PMA (50 nM) (*lane 3*) overnight, the cells were lysed and probed with anti-FLAG-M2 mAb. *B*, PMA had no effect on the processing at cysteine-rich domain of soluble hADAM19. The conditioned media were collected from COS1 cells transfected with the blank vector (*lane 1*) or D52 (*lanes 2* and *3*) after overnight treatment with 50 nM PMA. The results were obtained from Western blots using anti-FLAG-M2 mAb.

processed N-terminal fragments in the conditioned media from the F46-transfected COS1 cells, even after PMA treatment (data not shown), suggesting that the processed N-terminal fragment may be still associated with the remaining C-terminal fragment or full-length of hADAM19 via one or more disulfide bonds. Therefore, according to the definition of ectodomain shedding, membrane-bound proteins release their soluble forms and the processing of hADAM19 within its cysteine-rich domain is not technically a form of shedding. To test if PMA-enhanced processing relies on the transmembrane and cytoplasmic domains, we treated the COS1 cells expressing D52 with PMA. As shown in Fig. 2*B*, PMA had a negligible effect on the processing of soluble hADAM19, suggesting that PMA might enhance the processing of hADAM19 via interaction with the cytoplasmic domain, transmembrane domain, or both. As a note, we used the processed C-terminal fragment at 26 kDa as a marker for the processing of soluble hADAM19 throughout this study.

The Processing at Glu^{586} ↓ Ser^{587} within the Cysteine-rich Domain Occurs by an Autolytic Mechanism—A recent report showed that ADAM13 shedding is dependent on its own metalloproteinase activity (48). We therefore hypothesized that the metalloproteinase activity of hADAM19 is also involved in its processing. To test this hypothesis, we used several independent approaches. As shown in Fig. 3*A*, rare processing was detected in the conditioned media from D52-transfected COS1 cells that were treated with decRVKR-CMK, which blocks the activation of hADAM19 (46). This indicates that prodomain removal is necessary for the processing at its cysteine-rich domain of hADAM19. Furthermore, ^{199}RA-D, an inactive mutant of soluble hADAM19 resistant to furin-mediated removal of its prodomain (46), displayed no processing, confirming that the soluble pro-form of hADAM19 lacks the capacity to process at its cysteine-rich domain (Fig. 3*A*). (The total protein level in the media was comparable to that in the media of the D52 cells.) We also generated another soluble inactive form of hADAM19 (E346A-D), in which the active residue Glu at 346 in the metalloproteinase domain was mutated to Ala, and transfected it into COS1 cells. Obviously, no processing at its cysteine-rich domain was observed in the media of these cells. Once again, the total protein level in the media was almost equal to that in the media of the D52 cells (Fig. 3*A*). These results strongly argue that the processing at its cysteine-rich domain of soluble hADAM19 depends on its own metalloproteinase activity. However, neither GM6001 nor TIMP-3, inhibitors that typically block sheddase activity (26–30), inhibited the autolytic processing of hADAM19 at its cysteine-rich domain (data not shown).

To further confirm that autolytic processing at its cysteine-rich domain occurs in soluble hADAM19, we generated stable MDCK cell lines called D52-5, ^{199}RA-D-6, and E346A-D-17, which expressed soluble D52, ^{199}RA, and E346A, respectively. As we expected, the 26-kDa-processed fragment was clearly detectable in the conditioned media from D52-5; the processing was dramatically inhibited by decRVKR-CMK (Fig. 3*B*). Furthermore, there was no processing at the cysteine-rich domain in ^{199}RA-D-6 and E346A-D-17 (Fig. 3*B*). (The total amount of soluble protein was comparable among the conditioned media of these cell lines). Once again, PMA, GM6001, and TIMP-3 failed to affect the processing of D52-5 (Figs. 2*B* and 3*B*, data not shown).

N-terminal sequencing revealed that the starting sequence of the purified 26-kDa protein was SNAVPIDT, which is identical to ^{596}SNAVPIDT^{594} within the cysteine-rich domain of hADAM19. This suggests that the processing of hADAM19 occurs at Glu^{586} ↓ Ser^{587} within its cysteine-rich domain (Fig. 3*B*).

The Sizes of Glu^{586} and Ser^{587} Are Critical for the Processing at Glu^{586} ↓ Ser^{587} of Soluble hADAM19—To examine the importance of the Glu^{586} (P1) and Ser^{587} (P1′) sites in the processing at Glu^{586} ↓ Ser^{587} of soluble hADAM19, we changed the size, charge, and polarity of these two residues by mutagenesis. The constructs are shown in Fig. 4. Shown in Fig. 5*A*, there was no or little detectable processing in the COS1 cells transfected with either E586D-D or S587T-D, suggesting that subtle changes in the sizes of residues at the P1 and P1′ positions can dramatically impair the processing. Furthermore, rare or little processing was observed when significant changes were made to the side chains of the residues at these sites, as in the cases of the Glu^{586} to Ala or Gly, and Ser^{587} to Phe mutants (Fig. 5*A*). On the other hand, the amount of processing product in the E586Q-D or S587A-D mutant, in which the side chain of the amino acid residue was almost the same size as that in wild type soluble hADAM19, was almost equal (Fig. 5*A*).

To further confirm the fate of processing at Glu^{586} ↓ Ser^{587} upon changes at the P1 and P1′ sites, we chose mutants with subtle changes and, presumably, structural similarities, Glu^{586} to Asp and Ser^{587} to Thr, to generate stable transfectants in MDCK cells. Shown in Fig. 5*B*, rare or little processing was detectable in the conditioned media from these stable MDCK transfectants, confirming that both soluble E586D-D and S587T-D have undetectable or little ability to process, even if they are cleaved by furin in the MDCK transfectants. (There were no significant differences in protein levels among the transfectants.)

The Signals of CaM and Calcium May Be Involved in the Processing at Glu^{586} ↓ Ser^{587} of hADAM19—In addition to the PKC pathway, there are several other signal pathways, involving tyrosine kinase, MAPKs, phosphatase, phosphatidylinositol 3-kinase, calcium, and CaM, which regulate the ectodomain-shedding process (10, 13–19, 21–23). Specific inhibitors were used to determine whether or not these signal pathways regulate the processing at Glu^{586} ↓ Ser^{587} of hADAM19. As shown in Fig. 6, only CaM inhibitors impaired the processing of soluble hADAM19. The other inhibitors, including genistein, PD98059, pervanadate, wortmannin, and LY290042, had no significant effects on the processing of soluble or full-length hADAM19 (data not shown). In addition, both A23187 and brefeldin A block the activation of both soluble and full-length

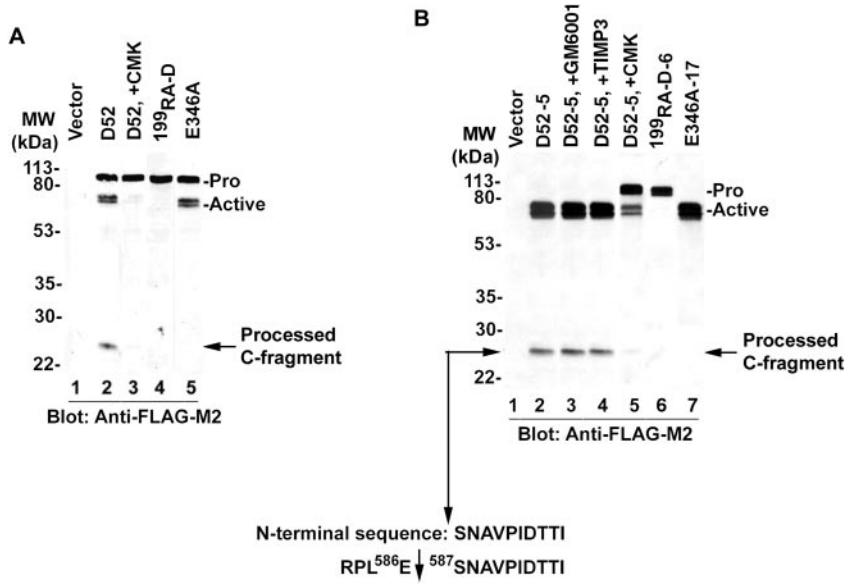

FIG. 3. **hADAM19 autolytically process at Glu^{586} ↓ Ser^{587} within the cysteine-rich domain.** *A*, autolytic processing at the cysteine-rich domain of soluble hADAM19 in COS1-transfected cells. COS1 cells were transfected with the blank vector (*lanes 1*), D52 (*lanes 2* and *3*), soluble mutant with ^{199}RR to ^{199}AA (^{199}RA-D) (*lane 4*), or soluble inactive mutant (E346A-D) (*lane 5*) and incubated without (*lanes 1*, *2*, *4*, and *5*) or with 100 μM CMK (*lane 3*) for 16 h. The conditioned media were analyzed by Western blotting with anti-FLAG-M2 mAb. *B*, processing at cysteine-rich domain of soluble hADAM19 in the stable MDCK transfectants. MDCK cells stably expressing soluble hADAM19 (D52-5) (*lanes 2–5*), soluble ^{199}RA mutant (^{199}RA-D-6) (*lane 6*), or the soluble inactive mutant (E346A-D-17) (*lane 7*) were treated without (*lanes 1*, *2*, *6*, and *7*) or with 5 μM GM6001 (*lane 3*), 100 nM TIMP-3 (*lane 4*), or 100 μM CMK (lane 5) overnight. The conditioned media were analyzed by Western blotting using anti-FLAG-M2 mAb. MDCK cells transfected with the blank vector were used as a control (*lane 1*). The sequence for the shed C-terminal protein is shown at the *bottom*.

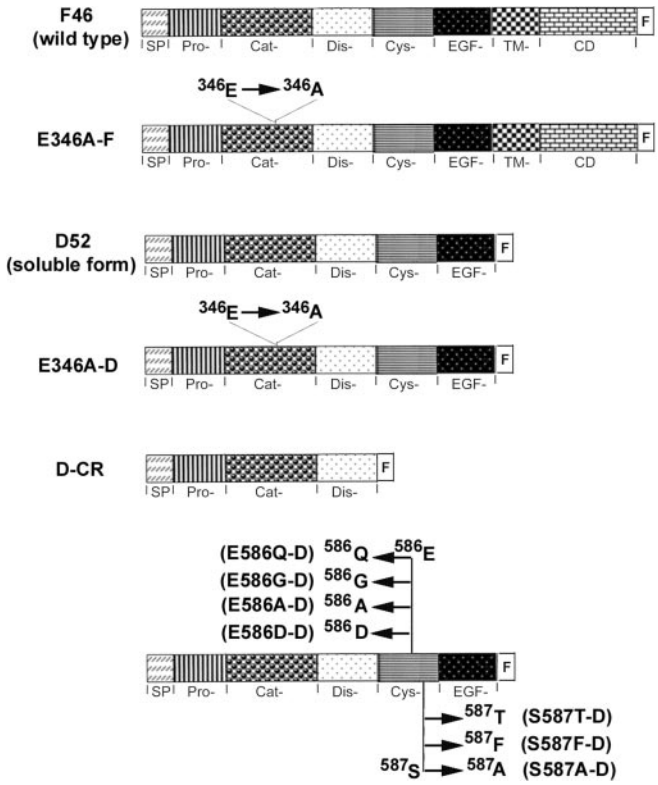

FIG. 4. **A schematic illustration of the wild type expression vector pCR3.1hADAM19 and its mutant constructs.** All of the constructs have a C-terminal FLAG tag. *SP*, signal peptide; *Pro-*, prodomain; *Cat-*, catalytic domain; *Dis-*, disintegrin domain; *Cys-*, cysteine-rich domain; *EGF-*, EGF-like domain; *TM*, transmembrane domain; *CD*, cytoplasmic domain; *F*, FLAG tag.

hADAM19 (46). There was no processing at Glu^{586} ↓ Ser^{587} detectable under the treatment of either A23187 or brefeldin A (data not shown), indicating that hADAM19 is activated and processed in the secretory pathway. Taken together, our results suggest that PKC, CaM, and calcium signal pathways may be related to the processing at Glu^{586} ↓ Ser^{587} of hADAM19.

The Processing at Glu^{586} ↓ Ser^{587} Is Necessary for hADAM19 to Exert Its Proteolytic Activity against α2-M—To assess the significance of the processing at Glu^{586} ↓ Ser^{587} of hADAM19, we purified the proteins from D52-5, E586D-D, and S587T-D, respectively. Intriguingly, the processed N-terminal fragments, containing the metalloproteinase, disintegrin, and parts of the cysteine-rich domain, were detected as mature forms using anti-disintegrin antibody (data not shown). This suggests that the processed N-terminal segments bind with unprocessed soluble forms or processed C-terminal-soluble fragments by one or more disulfide bonds, consistent with the results obtained early from the full-length hADAM19 (data not shown). When we probed the purified proteins with anti-FLAG-M2, as shown in Fig. 7*A*, S587T-D displayed relatively more processing than E586D-D, in which a very low level of processing was detected. D52-5 showed much more processing than E586D-D and S587T-D. As shown in Fig. 7*B*, D52-5 protein had a much greater ability to form a complex with α2-M and generate two products compared with both E586D-D and S587T-D proteins, which displayed much lower activities. Obviously, S587T-D had a relatively higher activity than E586D-D, which showed very low activity. These results perfectly coincide with the Western blot shown in Fig. 7*A*, indicating that the more that hADAM19 processed at Glu^{586} ↓ Ser^{587}, the more proteolytic activity it exerted against α2-M *in vitro*.

Ac-RPLE-SNAV Is Cleaved by Active hADAM19—To test hADAM19 activity, we synthesized a new peptide, Ac-RPLE-SNAV, which encompasses the processing site of hADAM19 and is conserved among humans and mice (42, 45). Because the processing at Glu^{586} ↓ Ser^{587} is mediated by its own metalloproteinase activity (Fig. 3), we were able to use this peptide to assay soluble hADAM19 activity and obtained the similar results as from the α2-M assay (Fig. 7*B*). Once again, the highest

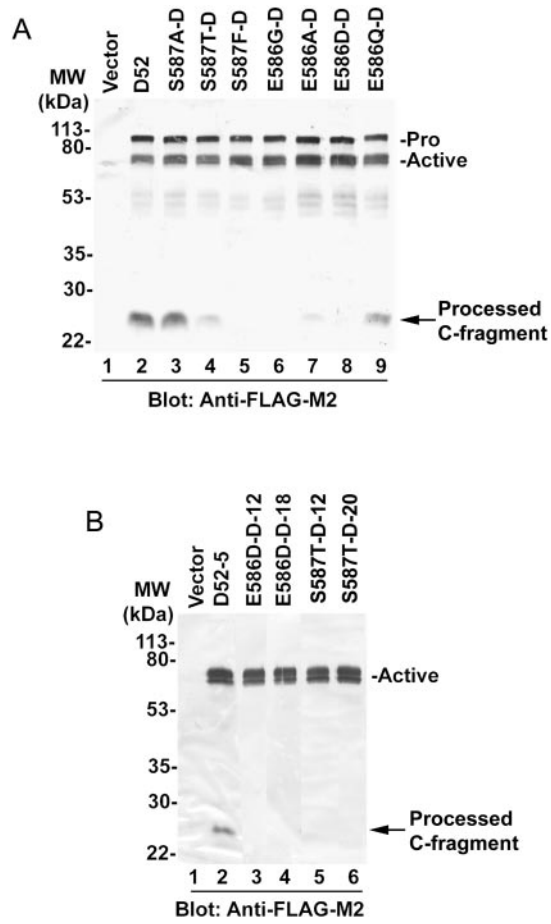

FIG. 5. **The residue sizes at both Glu^{586} and Ser^{587} are critical for the processing at Glu^{586} ↓ Ser^{587} of hADAM19.** *A*, processing profile at Glu^{586} ↓ Ser^{587} of soluble mutants in COS1-transfected cells. COS1 cells were transfected with the blank vector (*lane 1*), D52 (*lane 2*), soluble mutants with Ser^{587} to Ala (S587A-D) (*lane 3*), Thr (S587T-D) (*lane 4*), or Phe (S587F-D) (*lane 5*), or Glu^{586} → Gly (E586G-D) (*lane 6*), Ala (E586A-D) (*lane 7*), Asp (E586D-D) (*lane 8*), or Gln (E586Q-D) (*lane 9*). The conditioned media were subjected to SDS-PAGE and Western blotting with anti-FLAG-M2 mAb. *B*, detection of the processing at Glu^{586} ↓ Ser^{587} of hADAM19 in MDCK cells stably expressing soluble hADAM19 mutants. Four MDCK cells stably expressing soluble hADAM19 with Glu^{586} → Asp (E586D-D-12 and E586D-D-18) (*lanes 3* and *4*) or Ser^{587} to Ala (S587A-D-12 and S587A-D-20) (*lanes 5* and *6*) were prepared in serum-free media overnight. The conditioned media were analyzed by Western blotting using anti-FLAG-M2 mAb. MDCK cells transfected with the blank vector (*lane 1*) and D52-5 cells (*lane 2*) were used as controls.

activity was seen in D52-5 with 1% cleavage after incubation overnight. E586D-D and S587T-D only showed 30 and 10% the activity exerted by D52-5, respectively (data not shown). As we knew, the activity at 1% was not enough for a fluorescence assay. However, given that D52-5 was not fully processed (maybe 25% of the mature hADAM19 got processed according to the Western blotting result as shown in Fig. 7*A*), we decided to generate another stable line in MDCK cells expressing D-CR (Fig. 4). The reason we deleted the whole cysteine-rich domain is that we surmised that this domain has a potential interaction with the metalloproteinase domain, disintegrin domain, or both that might decrease the activity. Fortunately, purified D-CR from the MDCK transfectants had a higher activity for cleaving our new peptide substrate, and the cleavage product detected by N-terminal sequencing was peptide SNAV, confirming that the peptide substrate was cleaved at the E-S bond, which is identical to the processing site in the hADAM19 protein. Furthermore, as shown in Fig. 8, the k_{cat}, K_m, and k_{cat}/K_m values for 150-min incubation were 2.4 min^{-1}, 2.0 mM, and

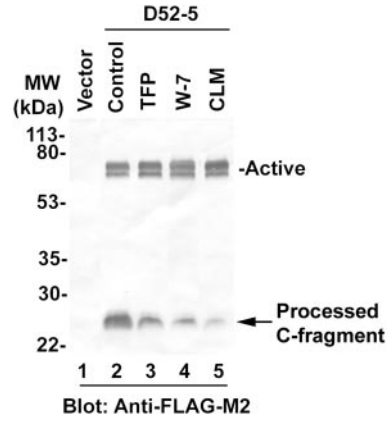

FIG. 6. **Processing at Glu^{586} ↓ Ser^{587} of hADAM19 is inhibited by calmodulin inhibitors.** MDCK cells stably expressing soluble hADAM19 (D52-5) (*lanes 2–5*) were treated without (*lanes 1* and *2*) or with 100 μM trifluoperazine (*lane 3*), 25 μM W7 (*lane 4*), or 50 μM calmidazolium (*lane 5*) for 16 h. The conditioned media were then analyzed by Western blotting using anti-FLAG-M2 mAb. MDCK cells transfected with the blank vector were used as a control (*lane 1*).

1200 M^{-1} min^{-1}, respectively. Thus, we developed a peptide substrate for determining soluble hADAM19 activity by a fluorescamine assay.

DISCUSSION

In the current report, we have demonstrated that processing of hADAM19 occurs at Glu^{586} ↓ Ser^{587} within the cysteine-rich domain by its own metalloproteinase activity and is a necessary step to display its proteolytic activity against both a peptide substrate and α2-M *in vitro*. We have also revealed that the processing at Glu^{586} ↓ Ser^{587} of hADAM19 is regulated by a unique pathway, distinguishable from those shown for other ADAMs, MT-MMPs, and other membrane-bound proteins.

Shedding or Processing of Metalloproteinases—Growing evidence suggests that shedding is of vital importance for the regulation of metalloproteinase activity. For MT-MMPs, Pei's group reported that MT5-MMP is shed by furin, down-regulating its activity, and that interleukin-8 triggers the signal for both release and activation of MT6-MMP by an unknown mechanism (47, 52). The activity of MT1-MMP can be autolytically terminated directly on the cell surface or via production of a soluble functional fragment, consequently down-regulating enzyme activity on the cell surface (40). Among ADAMs, ADAM13 is the only one that has been shown to shed its ectodomain intracellularly by an autolytic mechanism, producing an active enzyme able to bind with α2-M and integrins (48). In addition, truncation of mature ADAMTS4 at its C terminus is required for its aggrecanase activity (49). In this report, we demonstrate that hADAM19 carries out processes within its cysteine-rich domain, resulting in an active enzyme shown by both α2-M and peptide substrate assays *in vitro* (Figs. 3, 7, and 8). It might, therefore, be a general regulatory mechanism that MT-MMPs, such as MT1-MMP and MT5-MMP, are down-regulated by shedding to release active forms from the cell surface, whereas ADAMs must shed, carry out process, or become truncated at the C terminus to exert their functions, such as acting as a sheddase, binding with integrins on the cell surface, or digesting components of the extracellular matrix.

Regulation of the Processing at Glu^{586} ↓ Ser^{587} of hADAM19—The signal pathways involved in shedding or truncation processes, especially of ADAMs, are poorly understood. In the present report, we provide unique characteristics of the regulation of the processing at Glu^{586} ↓ Ser^{587} of hADAM19. We found that PMA, a common inducer of shedding, also enhances the processing at Glu^{586} ↓ Ser^{587} of hADAM19 (Fig.

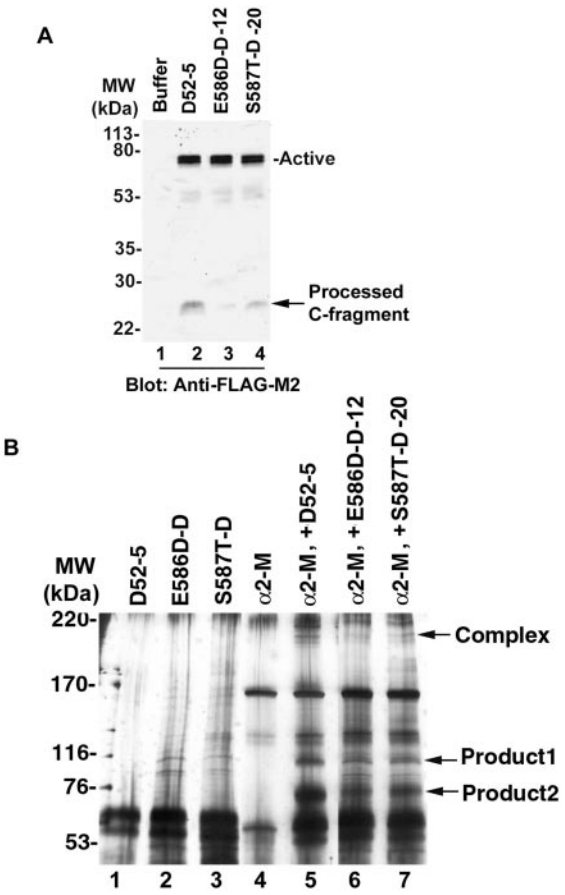

FIG. 7. **Requirement of the processing at Glu^{586} ↓ Ser^{587} for the proteolytic activity of hADAM19.** *A*, processing status at Glu^{586} ↓ Ser^{587} of purified proteins from the stable MDCK transfectants. MDCK cells stably expressing soluble hADAM19 (D52-5) (*lane 2*), soluble Glu^{586} → Asp (E586D-D-12) (*lane 3*), or soluble Ser^{587} to Ala (S587A-D-20) (*lane 4*) were prepared for purification as described under "Materials and Methods." Western blots using anti-FLAG-M2 mAb were performed on equal amounts of these purified proteins. *B*, the proteolytic activity of soluble hADAM19 using α2-M *in vitro*. The purified hADAM19 from D52-5 (*lanes 1* and *5*), E586D-D-12 (*lanes 2* and *6*), or S587A-D-20 (*lanes 3* and *7*) were normalized and incubated in reaction buffer alone (*lanes 1–3*) or with α2-M (*lanes 5–7*) for 24 h. α2-M in reaction buffer alone (24 h) was a control (*lane 3*). The α2-M·hADAM19 complex and the cleavage products of α2-M by hADAM19 are labeled on the *right*.

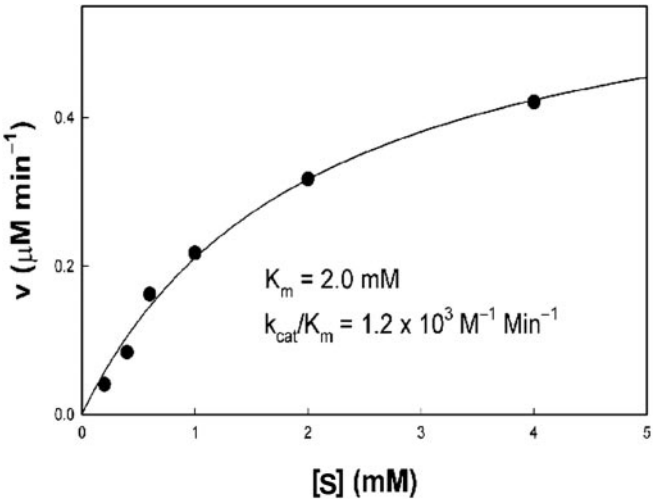

FIG. 8. **The hydrolysis of Ac-RPLE-SNAV by hADAM19.** The peptide (Ac-RPLE-SNAV) (0.2–4.0 mM), which mimics the processing site of hADAM19, was incubated with 0.26 μM soluble, active ADAM19 for 150 min. The formation of product was measured by monitoring the coupling of fluorescamine with the amino group newly formed from the cleavage. The nonlinear regression analysis of the data indicates k_{cat} = 2.4 min^{-1}, K_m = 2.0 mM, and k_{cat}/K_m = 1200 M^{-1} min^{-1}.

2*A*). The mechanism probably involves the cytoplasmic domain, transmembrane domain, or both, because PMA did not alter the processing of soluble hADAM19 (Fig. 2*B*). This is consistent with reports showing that the cytoplasmic domain of ADAM9 is required for PMA-induced shedding (34) and that the membrane anchor of TACE is necessary for its processing of TNF-α (53, 54). In addition, there are a few reports that have demonstrated the requirement of the cytoplasmic tail for shedding of pro-NRG, APP, pro-TGF, and L1 adhesion molecules (17, 55, 56). However, in most cases, endogenous and/or inducer-mediated shedding is independent of the cytoplasmic domain (Fig. 2*B*) (14, 17, 19, 22, 24). Calcium ionophore, A23187, is another potent inducer of most protein-shedding processes (13–16). Nevertheless, we found that A23187 and brefeldin A block the activation of both full-length and soluble hADAM19. Subsequently, no processing at Glu^{586} ↓ Ser^{587} was detectable, suggesting that hADAM19 is activated and processed in the secretory pathway (46, data not shown). Inhibitors of CaM have been shown to stimulate the shedding of several proteins, including MT1-MMP, pro-TGFα, pro-NRG, and APP, by a mechanism independent of both PKC and calcium (17–20). However, in our study, a reverse result was obtained; the processing at Glu^{586} ↓ Ser^{587} was inhibited by the CaM inhibitors (Fig. 6). Finally, we found that tyrosine kinase, MAPK, phosphatase, or phosphatidylinositol 3-kinase do not seem to play roles in the processing at Glu^{586} ↓ Ser^{587} of hADAM19 (data not shown), although they have been previously shown to participate in some shedding processes (9–12, 21–23, 29).

In addition, the proteinases responsible for the shedding of many cell surface molecules seem to have broad sequence specificity as revealed by mutational analysis of residues around the cleavage site of pro-TGFα, APP, IL-6 receptor, L-selectin, and pro-TNFα (57–61). In this report, we used mutagenesis to show that the residue sizes of the side chains at both the Glu^{586} and Ser^{587} sites are extremely important for normal processing at Glu^{586} ↓ Ser^{587} of hADAM19. Even delicate changes, such as Glu^{586} → Asp and Ser^{587} → Thr, caused dramatic decreases in the processing. Notably, many studies have shown that some potent synthetic inhibitors of metalloproteinases, such as TAPI, BB94, and GM6001, can block most, if not all, shedding processes and that many shedding processes are sensitive to TIMP-3, a matrix-associated TIMP that preferably inhibits ADAMs (5–8, 19, 24–30). However, neither GM6001 nor TIMP-3 inhibits the autolytic processing at Glu^{586} ↓ Ser^{587} of hADAM19 (Fig. 3*B*), which is consistent with some reports showing that the shedding of MT5-MMP, MT6-MMP, and IL-6 receptor is not affected by metalloproteinase inhibitors (47, 52, 62). One possibility, we speculate, is that shedding, in most cases, occurs at membrane-proximal regions on the cell surface, which are easily accessible to hydroxamate-based inhibitors and TIMP-3 (5, 25, 60, 63). Human ADAM19 processing takes place at a region distal from the transmembrane domain in the secretory pathway, which is less accessible to GM6001 and TIMP-3 (Fig. 3*B*). How hADAM19 initiates the processing at Glu^{586} ↓ Ser^{587} and what the roles for the disintegrin- and cysteine-rich domains are during the processing at Glu^{586} ↓ Ser^{587} remain to be uncovered. Perhaps dimerization through the disintegrin- and/or cysteine-rich domains is the key step for the processing as proposed for other ADAMs (3). This might also be an explanation for the fact that the processed N-terminal fragments, containing the metalloproteinase, disintegrin, and parts of the cysteine-rich domain, were not detected in the conditioned medium from the transfected COS1 cells with full-

length hADAM19 and were present among the purified soluble hADAM19 proteins (data not shown).

A New Peptide Substrate Based on the Processing Site Sequence for hADAM19—The peptide substrates currently used to measure MMP activity are synthesized based on the cleavage sites of protein substrates. Because hADAM19 processes at $Glu^{586} \downarrow Ser^{587}$ by its own metalloproteinase activity, we surmised that a peptide encompassing the processing site would be an ideal substrate. Indeed, this peptide, Ac-RPLE-SNAV, was suitable for a fluorescamine assay of enzyme activity and was cleaved at the *E*-S site as determined by peptide N-terminal sequencing. Although the wild type hADAM19 showed a minimally detectable activity by this method, D-CR, containing the metalloproteinase and disintegrin domains, displayed a higher activity to determine kinetic parameters after incubation for 150 min using our fluorescamine assay (Fig. 8). The kinetic parameters, k_{cat}, K_m, and k_{cat}/K_m, were 2.4 min^{-1}, 2.0 mM, and 1200 M^{-1} min^{-1}, respectively, demonstrating that this is a poor substrate and will have a limited usage. It is necessary that the peptide substrates will be optimized by creating analogs that are hydrolyzed more efficiently by hADAM19 in the near future. Our preliminary data show that TIMP-3 can inhibit hADAM19 activity in this peptide-based assay,[2] which is similar to the results for ADAM10, -12, and -17 and ADAMTS4 and -5 *in vitro* (64–67), and confirm that the TIMP-3 we used was active. However, TIMP-3 had no effect on the cellular processing at $Glu^{586} \downarrow Ser^{587}$ of soluble hADAM19 (Fig. 3*B*), indicating that this processing occurred intracellularly, to where TIMP-3 molecules might not be accessible. Interestingly, ADAMTS4 is truncated at $Glu^{373} \downarrow Ala^{374}$, the site for the cleavage of ADAMTS4 and ADAMTS5, not MMPs. In contrast, the truncation site at $Asn^{341} \downarrow Phe^{342}$ is mediated by MMPs, not aggrecanases (49, 68). Our mutational data showed that the $Glu^{586} \downarrow Ala^{587}$ processing site was also optimal for autolytic processing of hADAM19 (Fig. 5*B*), indicating that our peptide substrate might be useful to determine the activity of other ADAMs, such as aggrecanases. This peptide substrate may also be used for testing metalloproteinase inhibitors against hADAM19 and other ADAMs.

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Activation of Pro-gelatinase B by Endometase/Matrilysin-2 Promotes Invasion of Human Prostate Cancer Cells*

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This work has explored a putative biochemical mechanism by which endometase/matrilysin-2/matrix metalloproteinase-26 (MMP-26) may promote human prostate cancer cell invasion. Here, we showed that the levels of MMP-26 protein in human prostate carcinomas from multiple patients were significantly higher than those in prostatitis, benign prostate hyperplasia, and normal prostate glandular tissues. The role of MMP-26 in prostate cancer progression is unknown. MMP-26 was capable of activating pro-MMP-9 by cleavage at the Ala^{93}–Met^{94} site of the prepro-enzyme. This activation proceeded in a time- and dose-dependent manner, facilitating the efficient cleavage of fibronectin by MMP-9. The activated MMP-9 products generated by MMP-26 appeared more stable than those cleaved by MMP-7 under the conditions tested. To investigate the contribution of MMP-26 to cancer cell invasion via the activation of MMP-9, highly invasive and metastatic human prostate carcinoma cells, androgen-repressed prostate cancer (ARCaP) cells were selected as a working model. ARCaP cells express both MMP-26 and MMP-9. Specific anti-MMP-26 and anti-MMP-9 functional blocking antibodies both reduced the invasiveness of ARCaP cells across fibronectin or type IV collagen. Furthermore, the introduction of *MMP-26* antisense cDNA into ARCaP cells significantly reduced the MMP-26 protein level in these cells and strongly suppressed the invasiveness of ARCaP cells. Double immunofluorescence staining and confocal laser scanning microscopic images revealed that MMP-26 and MMP-9 were co-localized in parental and *MMP-26* sense-transfected ARCaP cells. Moreover, MMP-26 and MMP-9 proteins were both expressed in the same human prostate carcinoma tissue samples examined. These results indicate that MMP-26 may be a physiological and pathological activator of pro-MMP-9.

During the initial phases of carcinoma cell invasion, as tumor cells begin to spread and infiltrate into the surrounding normal tissues, these cells must first degrade the basement membrane and other elements of the extracellular matrix (ECM),[1] including type IV collagen, laminin, and fibronectin (FN) (1). Multiple protease families, including the matrix metalloproteinases (MMPs), serine proteases, and cysteine proteases, are suspected of contributing to the invasive and metastatic abilities of a variety of malignant tumors (2–5), but the specific biochemical mechanisms that facilitate these invasive behaviors remain elusive.

More than 23 human *MMPs*, and numerous homologues from other species, have been reported (5), and matrix metalloproteinase-26 (*MMP-26*)/endometase/matrilysin-2 is a novel member of this enzyme family that was recently cloned and characterized by our group (6) and others (7–9). *MMP-26* mRNA is primarily expressed in epithelial cancers, such as lung, breast, endometrial, and prostate carcinomas, in their corresponding cell lines (6–9), and in a very limited number of normal adult tissues, such as the uterus (6, 8), placenta (7, 8), and kidney (9). Recently, we have found that the levels of *MMP-26* gene and protein expression are higher in a malignant choriocarcinoma cell line (JEG-3) than in normal human cytotrophoblast cells (10). Our preliminary studies indicate that expression of *MMP-26* may be correlated with the malignant transformation of human prostate and breast epithelial cells. The specific expression of *MMP-26* in malignant tumors and the proteolytic activity of this enzyme against multiple components of the ECM, including fibronectin, type IV collagen, vitronectin, gelatins, and fibrinogen, as well as non-ECM proteins such as insulin-like growth factor-binding protein 1 and α1-protease inhibitor (6–9), indicate that MMP-26 may possess an important function in tumor progression.

Another member of the *MMP* family considered to be an important contributor to the processes of invasion, metastasis, and angiogenesis exhibited by tumor cells is gelatinase B (*MMP-9*) (11–14). Uría and López-Otín (8) have demonstrated that MMP-26 is able to cleave MMP-9, and here we examine the possibility that MMP-26 facilitates tumor cell invasion through the activation of pro-MMP-9. The highly invasive and metastatic cell line utilized for this study, an androgen-re-

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[1] The abbreviations used are: ECM, extracellular matrix; ANOVA, analysis of variance; ARCaP, androgen repressed prostate cancer cells line; BPH, benign prostate hyperplasia; FN, fibronectin; IMA, integrated morphometry analysis; MMP-7, matrix metalloproteinase-7/matrilysin; MMP-9, matrix metalloproteinase-9/gelatinase B; MMP-26, matrix metalloproteinase-26/endometase/matrilysin-2; MMPs, matrix metalloproteinases; CAPS, 3-cyclohexylamino-1-propanesulfonic acid.

pressed human prostate cancer (ARCaP), was derived from the ascites fluid of a patient with advanced prostate cancer that had metastasized to the lymph nodes, lungs, pancreas, liver, kidneys, and bones (15). This cell line produces high levels of MMP-9 and gelatinase A (MMP-2) (15, 16).

In this study, we provide evidence that MMP-26 is capable of activating pro-MMP-9, and that once activated, MMP-9 cleaves fibronectin, type IV collagen, and gelatin with great efficiency. Both the MMP-26 and MMP-9 proteins were highly expressed in the ARCaP cells, and co-localization of their expression patterns was consistently observed. The invasiveness of ARCaP cells through FN or type IV collagen was significantly decreased in the presence of antibodies specifically targeting MMP-26 or MMP-9. In addition, cells transfected with antisense *MMP-26*, showing significant reduction of MMP-26 at the protein level, exhibited a reduction of invasive potential *in vitro* in addition to a significant diminution in observed levels of active MMP-9 protein. These results support the hypothesis that activation of MMP-9 by MMP-26 may promote the *in vitro* invasiveness of ARCaP cells through FN or type IV collagen, whereas the co-expression of MMP-26 and MMP-9 in many human prostate carcinoma tissues indicates that this relationship may also occur *in vivo*.

MATERIALS AND METHODS

Cell Culture—ARCaP, DU145, PC-3, and LNCaP, which are all established human prostate carcinoma cell lines, were routinely grown in low-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37 °C.

Silver Stain and Gelatin Zymography—Purified recombinant MMP-26 (6) or MMP-7 were incubated with purified pro-MMP-9 (17) or pro-MMP-2 (18) in HEPES buffer (50 mM HEPES, pH 7.5, 200 mM NaCl, 10 mM $CaCl_2$, and 0.01% Brij-35) at 37 °C. For the dosage dependence of MMP-9 activation, MMP-9 (0.2 μM, final concentration) was incubated with MMP-7 and MMP-26 at the indicated molar concentration ratio (2:1, 4:1, and 8:1) for 24 h. The MMP-9 activation was quenched by 2× SDS-PAGE sample buffer containing 50 mM EDTA. The resulting solution was further diluted five times and 5 μl of the diluted sample was loaded onto SDS-polyacrylamide gels (8%). For the time dependence of MMP-9 activation, MMP-9 (0.2 μM) was incubated with MMP-7 (0.05 μM) and MMP-26 (0.05 μM) for the indicated time periods (0, 4, 8, 24 and 48 h) before quenching with the sample buffer. For FN cleavage assays, 2 μl of FN (0.25 mg/ml) were incubated with 30 μl of MMP-26 (final concentration 0.05 μM), pro-MMP-9 (final concentration 0.2 μM), or MMP-26-activated MMP-9 solutions in 1× HEPES buffer at 37 °C for 18 h. For silver staining, the reaction was stopped by adding 4× reducing sample buffer (6% SDS, 40% glycerol, 200 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol, 200 mM EDTA, and 0.08% bromphenol blue) and boiled for 5 min. Following electrophoresis on a 9% SDS-polyacrylamide gel, the protein bands were visualized by silver staining (19). For gelatin zymogram, the gel was incubated for 3 h at 37 °C before it was stained with 0.1% Coomassie Blue solution (17, 20, 21).

Protein N-terminal Sequencing—Samples were separated by SDS-PAGE and transferred to ProBlott™ polyvinylidene difluoride membranes (Applied Biosystems) using CAPS buffer (10 mM CAPS, pH 11, 0.005% SDS). Proteins were visualized by staining with Coomassie Brilliant Blue R-250 solution (0.1% Coomassie Brilliant Blue R-250, 40% methanol, 1% acetic acid) and excised fragments were sent for sequencing. N-terminal sequencing was performed at the Bioanalytical Core Facility, Florida State University.

Reverse Transcriptase-PCR Analysis—RNA was extracted from the original cells by Trizol according to manufacturer protocols (Invitrogen, Carlsbad, CA), and 2 μg of total RNA were subjected to reverse transcriptase-PCR according to the standard protocol provided with the PCR kit (Invitrogen Corp., Carlsbad, CA). The *MMP-26* forward primer was 5′-ACCATGCAGCTCGTCATCTTAAGAG-3′; the reverse primer was 5′-AGGTATGTCAGATGAACATTTTTCTCC-3′; for glyceraldehyde-3-phosphate dehydrogenase the forward primer was 5′-ACGGATTTGGTCGTATTGGG-3′; the reverse primer was 5′-TGATTTTGGAGGGATCTCGC-3′. PCR reactions were performed using a Biometra Personal Cycler (Biometra, Germany) with 30 thermal cycles of 10 s at 94 °C denaturing, 30 s at 60 °C annealing, and 1 min at 72 °C elongation. Ten μl of the amplified PCR products were then electrophoresed on a 1.0% agarose gel containing 0.5 mg/ml ethidium bromide for analysis of size differences. To confirm the amplification of the required cDNA sequences, PCR products were digested with a restriction enzyme as directed by the manufacturer.

Generation and Characterization of Polyclonal Antibodies—Specific antigen peptides corresponding to unique sequences in the pro-domain and metalloproteinase domain of MMP-26 were synthesized by Dr. Umesh Goli at the Biochemical Analysis, Synthesis and Sequencing Services Laboratory of the Department of Chemistry and Biochemistry, Florida State University (Tallahassee, FL). The sequence selected from the pro-domain was Thr^{50}-Gln-Glu-Thr-Gln-Thr-Gln-Leu-Leu-Gln-Gln-Phe-His-Arg-Asn-Gly-Thr-Asp^{67}, and the sequence selected from the metalloproteinase domain was Asp^{188}-Lys-Asn-Glu-His-Trp-Ser-Ala-Ser-Asp-Thr-Gly-Tyr-Asn^{201} of the prepro-enzyme. Using the BLAST search method at the National Center for Biotechnology Information web site against all of the sequences in the data banks, no peptide with >45% level of identity was found (6), predicting the antibodies directed against these two peptides should be specific. The purity of these peptides was verified by reverse-phase high performance liquid chromatography and mass spectrometry. Rabbit anti-human antibodies were then generated, purified, and characterized as described previously (19, 21). Western blot analyses have demonstrated that these two antibodies are highly specific for MMP-26 because they do not cross-react with human matrilysin (MMP-7), stromelysin (MMP-3), gelatinase A (MMP-2), gelatinase B (MMP-9), and some other proteins tested (data not shown).

Western Blotting—Western blotting for MMP-26 was performed by lysing the cells with Tris-buffered saline (50 mM Tris and 150 mM NaCl, pH 7.4) containing 1.5% (v/v) Triton X-114 as described previously (21). Aliquots (20 μl) of cell lysate and media containing equal volumes (20 μl) from each treatment treated with SDS sample buffer were then loaded onto an SDS-polyacrylamide gel. Samples were electrophoresed and then electroblotted onto a nitrocellulose membrane. Immunoreactive MMP-26 bands were visualized using a horseradish peroxidase or alkaline phosphatase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA). Western blot analysis for MMP-9 was performed with a 1 μg/ml dilution of polyclonal anti-MMP-9 antibody (Oncogene Science, Cambridge, MA). MMP-9 bands were visualized using an alkaline phosphatase-conjugated secondary antibody (Jackson ImmunoResearch) followed by the addition of 5-bromo-4-chloro-3-indoyl phosphate and nitro blue tetrazolium. The blot membranes were then scanned, and the signal intensities were measured by integrated morphometry analysis (IMA) (Metamorph System, version 4.6r8, Universal Imaging Corporation, Inc., West Chester, PA). The signal intensities obtained were expressed as integrated optical density (the sum of the optical densities of all pixels that make up the object). All the bands used the same exclusive threshold for analysis.

Immunocytochemistry and Immunohistochemistry—Cells were fixed in 50% methanol, 50% acetone for 15 min and permeated with 1% Triton X-100 in Tris-buffered saline for 15 min. Formalin-fixed paraffin-embedded human prostate cancer tissues were sectioned to 4 μm thickness and fixed on slides. The sections were dewaxed with xylene and rehydrated in 100 and 95% ethanol. Nonspecific antibody binding in cells and sections was blocked with blocking buffer (0.2% Triton X-100, 5% normal goat serum, and 3% bovine serum albumin in Tris-buffered saline) for 1 h at room temperature prior to overnight incubation with affinity-purified specific rabbit anti-human MMP-26 antibody in the same buffer (5 μg/ml for immunocytochemistry and 10 μg/ml for inmmunohistochemistry) or goat anti-human MMP-9 antibody (25 μg/ml for immunohistochemistry, R&D Systems, Minneapolis, MN) at 4 °C. Cells and sections were incubated with alkaline phosphatase-conjugated secondary antibody (Jackson ImmunoResearch) diluted (1:5000) in the blocking buffer for 4 h at room temperature. The signals were detected by adding Fast-Red (Sigma). Purified preimmune IgGs from the same animal were used as negative controls for MMP-26. Normal goat serum was used as a negative control for MMP-9. The sections were counterstained lightly with hematoxylin for viewing negatively stained cells.

Preparation of MMP-26 Constructs—Full-length cDNA of *MMP-26* was amplified by PCR according to published sequences (6) and cloned into modified mammalian expression vector pCR™3.1-Uni with a FLAG tag at its C-terminal as described (22). Following confirmation of cDNA sequencing, plasmids containing correct inserts were used as sense vectors and plasmids with reversibly inserted cDNA were used as antisense vectors (22).

Transfections of ARCaP Cells and Isolation of MMP-26 Sense and Antisense Construct Stably Transfected Clones—ARCaP cells were

transfected with sense and antisense *MMP-26* cDNA-containing vectors using LipofectAMINE 2000 (Invitrogen) as described earlier (22, 23). Sense- and antisense-transfected cell lines were treated identically with regard to transfection conditions and maintenance in the selection medium. Stable transfectants were selected by growing the cells in 400 μg/ml Geneticin (G418; Invitrogen). Cells that survived were then expanded in the absence of G418 for additional studies. Stable transfectants were screened on the basis of FLAG and MMP-26 expression. Clones with *MMP-26* sense- and antisense-integrated constructs were selected and analyzed for MMP-26 expression, invasive capabilities in modified Boyden chamber invasion assays, and co-localization with MMP-9. Parental ARCaP cells served as controls.

Cell Invasion Assay—The invasiveness of ARCaP cells cultured in the presence of MMP-26 or MMP-9 functional blocking antibodies, parental ARCaP cells, sense *MMP-26*- and antisense *MMP-26*-transfected cells through reconstructed ECM was determined as per our previous report (24). The final concentrations of MMP-26 antibody were 10 and 50 μg/ml. The preimmune IgG from the same animal was used as control for MMP-26 antibody, and the final concentration was 50 μg/ml. The mouse anti-human MMP-9 monoclonal antibody is Ab-1, clone 6-6B, which is a functional neutralizing antibody that inhibits the enzymatic activity of MMP-9 (25) (Oncogene Research Products, CalBiochem, La Jolla, CA). The final concentrations of MMP-9 monoclonal antibody were 10 and 25 μg/ml. The preimmune mouse IgG (Alpha Diagnostic Intl. Inc., San Antonio, TX) was used as control, and the concentration was 25 μg/ml. Briefly, modified Boyden chambers containing polycarbonate filters with 8-μm pores (Becton Dickinson, Boston, MA) were coated with 0.5 mg/ml human plasma FN (Invitrogen) or 0.5 mg/ml type IV collagen (Sigma). Three-hundred μl of prepared cell suspension (1×10^6 cells/ml) in serum-free medium was added to each insert, and 500 μl of media containing 10% fetal bovine serum was added to the lower chamber. After 60 h of incubation, invasive cells that had passed through the filters to the lower surface of the membrane were fixed in 4% paraformaldehyde (Sigma). The cells were then stained with 0.1% crystal violet solution and photographed with an Olympus DP10 digital camera (Melville, NY) under a Nikon FX microscope (Melville, NY). The cells were then counted by IMA. For statistical analyses, the number of invasive cells treated with preimmune IgG was assumed to reflect 100% cell invasion. The ratio of the number of invaded cells that were treated with antibody or the *MMP-26* gene-transfected cells to preimmune IgG or parental cells, respectively, was used for subsequent comparative analyses by analysis of variance (ANOVA). Media from each insert was collected for Western blot and gelatin zymogram analyses.

Immunofluorescence and Confocal Laser Scanning Microscopy—Cells were cultured on 8-well slides for 24 h, then fixed in fresh 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.2% Triton X-100 in 10% normal goat serum in phosphate-buffered saline. The fixed, permeabilized cells were stained for 1 h at room temperature with anti-human MMP-26 (25 μg/ml) or a goat anti-human antibody targeting MMP-9 (R&D Systems, Minneapolis, MN) (1:200 dilution). Secondary rhodamine red-X-conjugated mouse anti-rabbit IgG for MMP-26 or fluorescein-conjugated donkey anti-goat IgG (Jackson ImmunoResearch) for MMP-9 were subsequently applied at a 1:200 dilution for 1 h at room temperature. Slow Fade mounting medium was added to the slides, and fluorescence was analyzed using a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss, Germany) equipped with a multiphoton laser according to our previous report (23). Images were processed for reproduction using Photoshop software version 6.0 (Adobe Systems, Mountainview, CA). Purified preimmune IgGs from the same animal were used as negative controls for MMP-26, and normal goat serum was used as a negative control for MMP-9.

Densitometric and Statistical Analysis—Samples were simultaneously stained with antibody and preimmune IgG on the same slide, and the areas of MMP-26 immunostaining were quantified by IMA. Four photographs were taken from each sample with an Olympus DP10 digital camera under a Nikon FX microscope. An appropriate color threshold was determined (color model, HSI; hue, 230–255; saturation and intensity, full spectrum), the glandular epithelia from each image was isolated into closed regions, and all areas of staining in compliance with these specific parameters were measured by IMA. The total area of these closed regions was determined by region measurement, and the ratio of signal area to total area was then determined. The average of the four ratios obtained from each sample was then used for subsequent analysis. The same color threshold was maintained for all samples. The preimmune staining ratio was subtracted from the antibody-staining ratio, and this value was then divided by the preimmune staining ratio to yield the reduced signal to background ratios used for subsequent comparative analyses by ANOVA. Statistical analysis of all samples was performed with the least significant difference correction of ANOVA for multiple comparisons. Data represent the mean ± S.D. from three experiments where differences with $p < 0.05$ were considered to be significant.

RESULTS

Activation of Pro-MMP-9 by MMP-26 and Cleavage of Substrates by Activated MMP-9—Gelatin zymography was utilized for determination of MMP-9 activity levels following cleavage by MMP-26. Zymography revealed that pro-MMP-9 presented as 225-, 125-, and 94-kDa gelatinolytic bands under non-reducing conditions (Fig. 1, *A*, *lane 1*, and *B*, *lanes 1* and *6*). The 225-kDa band is a homodimer of pro-MMP-9, the 125-kDa band is a heterodimer of pro-MMP-9 and neutrophil gelatinase-associated lipocalin, and the 94-kDa band is a monomer of pro-MMP-9 (17, 26, 27). New 215-, 115-, and 86-kDa bands were generated after incubation with MMP-26 (Fig. 1, *A* and *B*), and their activities were increased in a dose- and time-dependent manner (Fig. 1, *A* and *B*). Compared with MMP-7, the cleavage products generated by MMP-26 at the concentrations tested appear more stable (Fig. 1, *A* and *B*). However, pro-MMP-2 was not activated after incubation with identical concentrations of MMP-26 (data not shown).

MMP-26 cleaved pro-MMP-9 (94 kDa) to yield a new 86-kDa band on a silver-stained gel under reducing conditions (Fig. 1*C*, *lane 4*). N-terminal sequencing showed that the 86-kDa protein had the sequence of MRTPR*X*G, which is the same N terminus as reported during activation of pro-MMP-9 by $HgCl_2$ (28), human fibroblast-type collagenase (MMP-1) (17), phenylmercuric acid (29), and aminophenylmercuric acid (30). For further confirmation of MMP-9 activity, digestive assays were performed utilizing FN as a substrate. MMP-26 alone demonstrated weak cleavage of FN (Fig. 1*C*, *lane 6*), whereas pro-MMP-9 exhibited no cleavage of FN (Fig. 1*C*, *lane 7*). Once activated by MMP-26, MMP-9 cleaved FN very effectively, generating at least 6 new bands (Fig. 1*C*, *lane 8*).

Expression of MMP-26 in Human Prostate Gland and ARCaP Cells—Immunohistochemistry staining revealed that the intensity of MMP-26 staining was the highest in human prostate carcinoma (15 patient cases, Gleason grades 5–7), was low in prostatitis (9 cases), and was very low in benign prostate hyperplasia (BPH) (12 cases) and normal prostate gland tissues (7 cases) (Fig. 2*A*). Densitometric and statistical analysis (Fig. 2*B*) showed that the intensities of the immunostaining signals were significantly different between normal prostate gland and prostate cancer samples ($p = 0.0007$), between BPH and prostate cancer ($p = 0.0025$), and also between prostatitis and prostate cancer ($p = 0.0043$). However, there were no significant differences between normal and BPH ($p > 0.05$), normal and prostatitis ($p > 0.05$), or BPH and prostatitis tissues ($p > 0.05$) (Fig. 2*B*).

For selection of a prostate cancer cell line that expressed *MMP-26* for use as a working model, reverse transcriptase-PCR and Western blot analyses were used to detect MMP-26 expression in four human prostate cancer cell lines. *MMP-26* mRNA was identified in the ARCaP, DU145, and LNCaP cell lines, but not in the PC-3 cell line (Fig. 3*A*). Whereas the 20-kDa form of MMP-26 was detected in the ARCaP detergent phase, a doublet between 30 and 40 kDa of pro-MMP-26 was located in the ARCaP aqueous phase (Fig. 3*B*). This doublet might be two *N*-glycosylated forms of pro-MMP-26 predicted according to the ScanProsite program, with two possible *N*-glycosylation sites at $N^{64}GTD^{67}$ and $N^{221}QSS^{224}$. MMP-26 may have *N*-linked sugars according to the results obtained from *N*-glycosidase F (PNGase F, Roche Molecular Biochemicals) digestion experiments (data not shown). MMP-26 protein was

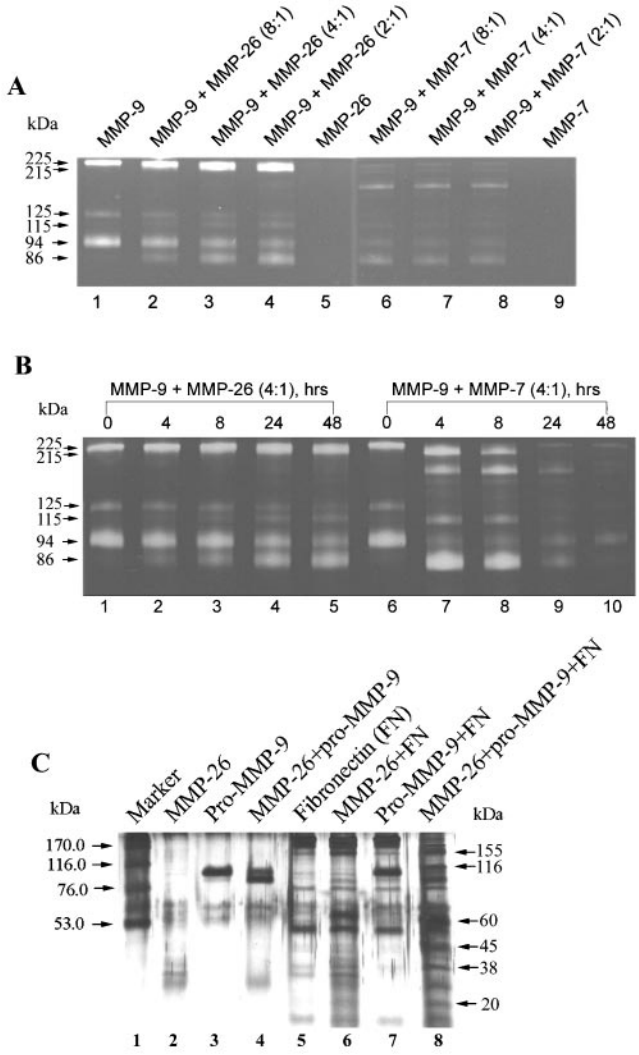

FIG. 1. **Activation of pro-MMP-9 by MMP-26.** *A* and *B*, gelatin zymogram of MMP-9 activity before and after activation of pro-MMP-9 by MMP-26 under non-reducing conditions. The 225-kDa band is a homodimer of pro-MMP-9, the 125-kDa band is a heterodimer of pro-MMP-9 and neutrophil gelatinase-associated lipocalin, and the 94-kDa band is a monomer of pro-MMP-9 (17, 26, 27). The activation reactions were incubated at 37 °C. *A*, dose-dependent analysis of pro-MMP-9 activation by MMP-26 (*lanes 1–4*) and MMP-7 (*lanes 6–8*). The activation reaction was incubated 37 °C for 24 h. The gelatin zymogram reaction was incubated at 37 °C for 3 h. The 86-kDa band was sequenced and the sequence is MRTPRXG, which is a product cleaved at the Ala^{93}–Met^{94} site. *B*, time-dependent analysis of pro-MMP-9 activation by MMP-26 (*lanes 1–5*) and MMP-7 (*lanes 6–10*). The gelatin zymogram reaction was incubated at 37 °C for 3 h. The ratio labeled in *A* and *B* is the molar concentration ratio. *C*, pro-MMP-9 activated by MMP-26 and cleavage of FN by MMP-26 and MMP-9 as detected by a silver-stained gel under reducing conditions. The molar concentration ratio for pro-MMP-9:MMP-26 is 4:1 and the reaction was incubated at 37 °C for 24 h. The molecular mass standards are labeled on the *left* and the estimated molecular masses of the FN cleavage products are labeled on the *right*.

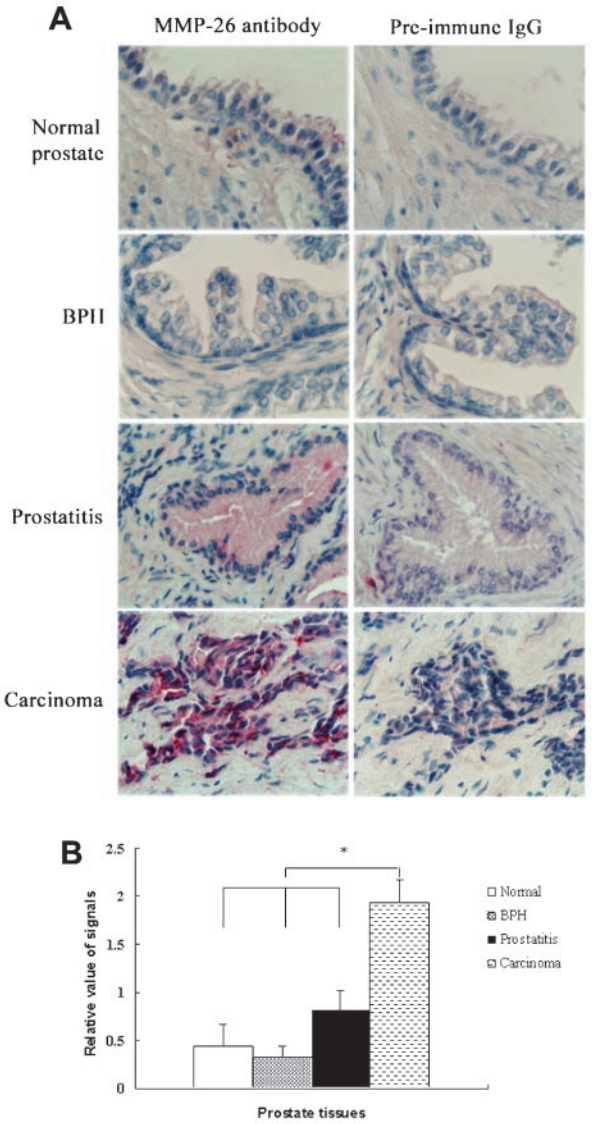

FIG. 2. **Comparison of MMP-26 expression in human normal and pathological prostate tissues.** *A*, immunohistochemistry and localization of MMP-26 in human prostate carcinoma (15 patient cases), prostatitis (9 cases), benign prostate hyperplasia (12 cases), and normal prostate gland tissues (7 cases). Cells stained *red* indicate MMP-26 expression. Photographs were taken under a microscope with ×400 magnification. *B*, densitometric analysis of MMP-26 expression in human prostate tissues. The quantification analysis was described under "Materials and Methods." Four pictures were taken from each sample with ×200 magnification. The epithelial regions were selected and the staining area and total selected area were obtained by IMA and analyzed by one-way ANOVA with LSD correction. Data shown are the mean ± S.D. values from the different prostate tissues. *, $p < 0.01$. *BPH*, benign prostate hyperplasia; *Normal*, normal prostate tissue; *Carcinoma*, prostate adenocarcinoma.

not detected in the DU145, LNCaP, or PC-3 cell lines (Fig. 3*B*), or in the ARCaP media under these experimental conditions (data not shown). Immunocytochemistry data confirmed that MMP-26 was localized inside the ARCaP cells (Fig. 3*C*) in a polarized manner.

Inhibitory Effects of Anti-MMP-26 and Anti-MMP-9 Antibodies on the Invasiveness of ARCaP Cells—To determine the role of MMP-26 and MMP-9 in ARCaP cell invasiveness, antibodies targeting the metalloproteinase domain of MMP-26 and targeting MMP-9 were utilized during *in vitro* cell invasion assays. We found significant ($p < 0.01$) reduction in the invasive potential of ARCaP cells through FN at concentrations of 10 (62.4%) and 50 μg/ml (46.0%) for the MMP-26 antibody (Fig. 4*A*), and at concentrations of 10 (55.9%) and 25 μg/ml (53.1%) for the MMP-9 antibody (Fig. 4*B*), when compared with the preimmune IgGs. We also found significantly ($p < 0.01$) reduced invasive potential in the movement of ARCaP cells through type IV collagen at concentrations of 10 (29.3%) and 50 μg/ml (18.8%) for the MMP-26 antibody (Fig. 4*A*), and at concentrations of 10 (52.2%) and 25 μg/ml (28.0%) for the MMP-9 antibody (Fig. 4*B*), when compared with the preimmune IgG. Antibody targeting the pro-domain of MMP-26 also significantly decreased the invasive potential of ARCaP cells through

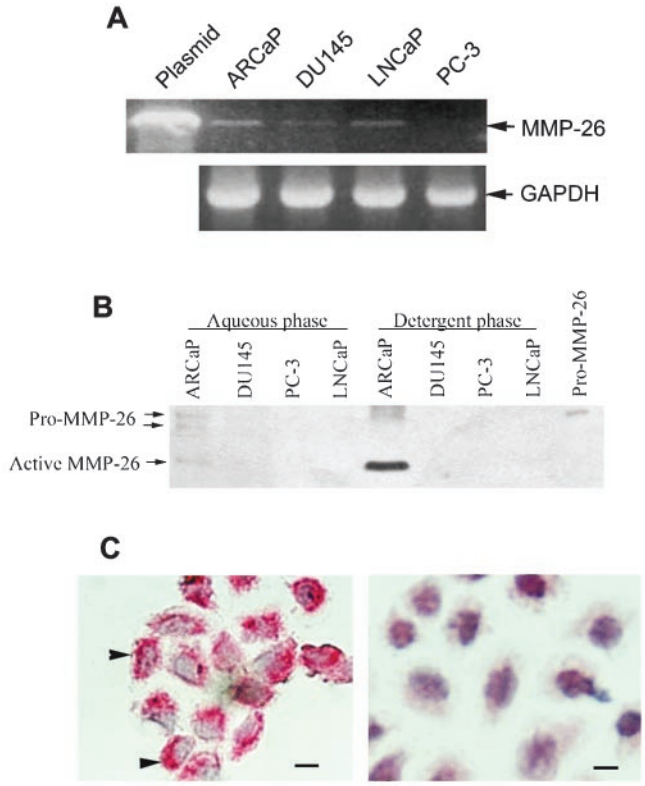

FIG. 3. ***MMP-26* mRNA and protein expression in ARCaP cells.** *A*, reverse transcriptase-PCR analysis of *MMP-26* mRNA in ARCaP, DU145, LNCaP, and PC-3 cell lines. MMP-26 plasmid is used as control (*top panel*, *lane 1*). The mRNA levels of a glycolysis pathway enzyme, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), are shown in the *bottom panel* as a positive control to normalize cellular mRNA concentration. *B*, Western blot analysis of MMP-26 protein in ARCaP, DU145, LNCaP, and PC-3 cell lines. The *last lane* is recombinant pro-MMP-26 as a control. *C*, immunocytochemistry localization of MMP-26 in ARCaP cells. *Left panel*, the primary antibody is rabbit anti-MMP-26 antibody; *right panel*, the primary antibody is preimmune IgG from the same rabbit. *Red* staining indicates MMP-26 expression. *Scale bars* = 12 μm. *Arrows* show the positive staining signals. The cells were counterstained with hematoxylin for viewing of negatively stained cells (*purple*).

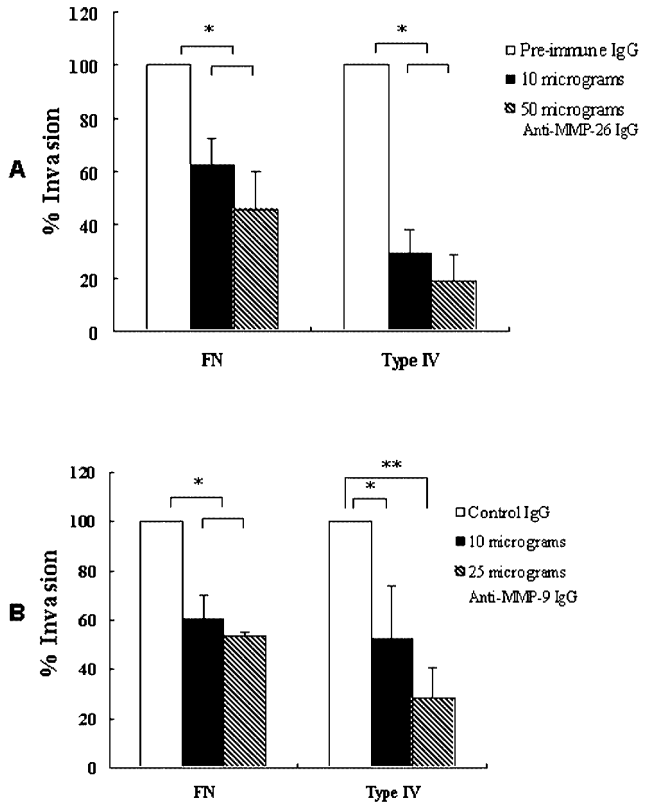

FIG. 4. **Blocking of ARCaP cell invasion through FN and type IV collagen by MMP-26 and MMP-9 antibodies.** The invasion assay was performed with modified Boyden chambers. The MMP-26 antibody is a rabbit anti-human MMP-26 metallo-domain antibody. The MMP-9 antibody is a mouse anti-human MMP-9 monoclonal antibody. The percentage of invading cells was quantified as described under "Materials and Methods." *A*, comparison of the invaded cell number in the presence of MMP-26 antibody/preimmune IgG. *Control*, preimmune rabbit IgG and the final concentration is 50 μg/ml. Ten and 50 μg of IgG means the final concentrations are 10 and 50 μg/ml, respectively. *B*, comparison of the invaded cell number in the presence of MMP-9 antibody/preimmune IgG. *Control*, preimmune mouse IgG and the concentration is 25 μg/ml. Ten and 25 μg of IgG means that the concentrations are 10 and 25 μg/ml, respectively. The invaded cell numbers of the preimmune IgG treatment were used as the 100% invasiveness. *Type IV*, type IV collagen. Data shown are the mean ± S.D. values from four separate experiments for each group. *, $p < 0.01$; **, $p < 0.001$.

FN and type IV collagen (data not shown). These results show that both anti-MMP-26 and anti-MMP-9 antibodies significantly inhibit ARCaP cell invasion through FN and type IV collagen.

MMP-26 Protein Expression in Stable Transfectants by Immunocytochemistry and Western Blotting—To further confirm the role of MMP-26 in ARCaP cell invasion, we transfected pCR 3.1 vectors containing full-length *MMP-26* cDNA in both sense and antisense orientations into ARCaP cells. Immunocytochemistry and Western blotting were performed to determine MMP-26 protein expression levels in the parental cells in addition to the sense and antisense *MMP-26* construct-transfected cells. Immunocytochemistry showed very strong MMP-26 staining in both the parental ARCaP and sense *MMP-26* construct-transfected cells, whereas the antisense *MMP-26* construct-transfected cells exhibited only minimal staining for MMP-26 (Fig. 5*A*). Western blotting revealed strong MMP-26 bands in the parental ARCaP and sense *MMP-26* construct-transfected cells, whereas only a very faint band was detected in the antisense *MMP-26* construct-transfected cells. No MMP-26 was detected in the cell culture media (Fig. 5*B*).

Reduction of Invasiveness of MMP-26 Antisense Stable Transfectants—Both the parental ARCaP and sense *MMP-26* construct-transfected cell lines invaded through either FN or type IV collagen *in vitro* during cell invasion assays (Fig. 6*A*), but without a marked difference ($p > 0.05$) in their invasive potentials (Fig. 6*B*). Antisense *MMP-26* construct-transfected cells showed a significant ($p < 0.01$) decrease in invasive potential through the same materials (44.0 and 23.5%, respectively) when compared with parental ARCaP cells (Fig. 6, *A* and *B*). A significant ($p < 0.01$) difference between the sense and antisense *MMP-26* construct-transfected cells was also noted (Fig. 6, *A* and *B*).

Reduced Levels of Active MMP-9 in MMP-26 Antisense Stably Transfected Cells—To determine the role of MMP-26-mediated MMP-9 activation in ARCaP cell invasion, the level of MMP-9 in conditioned media samples collected from the Boyden chambers during *in vitro* cell invasion assays was detected. Western blotting revealed a strong 86-kDa band of active MMP-9 in the conditioned media from parental ARCaP and sense *MMP-26* construct-transfected cells. A similar band, but of weaker intensity, was detected in the conditioned media collected from the antisense *MMP-26* construct-transfected cells (Fig. 7*A*). Semiquantitative analysis revealed that the active form of MMP-9 was significantly decreased ($p < 0.01$) in both the FN and type IV collagen invasive assay media from the antisense *MMP-26* construct-transfected cells (Fig. 7*B*).

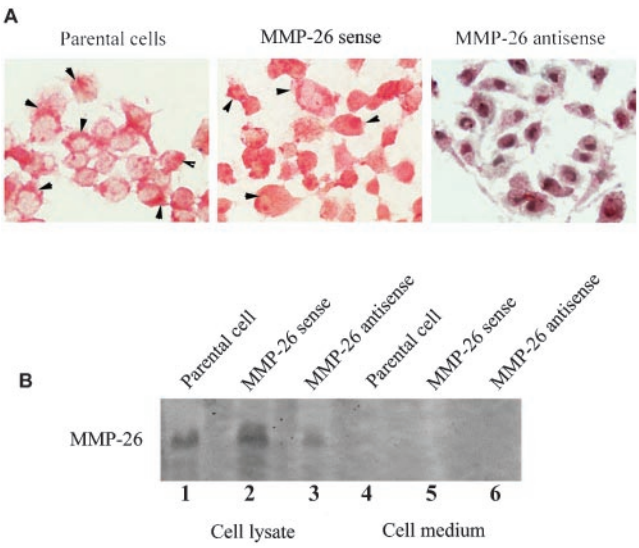

FIG. 5. **MMP-26 protein expression in parental ARCaP, sense *MMP-26* construct, and antisense *MMP-26* construct stably transfected cells.** *A*, immunocytochemistry of MMP-26 expression in parental ARCaP, sense *MMP-26* construct, and antisense *MMP-26* construct stably transfected cells. *Red* staining indicates MMP-26 expression. *Arrows* show examples of the positive staining signals. The cells were counterstained with hematoxylin for viewing of MMP-26 negative cells (*purple*). *B*, Western blot analysis of MMP-26 protein expression. Parental ARCaP, sense *MMP-26* construct, and antisense *MMP-26* construct stably transfected cells were cultured utilizing an equivalent number of cells. Conditioned medium samples were collected prior to cell lysis.

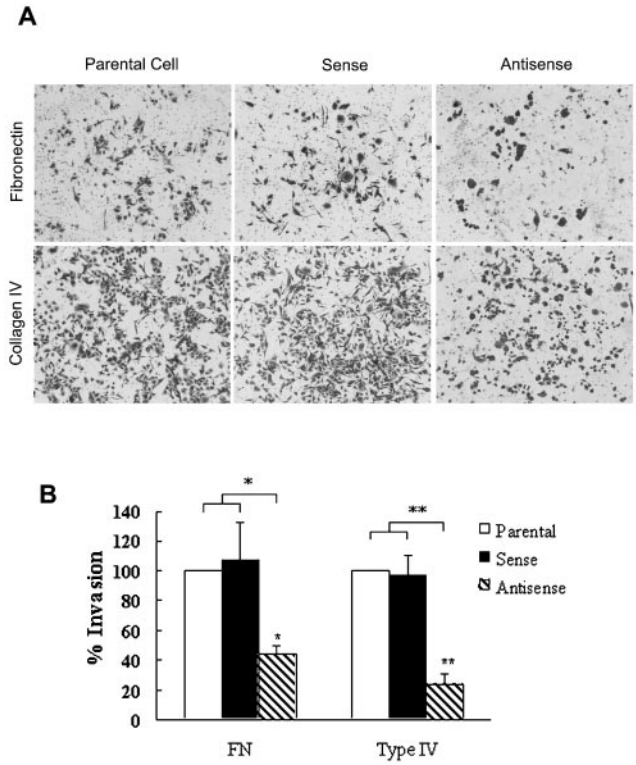

FIG. 6. **Invasion of parental ARCaP, sense, or antisense *MMP-26* construct stably transfected cells through FN and type IV collagen.** *A*, cells that invaded to the lower surface of the membrane were photographed under a microscope with ×40 magnification. *B*, the percentage of invading cells in parental and *MMP-26* sense or antisense construct stably transfected ARCaP cells. The cell numbers of the invaded parental cells were used as 100% invasiveness. The cells were counted and analyzed as described under "Materials and Methods." Data shown are the mean ± S.D. values from three separate experiments for each group. *, $p < 0.01$; **, $p < 0.001$.

Co-localization of MMP-26 with MMP-9 in Parental and MMP-26 Sense Gene Stably Transfected ARCaP Cells, and Co-expression of MMP-26 and MMP-9 in Human Prostate Carcinoma Tissue Samples—Double immunofluorescence experiments were performed in parental ARCaP and *MMP-26* stably transfected cells with human *MMP-26* sense or antisense genes. The red color indicates MMP-26 and the green color indicates MMP-9 protein staining. Merged images show a color shift to orange-yellow, indicating co-localization between MMP-26 and MMP-9. Confocal laser scanning microscopic analysis revealed co-localization of both proteins in the cytoplasm of parental ARCaP (Fig. 8*A*, *a–d*) and sense-transfected cells (Fig. 8*A*, *e–h*), but not in the antisense-transfected cells (Fig. 8*A*, *i–l*). Very weak signals were detected in parental ARCaP control cells using purified preimmune IgG for the detection of MMP-26 and nonimmune goat sera for the detection of MMP-9 (Fig. 8*A*, *m–p*). MMP-26 and MMP-9 proteins were also found to be co-expressed in human prostate carcinoma tissue samples (Fig. 8*B*).

DISCUSSION

MMP-26 is able to activate MMP-9 by cleavage at the Ala^{93}–Met^{94} site of the prepro-MMP-9, which is the same cleavage site detected previously during activation with $HgCl_2$ (28), human fibroblast-type collagenase (17), phenylmercuric acid (29), and aminophenylmercuric acid (30). This activation was confirmed by the effective cleavage of FN using MMP-9 activated by MMP-26. These results indicate that the zymogen form of MMP-9 can be transiently activated without the proteolytic loss of the cysteine (Cys^{99})-switch residue, even though these findings may appear to be in conflict with the original Cys-switch hypothesis (31). The 86-kDa form of MMP-9 may also be further activated to produce lower molecular mass active species similar to the process activated by other MMPs (17). Among all the MMPs, matrilysin (MMP-7) and MMP-26 share domain structures with pro- and metalloproteinase domains only and are both expressed in epithelial cells (6–9). Therefore, MMP-26 is also named as matrilysin-2 (8). Both MMP-26 and MMP-7 could activate MMP-9 but their cleavage sites in pro-MMP-9 are different. Matrilysin cleaved MMP-9 at two sites, Glu^{59}–Met^{60} and Arg^{106}–Phe^{107} of the prepro-MMP-9 (17). Our current results also demonstrated that the MMP-9 activation mediated by MMP-26 is much slower than that mediated by MMP-7, but the activation products are much more stable when compared with the products of activation by MMP-7. This indicates that activation of MMP-9 by MMP-26 is prolonged but persistent, which is consistent with the process of tumor cell invasion. MMP-26 did not cleave pro-MMP-2, another gelatinase, indicating that pro-MMP-9 activation by MMP-26 is highly selective. MMP-9 is a powerful enzyme, and is considered to be an important contributor to the processes of invasion, metastasis, and angiogenesis in various tumors (11–14, 32–36).

This work has tested the hypothesis that MMP-26 may enhance human prostate cancer cell invasion via the activation of pro-MMP-9 using an ARCaP cell line as a working model. The ARCaP cell line is a highly invasive and metastatic human prostate cancer cell line that expresses both MMP-9 (15) and MMP-26. We found that *MMP-26* mRNA was detected in the ARCaP cell line and two other human prostate carcinoma cell lines, DU145 and LNCaP, but the MMP-26 protein was only detected in ARCaP cells. More importantly, high levels of MMP-26 protein were also detected in human prostate carcinoma cells by immunohistochemistry, but only low expression was seen in prostatitis, benign prostate hyperplasia, and normal prostate tissues. This is in agreement with reports of

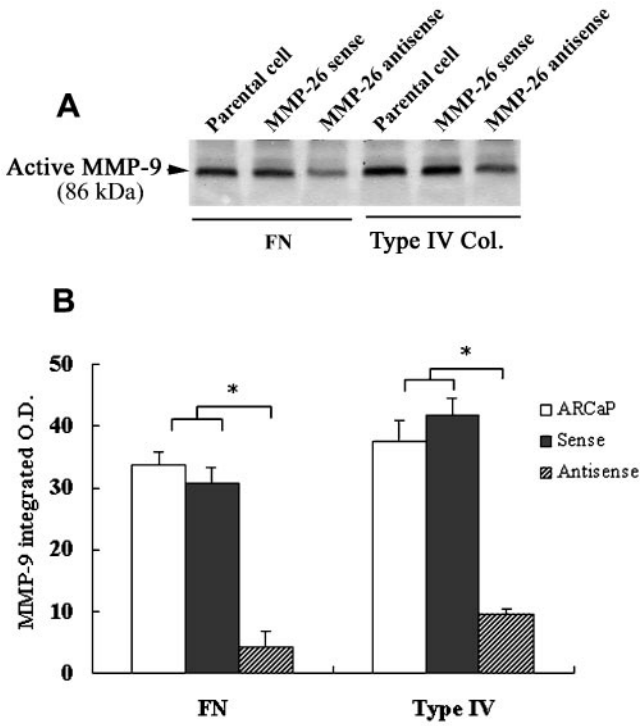

FIG. 7. **Detection of MMP-9 in the invasive assay media from parental ARCaP, sense *MMP-26* construct, or antisense *MMP-26* construct stably transfected cells.** *A*, Western blot of the invasive assay media. Media samples were collected from the upper compartments of Boyden chambers during cell invasion assays. *B*, densitometry scanning and semiquantitative analysis of the levels of MMP-9 in the invasive assay media. Data shown are the mean ± S.D. values from four separate experiments for each group. *, $p < 0.001$.

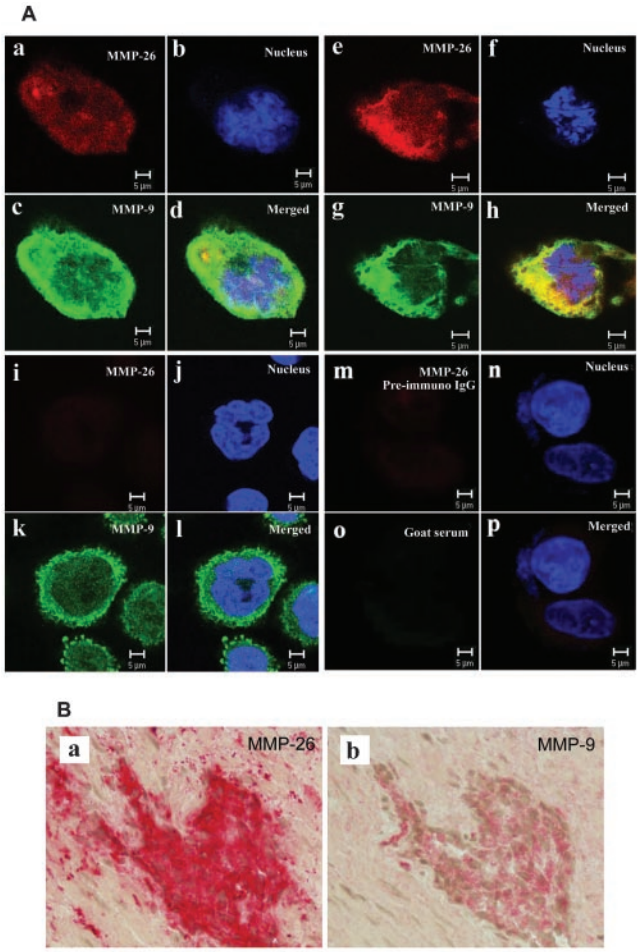

FIG. 8. **Co-localization of MMP-26 and MMP-9 in parental and sense *MMP-26*-transfected ARCaP cells and an example of MMP-26 and MMP-9 co-expression in the same human prostate carcinoma tissues.** *A*, double immunofluorescence staining and confocal laser scanning micrographs of parental, sense, and antisense *MMP-26*-transfected ARCaP cells. *Red* indicates MMP-26 signals and *green* indicates MMP-9 signals. *Yellow* reveals the co-localization of MMP-26 and MMP-9. *Blue* fluorescence represents the nuclei. *a–d*, parental ARCaP cells. *e–h*, sense *MMP-26* construct-transfected cells. *i–l*, antisense *MMP-26* construct-transfected cells. *m–p*, parental ARCaP cells with preimmune IgG and goat sera as controls. *Scale bars* = 5 μm. *B*, an example of co-expression of MMP-26 and MMP-9 proteins in the same human prostate carcinoma tissues. *a*, strong positive MMP-26 protein staining in epithelial cells of human prostate carcinoma (Gleason grade 3 + 3). *b*, positive MMP-9 protein staining in epithelial cells of the same human prostate carcinoma. Cells stained *red* indicate MMP-26 and MMP-9 expression. The sections were counterstained with hematoxylin for viewing negatively stained cells. Photographs were taken under a microscope with ×400 magnification.

MMP-26 gene expression in epithelial cancers (6–9). We have previously reported that the levels of *MMP-26* gene and protein expression are increased in a malignant choriocarcinoma cell line (JEG-3) to levels that are well in excess of that found in normal human cytotrophoblast cells (10). The majority of MMP-26 protein detected was in the detergent phase of the ARCaP cell lysates, not in the conditioned media, and only low levels were observed in the aqueous phase. This is in accordance with recent studies demonstrating that *MMP-26*-transfected COS-7 and HEK293 cells secrete the protein poorly (7–9). As MMP-26 was found in the detergent phase of the ARCaP cell lysates, it is possible that MMP-26 may be associated with cell membrane components via an unidentified mechanism. Membrane-associated MMP-26 may participate directly in degradation of the ECM, activating pro-enzymes, and releasing growth factors, partially accounting for the inhibition of ARCaP cell invasion by the MMP-26 antibody tested. These reports converge to suggest that MMP-26 may play an important role in human carcinoma invasion and tumor progression.

MMP-26 exhibits wide substrate specificity, and is capable of degrading many components of the basement membrane and other ECM components (6–9, 37). Although MMP-26 can cleave type IV collagen, fibronectin, and other proteins, it is a catalytically less powerful enzyme than gelatinase B/MMP-9. The inhibition of ARCaP cell invasion by MMP-26-specific antibodies suggests that MMP-26 may contribute to ARCaP cell invasion by cleaving ECM components directly and/or by activating pro-MMP-9 to cleave the ECM. Our FN cleavage assays with MMP-26 alone and MMP-26-activated MMP-9 show that once activated by MMP-26, MMP-9 cleaves FN more efficiently. This indicates that the activation of MMP-9 may be a major pathway for MMP-26 promotion of ARCaP cell invasion. Indeed, this hypothesis was further verified by ARCaP cell invasion inhibition in the presence of MMP-9 functional blocking antibodies. When the proteolytic activity of MMP-26 is combined with that of activated MMP-9, which digests ECM and basement membrane proteins in an even more aggressive fashion than MMP-26 alone, this hints at an amplification mechanism by which MMP-26 might contribute significantly to the processes of tumor cell invasion and subsequent metastasis.

Several lines of evidence have demonstrated that biochemical activation of pro-MMP-9 by MMP-26 may be a physiologically and pathologically relevant event. Our results demonstrated that antibodies directed against MMP-26 catalytic domain and prodomain both blocked the ARCaP cell invasion. Equally as significant, a function blocking monoclonal antibody that inhibits MMP-9 catalytic activity (25, 38) also prevented the invasion of ARCaP cells in patterns similar to a MMP-26 antibody. These results verify our hypothesis that activation of

pro-MMP-9 by MMP-26 promotes invasion of human prostate cancer cells.

Recently, our group has also determined that MMP-26 autodigested itself during the folding process. Two of the major autolytic sites were Leu^{49}–Thr^{50} and Ala^{75}–Leu^{76}, which left the "cysteine-switch" sequence ($PHC^{82}GVPD$) intact (37), and suggests that Cys^{82} may not play a role in the latency of the zymogen form. Another group has demonstrated that autolytic activation of MMP-26 occurred at $LLQ^{59} \downarrow Q^{60}FH$, which is upstream from the cysteine residue known to be responsible for the latency of many other MMPs (39). Interestingly, our pro-domain antigen peptide mimics the Thr^{50} to Asp^{67} region of MMP-26, and the resultant antibody complex shields the autocleavage sites (data not shown). This fortunate circumstance may account for the decreased invasiveness of ARCaP cells treated with our antibody targeting the pro-domain, while also suggesting that the catalytic activity of MMP-26 may not require the highly conserved cysteine-switch activation mechanism.

To further confirm the role of MMP-26 during ARCaP cell invasion, we generated stably transfected ARCaP cells with vectors containing full-length *MMP-26* cDNA in both the sense and antisense orientations. Our results show that transfection of the ARCaP cells with antisense *MMP-26* constructs leads to decreased levels of MMP-26 protein expression when compared with parental and sense controls, suggesting that this antisense construct is responsible for the observed decrease in MMP-26 protein expression, resulting in profound biological consequences. In cell invasion assays, antisense-transfected cells show a marked reduction in invasiveness over those of parental ARCaP and *MMP-26* sense gene-transfected ARCaP cells, suggesting that the modulation of *MMP-26* in ARCaP cells altered the invasive potential of these cells in our experimental model system, lending support to the hypothesis that MMP-26 activity may play a crucial role in facilitating the invasion of ARCaP cells through the ECM.

Western blotting of conditioned media collected from the upper compartments of the Boyden chambers during invasion assays reveals that the 86-kDa active form of MMP-9 is present in parental ARCaP and sense *MMP-26*-transfected ARCaP cell media, but very little active MMP-9 is present in the antisense *MMP-26*-transfected ARCaP cell media. These findings suggest that MMP-26 activated MMP-9 in parental ARCaP and sense *MMP-26*-transfected ARCaP cells, while very little activation took place in the antisense *MMP-26*-transfected ARCaP cells. When present, active MMP-9 accumulates in the cytosol of human endothelial cells, where it is eventually utilized by invading pseudopodia (40), and it is possible that endogenous, self-activated MMP-26 acts as an activator for intracellular pro-MMP-9. The active form of MMP-9 may then be stored inside the cell, ready for rapid release when it is required to facilitate the invasion of ARCaP cells.

Consistent with the above data, double immunofluorescence labeling and confocal laser scanning microscopy reveal that MMP-26 and MMP-9 were co-localized in parental ARCaP and sense *MMP-26*-transfected ARCaP cells, affording them ample opportunity to interact. Co-localization was not observed in antisense *MMP-26*-transfected ARCaP cells, as MMP-26 was not expressed in these cells. Immunohistochemistry revealed a similar relationship in human prostate tissue samples, demonstrating that MMP-26 and MMP-9 were also co-expressed in prostate carcinomas. Recently, Nemeth *et al.* (36) have reported that both *MMP-9* mRNA and protein were expressed in biopsy specimens from patients with documented, bone-metastatic prostate cancer. Thus, the biochemical activation mechanism of pro-MMP-9 that we observed *in vitro* might well be applicable to prostate cancer *in vivo*.

Although direct degradation of the ECM by MMP-26 may contribute to the processes of cell invasion and tumor metastasis, as the consequential relationship between MMP-26 and MMP-9 begins to emerge, we find evidence of coordination and a proteolytic cascade (activation of MMP-9) that may be a major pathway to promote the invasion of human prostate carcinoma. The specific expression of MMP-26 and its potential role in the invasion of cancer cells suggest that MMP-26 may be a new marker for certain types of prostate carcinomas, and perhaps a new therapeutic molecular target for prostate cancer.

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The Intermediate S_1' Pocket of the Endometase/Matrilysin-2 Active Site Revealed by Enzyme Inhibition Kinetic Studies, Protein Sequence Analyses, and Homology Modeling*

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Human matrix metalloproteinase-26 (MMP-26/endometase/matrilysin-2) is a newly identified MMP and its structure has not been reported. The enzyme active site S_1' pocket in MMPs is a well defined substrate P_1' amino acid residue-binding site with variable depth. To explore MMP-26 active site structure-activity, a series of new potent mercaptosulfide MMP inhibitors (MMPIs) with Leu or homophenylalanine (Homophe) side chains at the P_1' site were selected. The Homephe side chain is designed to probe deep S_1' pocket MMPs. These inhibitors were tested against MMP-26 and several MMPs with known x-ray crystal structures to distinguish shallow, intermediate, and deep S_1' pocket characteristics. MMP-26 has an inhibition profile most similar to those of MMPs with intermediate S_1' pockets. Investigations with hydroxamate MMPIs, including those designed for deep pocket MMPs, also indicated the presence of an intermediate pocket. Protein sequence analysis and homology modeling further verified that MMP-26 has an intermediate S_1' pocket formed by Leu-204, His-208, and Tyr-230. Moreover, residue 233 may influence the depth of an MMP S_1' pocket. The residue at the equivalent position of MMP-26 residue 233 is hydrophilic in intermediate-pocket MMPs (*e.g.* MMP-2, -8, and -9) and hydrophobic in deep-pocket MMPs (*e.g.* MMP-3, -12, and -14). MMP-26 contains a His-233 that renders the S_1' pocket to an intermediate size. This study suggests that MMPIs, protein sequence analyses, and molecular modeling are useful tools to understand structure-activity relationships and provides new insight for rational inhibitor design that may distinguish MMPs with deep *versus* intermediate S_1' pockets.

Matrix metalloproteinases (MMPs,[1] matrixins) are believed to participate in angiogenesis, embryonic development, morphogenesis, reproduction, tissue resorption and remodeling, and tumor growth, progression, invasion, and metastasis through breakdown of the extracellular matrix, cell surface proteins, and processing growth factors, cytokines, and chemokines (1–3). Recently, human MMP-26 (endometase/matrilysin 2) was identified and its mRNA expression was detected in normal tissues of the human uterus and placenta, and in many types of malignant tumors (4–7). Characterization of the MMP-26 promoter suggests that this proteinase may be expressed in cancer cells of epithelial origin (8). MMP-26 may play an important role in human prostate and breast cancer invasion (9–10).

MMP-26 cleaves type I gelatin, α_1-proteinase inhibitor, fibrinogen, fibronectin, vitronectin, type IV collagen, and insulin-like growth factor binding protein-1 (4, 7, 11). Studies of MMP-26 indicate that it has substrate specificity similar to other MMPs, with the exception of a preference for Ile at the P_2 and P_2' positions, for small residues at the P_3' and P_4' positions, and Lys at the P_4 position (11). MMP-26 also hydrolyzes several synthetic fluorogenic peptide substrates designed for stromelysin-1, gelatinases, collagenases, and tumor necrosis factor-α converting enzyme (4, 11). According to these peptide substrate studies, MMP-26 may be capable of cleaving a broad range of substrates, although it has less catalytic efficiency than other MMPs.

X-ray crystal structures of MMPs illustrate that overall topology and secondary structures are conserved (12–18). The S_1' pocket, a hydrophobic pocket of variable depth, is a well defined substrate P_1'-binding site in MMPs. Three types of S_1' pockets can be distinguished from the available structures of MMPs (19–20). One type is a shallow pocket, as found in MMP-1 (human fibroblast collagenase; 13) and MMP-7 (matrilysin; 16), where the pockets are limited by the side chains of Arg and Tyr, respectively, crossing the pockets. Many of the structurally known MMPs possess Leu at the corresponding site, and its side chain forms the top of the pocket rather than crossing the pocket. These Leu-containing MMPs may be further classified as deep and intermediate S_1' pocket MMPs. A deep, tunnel-like pocket is found in MMP-3 (stromelysin-1; 12), MMP-12 (metalloelastase; 17), and MMP-14 (MT1-MMP; 21), whereas MMP-2 (gelatinase A; 22), MMP-8 (human neutrophil collagenase; 15), and MMP-9 (gelatinase B; 23) possess an intermedi-

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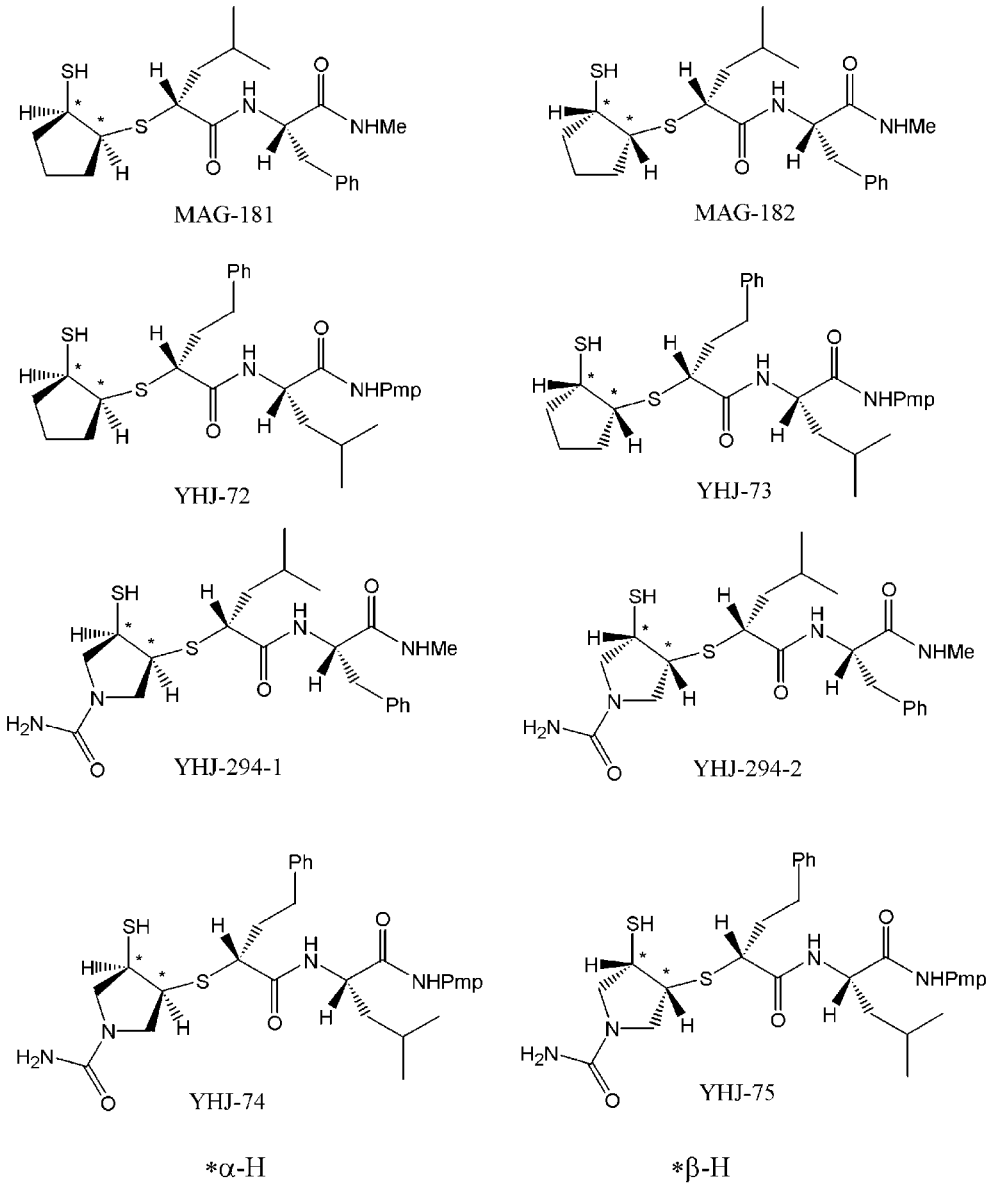
[1] The abbreviations used are: MMP, matrix metalloproteinase; Boc, *tert*-butoxycarbonyl; Brij-35, polyoxyethylene lauryl ether; Homophe, homophenylalanine; Mca, (7-methoxycoumarin-4-yl)acetyl; Dpa, *N*-3-(2,4-dinitrophenyl)-2,3-diaminopropionyl; MMPI, matrix metalloproteinase inhibitor.



Abbreviations: Me, Methyl; Ph, Phenyl; Pmp, *p*-methoxyphenyl.

FIG. 1. **Structures of new mercaptosulfide MMP inhibitors.** The diastereomer notation in the ring system is started from the α carbon of the mercapto group.

TABLE I
Inhibition of human MMPs by mercaptosulfide MMP inhibitors

Inhibitor	K_i^{app}								
	Shallow		Deep			Intermediate			
	MMP-1	MMP-7	MMP-3	MMP-12	MMP-14	MMP-2	MMP-8	MMP-9	MMP-26
	nM			*nM*				*nM*	
MAG-181	680	710	2500	1,300	259	85	4.1	44	81
MAG-182	49	40	470	130	24	1.1	0.89	0.57	17
YHJ-72	$>12 \times 10^3$	5,500	150	130	380	930	530	180	160
YHJ-73	$>12 \times 10^3$	1,000	100	13	16	20	70	8.6	28
YHJ-294–1	5,200	3,500	$>40 \times 10^3$	16,000	3,000	430	130	550	450
YHJ-294–2	100	26	360	93	13	6.1	1.2	1.2	2.8
YHJ-74		3600	270	117	20	88	300	220	82
YHJ-75	2400	300	21	5.6	3.7	6.9	44	3.0	8.6

ate-sized pocket, which is neither deep nor shallow. An enzyme with a shallow pocket prefers large, aliphatic residues in the P_1' position, such as Leu and Met (24–25). The remainder of the MMPs can accommodate larger amino acid derivatives, such as homophenylalanine, in the P_1' position (26).

MMP-26, composed of 261 amino acid residues and lacking a hemopexin-like domain, represents the smallest member of the MMP family. The primary structure of MMP-26 can be divided into three regions that include a signal peptide, a propeptide domain, and a catalytic domain. MMP-26 identification, ex-

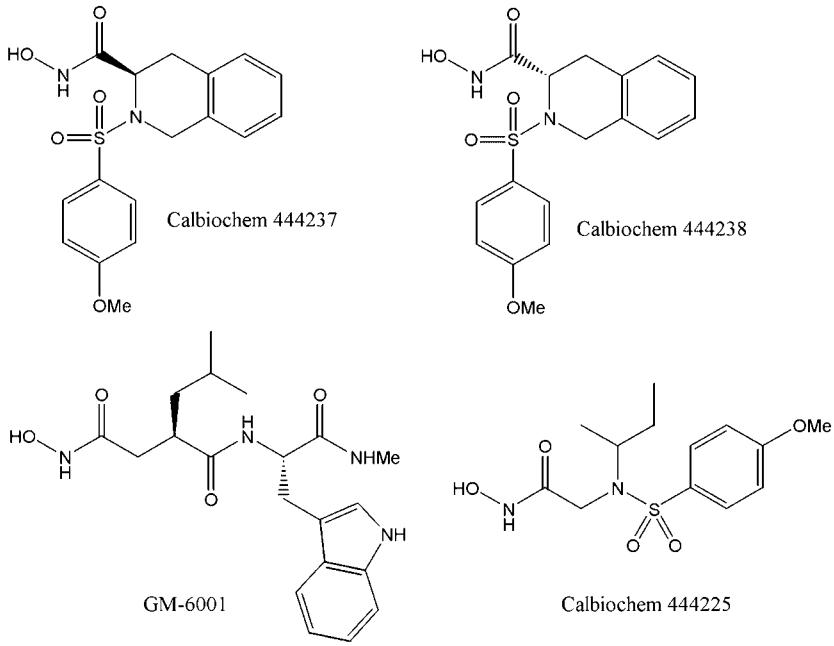


FIG. 2. **Structures of commercially available hydroxamate MMP inhibitors.** Calbiochem 444237, 444238, and 444225 are three known inhibitors of deep S_1' pocket MMPs.

TABLE II
Inhibition of MMP-26, MMP-7, and MMP-12 by hydroxamate MMP inhibitors

Inhibitor	K_i^{app}		
	MMP-26	MMP-7	MMP-12
		nM	
GM6001	0.36[a]	3.7	3.6
444237	1.5[a]	225	0.20
444238	60[a]	5.7×10^3	36
444225	43	2100	3.4

[a] Values from Ref. 11.

Shallow S_1' Pocket MMPs

```
      203                                        235
MMP-1   HRVAAHELGH SLGLSHSTDI GALMYPSYTFSGD
MMP-7   LYAATHELGH SLGMGHSSDP NAVMYPTYGNGDP
```

Deep S_1' Pocket MMPs

```
MMP-3   FLVAAHEIGH SLGLFHSANT EALMYPLYHSLTD
MMP-12  FLTAVHEIGH SLGLGHSSDP KAVMFPTYKYVDI
MMP-14  FLVAVHELGH ALGLEHSSDP SAIMAPFYQWMDT
```

Intermediate S_1' Pocket MMPs

```
MMP-2   FLVAAHEFGH AMGLEHSQDP GALMAPIYTYTKN
MMP-8   FLVAAHEFGH SLGLAHSSDP GALMYPNYAFRET
MMP-9   FLVAAHEFGH ALGLDHSSVP EALMYPMYRFTEG
MMP-26  FLVATHEIGH SLGLQHSGNQ SSIMYPTYWYHDP
```

FIG. 3. **The sequence alignment of eight MMPs.** The alignment was determined using the Genetic Computer Group (Wisconsin Package, version 10, Madison, WI, 2002) program PILEUP with a default gap weight of 8 and a gap length weight of 2 based on the full protein sequences without propeptide regions. *Boldface* amino acid residues form the S_1' pocket. *Italicized* sequences are metal binding consensus sequences. *Underlined* residues may determine S_1' pocket characteristics. To align MMP-2 and MMP-9, the 183-residue insert of fibronectin type II-like modules were deleted before the alignment. The residue numbering system is based on the sequence of MMP-26 (4).

pression, and substrate specificity have been explored by several groups (4–11). However, the S_1' pocket characteristics of MMP-26 are unknown because of the absence of an MMP-26 x-ray crystallographic structure. Therefore, in this study we have utilized previously characterized and newly developed mercaptosulfide MMPIs (27–29) together with protein sequence analyses and molecular modeling to understand the S_1' pocket characteristics of MMP-26.

EXPERIMENTAL PROCEDURES

Materials—The fluorescent peptide substrates for MMPs used in this study were purchased from Bachem Chemical Co. The metal salts and Brij-35 were purchased from Fisher Scientific Inc. The hydroxamate MMPIs 444237, 444238, 444225, and GM6001 were purchased from Calbiochem. All other chemicals were purchased from Sigma.

The mercaptosulfide inhibitors were prepared and characterized as previously described (27–29). *cis*-1-Acetylthio-2-*tert*-butoxycarbonylthiocyclopentane and *cis*-3-acetylthio-4-*tert*-butoxycarbonylthio-*N*-*tert*-butoxycarbonylpyrrolidine were synthesized (29) and *S*-alkylated with (2*S*)-2-bromo-4-methylpentanoic acid or (2*S*)-2-bromo-4-phenylbutanoic acid; the latter bromoacids were derived from L-leucine and L-homophenylalanine, respectively (27). Subsequent coupling with L-PheNHMe or L-leucine-*p*-methoxyanilide (27) afforded the *S*-Boc and *N*-Boc protected inhibitors as mixtures of two diastereomers. The *N*-Boc group was selectively removed and replaced by the other acyl groups (29). The diastereomers were separated by flash chromatography on silica gel or by reverse-phase preparative high performance liquid chromatography on a C18 column. The slower-eluting *S*-Boc protected diastereomer exhibited the more potent MMP inhibition in each case. Its stereochemistry was assigned by ^{1}H NMR NOE analysis (MAG-182), x-ray crystallography (YHJ-294-2) (29), or by analogy. Finally, the *S*-Boc protecting groups were removed by brief treatment with 2 *N* HCl in acetic acid and the mercaptosulfide inhibitors were isolated by lyophilization of the reaction mixture.

MAG-181: m.p. 174–176 °C; $[\alpha]^{25}_D$ + 11.2° (*c* = 0.4, MeOH); analysis (CHNS). *S*-Boc derivative: m.p. 118–119 °C; $[\alpha]^{25}_D$ + 33.5° (*c* = 0.49, MeOH); analysis (CHNS).

MAG-182: m.p. 173–174 °C; $[\alpha]^{25}_D$ + 98.6° (*c* = 0.45, MeOH); analysis (CHNS). *S*-Boc derivative: m.p. 159–160 °C; $[\alpha]^{25}_D$ + 63.4 (*c* = 0.52, MeOH); analysis (CHNS).

YHJ-72: m.p. 136–137 °C; $[\alpha]^{20}_D$ − 67.9° (*c* = 0.14, $CHCl_3$); analysis (CHNS). *S*-Boc derivative: m.p. 94–95 °C; $[\alpha]^{25}_D$ +0.4° (*c* = 0.24, $CHCl_3$); analysis (CHNS).

YHJ-73: m.p. 145–146 °C; $[\alpha]^{20}_D$ − 0.7° (*c* = 0.14, $CHCl_3$); analysis (CHNS). *S*-Boc derivative: m.p. 126–127 °C; $[\alpha]^{25}_D$ − 8.8° (*c* = 0.25, $CHCl_3$); HRMS.

YHJ-294-1: m.p. 98–100 °C; $[\alpha]^{20}_D$ + 54.4° (*c* = 0.50, MeOH); analysis (CHNS). *S*-Boc derivative: m.p. 123–124 °C; $[\alpha]^{20}_D$ + 11.5° (*c* = 0.55, MeOH); analysis (CHNS).

YHJ-294-2: m.p. 128–130 °C; $[\alpha]^{20}_D$ + 38.5° (*c* = 0.40, MeOH); analysis (CHNS). *S*-Boc derivative: m.p. 173–175 °C; $[\alpha]^{20}_D$ + 82.6° (*c* = 0.50, MeOH); analysis (CHNS).

YHJ-74: m.p. 174–175 °C; $[\alpha]^{20}_D$ + 2.4° (*c* = 0.50, $CDCl_3$); HRMS. *S*-Boc derivative: m.p. 112–113 °C; $[\alpha]^{20}_D$ − 42.1° (*c* = 0.24, $CHCl_3$); analysis (CHNS).

YHJ-75: m.p. 105–106 °C; $[\alpha]^{20}_D$ − 35.4° (*c* = 0.24, $CHCl_3$); analysis (CHNS). *S*-Boc derivative: m.p. 171–172 °C; $[\alpha]^{20}_D$ + 17.2° (*c* = 0.25, $CHCl_3$); HRMS.

Enzyme Preparation and Folding of the Denatured Protein—MMP-7/matrilysin, MMP-3/stromelysin-1 (30), and MMP-12/metalloelastase (4) were kindly provided by Dr. Harold E. van Wart (Roche Diagnostics),

Professor L. Jack Windsor (Indiana University), and Dr. C. Bruun Schiødt (OsteoPro A/S), respectively. MMP-1/human fibroblast collagenase, MMP-2/human fibroblast gelatinase, MMP-8/human neutrophil collagenase, and MMP-9/human neutrophil gelatinase were described previously (30, 31). The catalytic domain of MT1-MMP/MMP-14 was provided by Professor Harald Tschesche (Bielefeld University) (32). MMP-26 was prepared as described previously (4, 11). Briefly, MMP-26 was expressed as inclusion bodies from a transformed BL-21 DE3 strain. After bacterial insoluble body preparation with B-Per™ reagent, the isolated insoluble protein was folded by following the procedures previously outlined (4–11). The total MMP-26 concentration was measured by UV absorption and calculated with the molar extinction coefficient $\epsilon_{280} = 57130\ \text{M}^{-1}\ \text{cm}^{-1}$. The active concentration of MMP-26 was determined by titration with GM6001, a tight-binding inhibitor, as described previously (11).

Kinetic Assays and Inhibition of Endometase—The substrate Mca-PLGLDpaAR-NH_2 was used to measure inhibition constants (11, 33). Enzymatic assays were performed at 25 °C in 50 mM HEPES buffer at pH 7.5 in the presence of 10 mM $CaCl_2$, 0.2 M NaCl, and 0.01 or 0.05% Brij-35 with substrate concentrations of 1 μM. The release of product was monitored by measuring fluorescence (excitation and emission wavelengths of 328 and 393 nm, respectively) with a PerkinElmer luminescence spectrophotometer LS 50B connected to a temperature controlled water bath. All stock solutions of inhibitors were in methanol. For inhibition assays, 10 μl of inhibitor stock solution, 176 μl of assay buffer, and 10 μl of enzyme stock solution were mixed and incubated for 30 to 60 min prior to initiation of the assay, which was accomplished by adding and mixing 4 μl of the substrate stock solution. Enzyme concentrations ranged from 0.2 to 7 nM during the assay. Apparent inhibition constant (K_i^{app}) values were calculated by fitting the kinetic data to the Morrison equation for tight-binding inhibitors (34, 35), where v_i and v_0 are the initial rates with and without inhibitor, respectively, and $[E]_o$ and $[I]_o$ are the initial (total) enzyme and inhibitor concentrations, respectively.

$$\frac{v_i}{v_o} = \frac{[E]_o - [I]_o - K_i^{app} + \sqrt{([I]_o + K_i^{app} - [E]_o)^2 + 4[E]_o K_i^{app}}}{2[E]_o} \quad \text{(Eq. 1)}$$

Determination of Mercaptosulfide Inhibitor Concentration—The active inhibitor concentrations were estimated by titrating the mercapto group with 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent) as described previously (36, 37). Briefly, the reaction of 5,5′-dithiobis(2-nitrobenzoic acid) with the mercapto group produces 2-nitro-5-thiobenzoic acid. The concentration of 2-nitro-5-thiobenzoic acid is then measured by monitoring the absorbance at 412 nm. Cysteine was used to generate the standard curve with a molar extinction coefficient of $14{,}000 \pm 500\ \text{M}^{-1}\ \text{cm}^{-1}$, which is close to the value in the literature (37).

Computational Protein Sequence Analyses and Homology Modeling Structure of MMP-26—The sequence alignment of MMP catalytic domains was performed by the PILEUP program in Genetics Computer Group (GCG) software (Wisconsin Package version 10), with a default gap weight of 8 and gap length weight of 2. To align MMP-2 and -9, the 183-residue inserts of fibronectin type II-like modules were deleted before the alignment. The homology modeling structure of the MMP-26 catalytic domain was constructed using the Swiss Model program (38–40) with the crystal structure of the MMP-12-inhibitor complex (Protein Data Bank number 1JK3) (17) as a template. The mercaptosulfide inhibitors were computationally docked into the active site of MMP-26 with MacroModel version 7.2 (41, 42). Global minimization calculations were performed by the Monte Carlo molecular mechanical minimization method (43) with the Amber force field modified to include parameters for zinc and calcium. Residues within 7 Å of the inhibitor were included in the minimizations. All modeling was performed using the continuum solvent model. The crystallographic structures of MMP-1 (Protein Data Bank number 1HFC) (44), MMP-7 (Protein Data Bank number 1MMQ) (16), MMP-8 (Protein Data Bank number 1BZS) (45), MMP-12 (Protein Data Bank number 1JK3) (17), and MMP-14 (Protein Data Bank number 1BUV) (21) were used for comparison of the S_1' pocket.

RESULTS

Inhibition of MMPs with Mercaptosulfide MMPIs—An inhibitor set consisting of eight mercaptosulfide inhibitors was chosen to evaluate the S_1' pocket of MMP-26 (Fig. 1). These inhibitors contain P_1' and P_2' residues and have a mercapto and a sulfide group as a possible bidentate metal-binding moiety. The inhibitors contain a Leu side chain (MAG-181 and -182 and YHJ-294-1 and -2) or a Homophe side chain (YHJ-72, -73, -74, and -75) at the P_1' site. These inhibitors were tested against MMPs with known pocket characteristics (MMP-1–3, -7–9, -12, and -14). The inhibition potency of this class of inhibitors for the MMPs is significantly enhanced with a *β-H* configuration at the five-membered ring containing the mercapto and sulfide groups. The inhibitors with a Leu side chain are more potent against the shallow pocket MMPs, MMP-1/human fibroblast collagenase, and MMP-7/matrilysin than those with a Homophe side chain. Inhibitors with a Homophe side chain (YHJ-72, -73, 74, and -75) were more potent against the known deep-pocket MMPs such as MMP-3, -12, and -14 than those

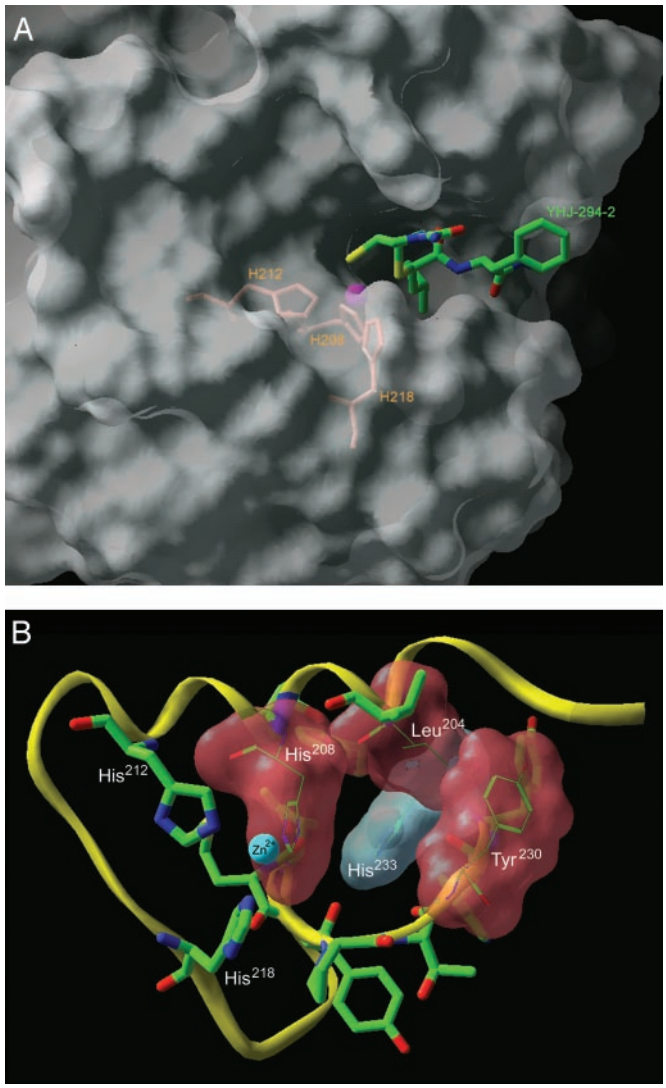


FIG. 4. **A modeled structure of MMP-26 complexed with YHJ-294-2.** *A*, the overall protein structure is shown as a molecular surface and the residues coordinating the catalytic Zn(II) (*magenta sphere*) are represented as *brown sticks* (His-208, His-212, and His-218). The inhibitor YHJ-294-2 is represented as a tube with atoms colored as follows: *green*, carbon; *red*, oxygen; *blue*, nitrogen; and *yellow*, sulfur. *B*, a close-up view of the S_1' pocket reveals that Leu-204, His-208, and Tyr-230 may be involved in formation of the pocket walls represented as *pink* molecular surfaces. The depth of the pocket may be limited by His-233 (*light blue* molecular surface). The three His residues coordinating the Zn(II) (*blue sphere*) are represented by tubes colored as described in *A*. The homology-modeled structure of MMP-26 was generated with the Swiss Model program (38–40) using the x-ray crystallographic structure of a cd-MMP-12-inhibitor complex as a template (Protein Data Bank code 1JK3) (17). The resulting MMP-26 structure was docked with YHJ-294-2 and energy-minimized as described under "Experimental Procedures."

FIG. 5. **The x-ray crystallographic structure MMP-8 (Protein Data Bank number 1BZS) (45) and homology modeled MMP-26 structure are shown after superimposition of zinc (*black sphere*) and histidine N ligands with MacroModel version 7.2.** The proteins are represented by a *flat ribbon* (MMP26) or by a *line ribbon* (MMP-8). Arg-233 and His-233 from MMP-8 and -26, respectively, may limit the depth of the S_1' pocket and are represented by *gray* and *black sticks*.

with Leu side chain. The inhibitors with the Leu side chain at the P_1' site (MAG-182 and YHJ-294-2) inhibit MMP-7 (40 and 26 nM, respectively) and MMP-12 (130 and 93 nM, respectively) without significant differences in K_i^{app} values. However, the presence of Homophe at the P_1' site dramatically distinguishes MMP-12 from MMP-7. YHJ-73 efficiently inhibits MMP-12 (13 nM), however, the potency is decreased to 1 μM against MMP-7. This trend is also displayed by YHJ-75, which has a high nM K_i^{app} value against MMP-7 (300 nM) but retains potency against MMP-12 (5.6 nM). This dramatic change of potency because of changes in the P_1' site of the inhibitors is consistently observed with the remaining shallow- and deep-pocket MMPs.

MMPs with an intermediate pocket can also accommodate the Homophe at the P_1' residue. However, the difference in inhibitor potency observed with Leu or Homophe at the P_1' residue is not as remarkable as that in the shallow- and deep-pocket MMPs. Inhibitors containing Leu at the P_1' site (MAG-182 and YHJ-294-2) are only slightly more potent against MMP-2 and MMP-9 than inhibitors with Homophe (YHJ-73 and -75). These Homophe inhibitors are still potent against MMP-8 with K_i^{app} values in the low nanomolar range. In general, these results indicate that mercaptosulfide inhibitors are suitable for characterizing the S_1' pocket of MMPs.

Characteristics of the S_1' Pocket of MMP-26 as Probed by Mercaptosulfide MMPIs—Inhibition constants for the inhibitors in Fig. 1 were measured with MMP-26 (Table I). YHJ-294-2 is the most potent inhibitor of MMP-26 among the mercaptosulfide inhibitors tested, with a K_i^{app} value of 2.8 nM. MMP-26 also favors the *β-H* configuration at the cyclopentyl or pyrrolidine ring moiety in the inhibitor. Addition of the urea-substituted pyrrolidine ring in place of the cyclopentyl ring (YHJ-294-1 and -2; YHJ-74 and -75) enhances the stereoselectivity for the *β-H* configuration. Importantly, MMP-26 prefers Leu over Homophe at the S_1' site, similar to the intermediate pocket MMPs, MMP-2, -8, and -9.

Characterization of MMP-26 S_1' Pocket Using Commercial Hydroxamate MMPIs—The S_1' site of MMP-26 was further investigated with commercially available inhibitors (Fig. 2). MMP-7/matrilysin was selected as a representative member of the shallow S_1' pocket MMPs and MMP-12/metalloelastase as one of the deep S_1' pocket MMPs for comparison purposes. The K_i^{app} values of the inhibitors with MMP-7, MMP-12, and MMP-26 are summarized in Table II. GM6001 is a broad-spectrum and potent inhibitor of MMPs (K_i^{app} = 0.4 nM for MMP-1, 0.5 nM for MMP-2, 27 nM for MMP-3, 0.1 nM for MMP-8, and 0.2 nM for MMP-9) (46). It is also the most potent synthetic MMP-26 inhibitor tested, with a K_i^{app} value of 0.36 nM. It contains a Leu residue at the P_1' site, and inhibits MMP-7 (3.7 nM) and MMP-12 (3.6 nM) with similar K_i^{app} values as observed in the mercaptosulfide inhibitors with a Leu side chain at the P_1' site. The potent inhibitor 444237 of deep S_1' pocket MMPs and its less potent stereoisomer 444238 were designed for human MMP-8 (IC_{50} = 4 nM and 1 μM, respectively; 45). Inhibitor 444225 was designed to be a potent deep S_1' pocket inhibitor of MMP-3 (K_i = 130 nM; 47). The 4-methoxybenzenesulfonyl group of these inhibitors binds at the deep S_1' pocket according to the crystallographic structure (45) and the structure-activity relationship of several derivatives (47). They inhibit MMP-7 and MMP-12 with at least 150-fold lower K_i^{app} values for MMP-12 than MMP-7. These deep S_1' pocket inhibitors effectively inhibited MMP-26 with at least 90-fold lower K_i^{app} values than those of MMP-7, but were more potent against MMP-12. These results are consistent with MMP-26 having an intermediate S_1' pocket.

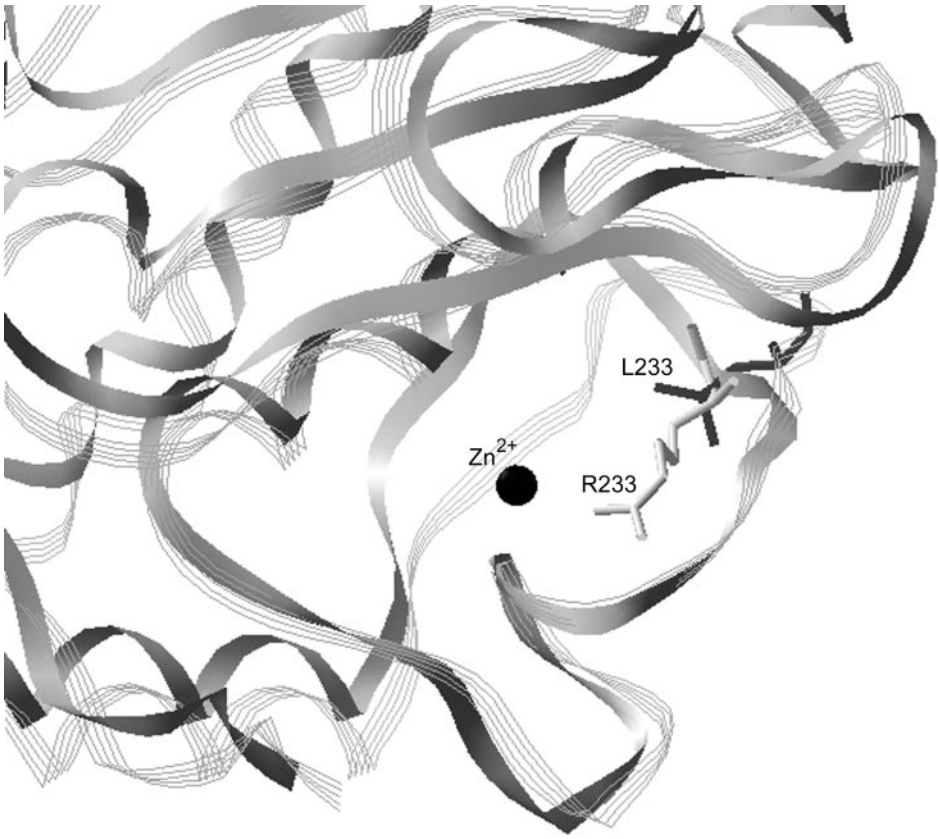


FIG. 6. **The x-ray crystallographic structures MMP-3 (Protein Data Bank number 1CIZ) (48) and MMP-8 (Protein Data Bank number 1BZS) (45) are shown after superimposition of zinc (*black sphere*) and histidine N ligands with MacroModel version 7.2.** The proteins are represented by a *flat ribbon* (MMP-8) or by a *line ribbon* (MMP-3). The Arg-233 in MMP-8 limits the depth of the S_1' pocket that is not restricted in MMP-3 by Leu-233.

Sequence Alignment and Homology Modeling Structure of MMP-26—The folding topology and patterns of all MMP catalytic domains are quite similar (19). Thus, homology modeling and protein sequence alignment may be useful tools to predict key residues involved in forming the S_1' pocket of MMP-26. Protein sequence alignment in Fig. 3 reveals a plausible explanation for residues participating in the formation of the S_1' pocket of MMP-26. According to the alignment, Leu-204, His-208, and Tyr-230 may be key residues in forming the S_1' pocket of MMP-26. To evaluate the prediction from the alignment, a homology modeled structure of the MMP-26 catalytic domain was constructed using the Swiss Model program (38–40) and the crystal structure of the MMP-12-inhibitor complex (Protein Data Bank number 1JK3) (17) as a template. The mercaptosulfide inhibitors were docked into the modeled MMP-26 structure using MacroModel version 7.2. The docked structures were further energy minimized as described under "Experimental Procedures." The overall MMP-26 structure complexed with YHJ-294-2 is shown in Fig. 4*A*. Consistent with other MMP family members (19), the non-primed (left) side of the MMP-26 active site is relatively flat. The primed (right) side extends deeper into the surface and the well defined S_1' pocket is clearly visible. The pocket that is formed by Leu-204, His-208, and Tyr-230 is illustrated in Fig. 4*B*. Interestingly, the depth of the pocket may be limited by His-233, consistent with the intermediate size prediction.

DISCUSSION

The inhibition characteristics of MMP-26 with mercaptosulfide inhibitors (Table I) and hydroxamate inhibitors (Table II) indicate that MMP-26 does not have a shallow S_1' pocket. According to the protein sequence alignment in Fig. 3 and the crystallographic structures of MMP-7 (16) and MMP-1 (13), Leu-204 in MMP-26 is substituted for Tyr and Arg at the equivalent position in MMP-7 and MMP-1, respectively. The side chains of Tyr and Arg terminate the S_1' pockets in these shallow-pocket MMPs. In the structure of MMP-26 (Fig. 4*B*), the side chain of Leu-204 forms the top wall of the S_1' pocket as found in most MMPs. Thus, MMP-26 appears to satisfy the requirement for a deep-pocket MMP. However, the inhibition profile of MMP-26 indicates a difference in the S_1' pocket of MMP-26 from those of other deep-pocket MMPs. The inhibitors with Homophe at the S_1' site (YHJ-73 and -75) do not show better potency than those with Leu (MAG-182 and YHJ-294-2). For the deep-pocket MMPs, the inhibition constants are consistently lower for the Homophe inhibitors than Leu inhibitors. The inhibition profile of MMP-26 with mercaptosulfide inhibitors is more similar to intermediate-pocket MMPs (MMP-2, -8, and -9) than deep-pocket MMPs (MMP-3, -12, and -14). These results suggest that MMP-26 may possess an intermediate pocket similar to those of MMP-2, MMP-8, and MMP-9.

A structural comparison of MMP-26 with MMP-8 further supports the similarity between the S_1' pockets of these two enzymes. The overlapping structures of MMP-8 (Protein Data Bank number 1BZS) (45) and MMP-26 at the S_1' pocket are displayed in Fig. 5. In MMP-8, it is known that the depth of the S_1' pocket is restricted by the Arg-233 side chain projecting toward the catalytic Zn(II) (14). In MMP-26, His-233 is present in place of Arg-233, which may restrict the depth of the pocket in a similar fashion, rendering the S_1' pocket to an intermediate size.

Based on the findings provided in this study and x-ray crystallographic structures of MMPs, the residue at the position

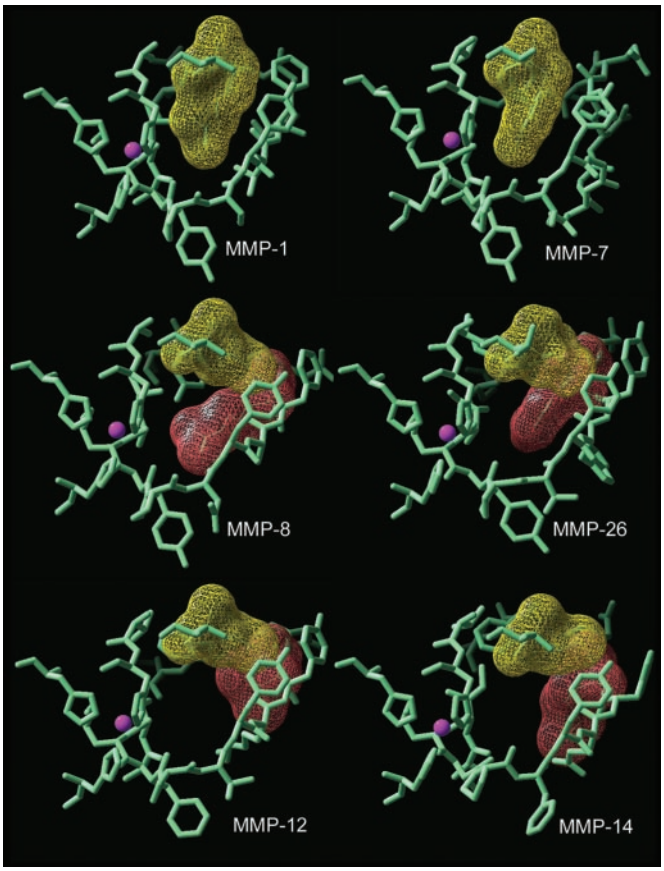


FIG. 7. **The residues forming the S_1' pocket of each enzyme are shown after superimposition of zinc (*magenta sphere*) and histidine N ligands with MacroModel version 7.2.** Inhibitors were removed from the x-ray crystallographic structures with Protein Data Bank accession numbers 1HFC (MMP-1) (44), 1MMQ (MMP-7) (16), 1BZS (MMP-8) (45), 1JK3 (MMP-12) (17), and 1BUV (MMP-14) (21). The MMP-26 structure is a homology model obtained as described under "Experimental Procedures." The key residue 204 that distinguishes a shallow pocket (MMP-1 and -7) is represented by a *yellow* molecular surface. Residue 233 may discriminate between the intermediate (MMP-8 and -26) and deep (MMP-12 and -14) pocket sizes and is represented by a *pink* molecular surface.

equivalent to His-233 of MMP-26 may play a key role in the determination of a deep or intermediate S_1' pocket. The sequence analyses (Fig. 3) showed that the residue at position 233 is hydrophobic in MMPs with deep S_1' pockets and hydrophilic in MMPs with intermediate pockets. The loop containing residue 233 may have a different orientation depending on the hydrophobicity of the side chain. The superimposed x-ray crystallographic structures of MMP-8 (Protein Data Bank number 1BZS) (45) and MMP-3 (Protein Data Bank number 1CIZ) (48) in Fig. 6 reveals this type of structural difference between an intermediate-pocket MMP (MMP-8) and a deep-pocket MMP (MMP-3). These investigations suggest that it is possible to predict the S_1' pocket properties by sequence analyses of the key residues at the Leu-204 and His-233 equivalent positions in other MMPs.

MMPs can be divided into three groups based on the characteristics of their S_1' pockets: shallow-, intermediate-, and deep-pocket MMPs (Fig. 7). Enzyme inhibition kinetic studies using MMPIs in combination with protein sequence analysis and homology modeling reveal that MMP-26 has an intermediate S_1' pocket. Our data may provide important mechanistic and structural information to design MMP-26-specific inhibitors. As the need for innovations and new strategies for MMP inhibition in cancer and inflammation is increasing (49, 50), this study may shed light on the molecular mechanisms by which highly selective and specific inhibitors targeting an individual MMP or subgroups of MMPs may be rationally designed and developed.

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Androgen Stimulates Matrix Metalloproteinase-2 Expression in Human Prostate Cancer

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Prostate growth and differentiation is androgen dependent, and increased expression of matrix metalloproteinase 2 (MMP-2) has been found in more aggressive prostate cancers. As part of our efforts to elucidate the mechanisms responsible for prostate cancer progression, we evaluated the MMP-2 expression after androgen stimulation in human prostate cancer LNCaP and LAPC-4 cells, which express a functional androgen receptor. Treatment of the cells with a synthetic androgen R1881 resulted in an increase of pro-MMP-2 expression assessed by Western blot and gelatinolytic zymography in both cell lines. R1881-stimulated pro-MMP-2 expression occurred in a dose-dependent manner, which was completely abrogated in the presence of the nonsteroid androgen antagonist bicalutamide. In accordance with the protein expression, MMP-2 promoter activity was also increased by R1881 in a cell-based luciferase reporter assay. However, R1881 treatment did not significantly affect either the pro-MMP-9 expression or its promoter activity. Although we observed an appearance of active form of MMP-2, its activator MT1-MMP was not changed after R1881 treatment. Pretreatment of the cells with inhibitors of RNA transcription, actinomycin D, or protein translation, cycloheximide, significantly suppressed R1881-induced pro-MMP-2 expression in LNCaP cells, indicating that androgen stimulates pro-MMP-2 gene expression. In addition, phosphatidylinositol 3′-kinase inhibitor, LY294002 or wortmannin, strongly inhibited R1881-induced pro-MMP-2 expression. Finally, R1881-enhanced LNCaP cell migration was clearly suppressed by LY294002 or the MMP-2 inhibitor OA-Hy in an *in vitro* migration assay. In conclusion, our data demonstrated that androgen stimulates pro-MMP-2 expression in LNCaP cells via phosphatidylinositol 3′-kinase-dependent androgen receptor transactivation. (*Endocrinology* 144: 1656–1663, 2003)

PROSTATE CANCER IS the second most frequently diagnosed cancer in men after skin cancer in the United States and is second only to lung and bronchus cancer in the frequency of mortality (1). Since the seminal work of Huggins and Hodges in 1941 (2), it has been widely accepted that prostate growth and differentiation is androgen dependent. As a result of this insight, medical treatment for metastatic prostate cancer has relied heavily on androgen ablation. However, most patients treated by androgen ablation ultimately relapse to more aggressive androgen-refractory prostate cancer with no means to cure (reviewed in Ref. 3).

The matrix metalloproteinase (MMP) family is comprised of secreted and membrane-associated zinc-dependent endopeptidases that can selectively degrade extracellular matrix (ECM) protein and nonmatrix proteins. Currently, up to 25 members of the MMP family have been reported, and the broad range of their substrates conveys a pivotal role for the MMP involvement in normal physiological processes and pathological states including tumor metastasis and angiogenesis (reviewed in Ref. 4). MMP-2, also called gelatinase A, is produced as a latent form (pro-MMP-2), and the activation process is mediated at least partially by MT1-MMP on the cell surface (5). It has been shown that MMP-2 is secreted by the human prostate gland, both *in vivo* and *in vitro*, and higher expression levels of MMP-2 are associated with increasing Gleason score, tumor metastasis, and aggressive behavior of prostate cancer (Refs. 6 and 7 and reviewed in Ref. 8).

To understand the role of the androgen receptors (ARs) in prostate cancer development and progression, it is important first to determine the AR signaling cascades and the genes that are regulated by AR. In view of the evidence for the association of MMP-2 expression and prostate cancer behavior, we evaluated the expression of pro-MMP-2 after androgen treatment in human prostate cancer LNCaP and LAPC-4 cells, which express a functional AR.

Materials and Methods

Cell culture and reagents

The LNCaP cell line was obtained from the American Type Culture Collection (Manassas, VA) and was maintained in a humidified atmosphere of 5% CO_2, RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics (Invitrogen, Carlsbad, CA). The LAPC-4 cells were obtained from Dr. Charles L. Sawyers (9) and maintained in Iscoves medium with 15% FBS/1% L-glutamine and antibiotics. The inhibitors of LY294002, rapamycin, PD98059, MMP-2 inhibitor I (OA-Hy), and PP2 were purchased from Calbiochem (San Diego, CA). R1881 and cycloheximide were obtained from ICN (Aurora, OH). Actinomycin D, type-1 rat-tail collagen, and wortmannin were purchased from Sigma (St. Louis, MO). Bicalutamide was a gift from AstraZeneca. Where indicated, the inhibitor was added from a 1000-fold concentrated stock in the solvent, dimethylsulfoxide, or ethanol. Control cultures received

Abbreviations: AR, Androgen receptor; ARE, androgen-responsive element; cFBS, charcoal-stripped FBS; CMV, cytomegalovirus; ECM, extracellular matrix; FBS, fetal bovine serum; FGF, fibroblast growth factor; MEK, MAPK and ERK kinase; MMP, matrix metalloproteinase; MMP9-LUC, human MMP-9 gene promoter-luciferase vector; PI3K, phosphatidylinositol 3′-kinase; PMA, phorbol 12-myristate 13-acetate; pro-MMP-2, latent form of MMP-2; PSA, prostate-specific antigen; SEAP, secreted alkaline phosphatase.

similar amounts of the solvent only. Final concentrations of the solvent did not exceed 0.1%. The antibodies against MMP-2 and MMP-9 were purchased from Chemicon (Temecula, CA). MT1-MMP antibody was described previously (10). The antibodies against AR, prostate-specific antigen (PSA), and actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Charcoal-stripped FBS (cFBS) was obtained from Atlanta Biologicals (Norcross, GA). Phorbol 12-myristate 13-acetate (PMA) and fibroblast growth factor 2 (FGF-2) were obtained from Sigma.

Western blot analysis

For immunoblot analysis, cells were washed in PBS and lysed in a radioimmunoprecipitation assay buffer supplied with protease inhibitors (CytoSignal, Irvine, CA). Equal amounts of protein were separated on an 8% sodium dodecyl sulfate-polyacrylamide gel and blotted onto a polyvinyl difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA). Membranes were blocked in a Tris-buffered saline solution with 5% nonfat dry milk and incubated with antibodies overnight at 4 C. Immunoreactive signals were detected by incubation with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) followed by chemiluminescent detection (SuperSignal West Dura substrate kit, Pierce Chemical Co., Rockford, IL).

Assay of gelatin-degrading MMPs by zymography

Unconcentrated conditioned media from the cell cultures were analyzed for MMP gelatinolytic activities by gelatin zymography as described previously (11). Briefly, conditioned media (mixed with 5× sample buffer) were fractionated by SDS-PAGE on a 10% gel containing 1.0 mg/ml gelatin (Sigma) under nonreducing conditions. After two washes in Tris buffer (50 mM Tris, 200 mM NaCl, 10 mM $CaCl_2$, 1 mM $ZnCl_2$, 1% Triton X-100, pH 7.5), the gel was incubated in the same buffer in the absence of Triton X-100 for 18 h at 37 C. After being stained with Coomassie Brilliant Blue R-250, the gel was destained with 10% (vol/vol) acetic acid, and the nonstaining bands resulting from digestion of the substrate by gelatinase enzymes were then visualized.

Cell migration assay

After serum starvation, the cells were trypsinized and resuspended in RPMI 1640 with 5% cFBS. A Transwell insert with 8-μM pore (Nunc, Naperville, IL) was coated with collagen (50 μg/ml in PBS) for 2 h at 37 C. A total of 1.0×10^5 cells were then seeded in the upper chambers of the Transwells. R1881 was added in both the upper and lower chambers containing RPMI 1640 supplied with 5% cFBS. Where indicated, cells were preincubated with inhibitors (5.0 μM OA-Hy or 10 μM LY294002) for 30 min at room temperature before seeding in the Transwell. Cells were incubated for 48 h, and then the chamber was disassembled. Cells on the upper surface were removed, and cells invaded to the lower surface of the filters were fixed, stained, and counted as described (12).

Luciferase and SEAP reporter assay

A luciferase reporter plasmid controlled by the 1716-bp length of the human MMP-2 promoter (MMP2-LUC) was obtained from Dr. Yi Sun (13, 14). The human MMP-9 gene promoter-luciferase vector (MMP9-LUC) was obtained from Dr. Yasuyuki Sasaguri (15). The reporter vector pCMV-SEAP, expressing secreted alkaline phosphatase (SEAP) under the control of the cytomegalovirus (CMV) promoter, was a kind gift from Dr. David Spencer (16) and was used as an internal reference control. The cells were plated in 6-well tissue culture plates and transfected the following day with 2.0 μg MMP-2 reporter construct and 0.5 μg pCMV-SEAP construct by using the Cytofectene reagent (Bio-Rad Laboratories, Inc.) according to manufacturer's protocol. After 24 h, the cells were serum starved for another 24 h and then treated with R1881 (1.0 nM) or PMA (50 μM) in 2% cFBS. After 24 h, culture supernatants were harvested and assayed for SEAP activity as described previously (17). Cells were lysed with a lysis buffer supplied by a luciferase assay system (catalog no. 4030, Promega Corp., Madison, WI). Protein concentration in the cell lysates was measured by a protein assay kit (Bio-Rad Laboratories, Inc.). An equal amount of protein from each cell lysate was assayed in triplicate for luciferase enzyme activity by using the luciferase assay system (Promega Corp.) and Lumat LB9501 reader (Berthold, Oak Ridge, TN). The luciferase activity of each sample was normalized against the corresponding SEAP activity before the fold induction value relative to control cells was calculated.

Statistical analysis

All experiments were repeated two or three times. Zymographic data and Western blot results are presented from a representative experiment. The mean and SD from two experiments for cell migration and luciferase assay are shown. The number of migrating cells in the absence of either R1881 or inhibitors is assigned a relative value of 100%. The significant differences between groups were analyzed using the SPSS computer software (SPSS, Inc., Chicago, IL).

Results

Androgen stimulates pro-MMP-2 expression

We determined the effect of androgen stimulation on MMP expression/activation in human prostate cancer LNCaP and LAPC-4 cells. The LNCaP cell line is a commonly used *in vitro* model with well-characterized features of androgen responsiveness for prostate cancer research (18). It was originally derived from a lymph node metastatic prostate cancer and secretes PSA. The LAPC-4 cell line is a recently established androgen responsive cell line (9), similar to LNCaP cells, but LAPC-4 cell harbors a wild-type *AR* gene and LNCaP cell has a mutated one. MMP activity is usually analyzed in cell culture-conditioned medium by gelatinolytic zymography (11) because most MMP family members are secreted proteases. Following serum starvation for 24 h, the cells were treated with increasing doses (0.01–10 nM) of the synthetic androgen R1881 in serum-free condition for another 24 h. The conditioned media were analyzed by gelatinolytic zymography without concentrating. Meanwhile, the cells were harvested and the cellular content of MMP-2 protein in whole-cell lysates was determined by Western blot. The conditioned media from the HT-1080 cell culture (RPMI 1640 without serum), which contains high levels of MMP-2 and -9, were used as a positive control (19).

Under serum-free condition and the solvent control, there was no detectable MMP-2 gelatinolytic activity in the conditioned media from the cell culture (Fig. 1, A and E, lanes 1 and 2). On R1881 addition, MMP gelatinolytic activity corresponding to pro-MMP-2 was gradually increased in a dose-dependent manner (lanes 3–6). Furthermore, an additional active form of MMP-2 appeared after higher doses of R1881 in LNCaP cells (1.0–10 nM in lanes 5 and 6) but not in LAPC-4 cells, which may reflect a cell-based specificity. To confirm the MMP-2 induction by R1881 stimulation, MMP-2 protein levels in the whole-cell lysates were determined by Western blot analysis. As shown in Fig. 1B, on R1881 stimulation, the cellular level of pro-MMP-2 protein was increased in a dose-dependent manner. The protein levels of pro-MMP-2 matched well with the gelatinolytic activities secreted into the conditioned media. However, MMP-9 gelatinolytic activity was not detectable under our experimental conditions (Fig. 1, A and E), which is consistent with our previous report (20). The protein level of cellular pro-MMP-9 (Fig. 1C) was unchanged by R1881 treatment. These data indicate that androgen induces pro-MMP-2 but not pro-MMP-9 expression in LNCaP and LAPC-4 cells.

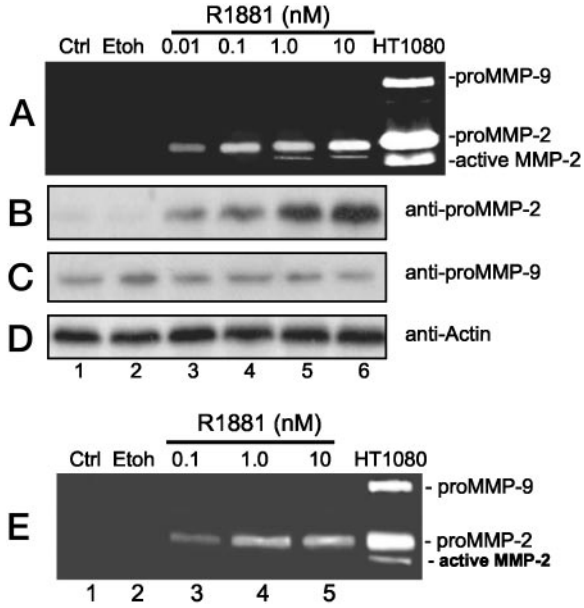

FIG. 1. R1881 stimulates pro-MMP-2 expression. Following serum starvation for 24 h, LNCaP cells were left untreated (lane 1) or treated with solvent ethanol (lane 2, Etoh), increasing dose of R1881 (lane 3–6, 0.01–10 nM) for 24 h in serum-free media. Twenty-four hours later, the MMP gelatinolytic activities secreted into media were examined by zymography (A). Conditioned media from HT1080 cell culture served as positive control. Cells were harvested and protein levels of pro-MMP-2 (B) and pro-MMP-9 (C) were determined by Western blot. Immunoblot for actin served as loading control (D). E, MMP gelatinolytic zymography was also performed using the conditioned media from LAPC-4 cell culture in the same way as used in LNCaP cells. Data represent two independent experiments. Ctrl, Control.

Androgen induction of pro-MMP-2 expression is mediated through AR transactivation

The biological effects of androgens are believed to be mediated through the intracellular AR, which is a ligand-activated transcription factor that regulates gene expression (4), although the nongenomic effect of androgens has been reported recently (Ref. 21 and reviewed in Ref. 22). Androgen action can be abolished by pretreatment of the cells with androgen antagonist bicalutamide (23). To examine whether androgen induction of pro-MMP-2 is mediated through AR transactivation, we pretreated LNCaP cells with a maximally effective dose of bicalutamide (10 μM) for 30 min followed by R1881 (1.0 nM) addition. MMP-2 protein levels in the whole-cell lysates and its gelatinolytic activity in the conditioned media were assessed 24 h later. The well-known AR target gene product PSA served as a positive control. As shown in Fig. 2A, as is the case for PSA, pretreatment with bicalutamide completely abolished androgen-induced pro-MMP-2 expression in LNCaP cells (lane 3 *vs.* lane 2). Paralleling the protein expression, androgen-induced MMP-2 gelatinolytic activity was also suppressed in the presence of bicalutamide (Fig. 2A, *zymography panel*). However, bicalutamide itself when added alone had no effect on MMP-2 expression (data not shown).

To examine whether androgen induction of pro-MMP-2 expression is mediated via AR transactivation at the gene transcription/translation level, an inhibitor for RNA transcription (actinomycin D) and an inhibitor for protein translation (cycloheximide) were used at a level of none-cytotoxic dose (1.0 μg/ml, data not shown). Similar to the effect of bicalutamide on the expression of PSA and pro-MMP-2, these two inhibitors significantly suppressed R1881-stimulated pro-MMP-2 expression (in both Western blot and zymography assays, Fig. 2A, lanes 4 and 5 *vs.* lane 2). However, addition of actinomycin D or cycloheximide did not affect AR protein levels (Fig. 2A, *AR panel,* lanes 4 and 5 *vs.* lane 2), indicating that suppression of R1881-induced pro-MMP-2 expression by the two inhibitors is not due to reduced expression of AR protein. In contrast, AR protein was slightly increased in the presence of R1881 (Fig. 2A, *AR panel,* lane 1 *vs.* lanes 2–5), which is consistent with a previous report (24). These results indicate that androgen induction of MMP-2 is due to AR-mediated gene expression (in other words, AR transactivation).

To further confirm the involvement of AR transactivation in androgen induction of pro-MMP-2, we used a luciferase reporter construct under the control of human MMP-2 promoter (1659 bp of the 5′ region on *MMP-2* gene) to define the stimulating effect of androgen on MMP-2 promoter activity (13). After transfection with the reporter constructs, LNCaP cells were serum starved for 24 h and then stimulated with R1881 (1.0 nM). Luciferase activity in the cell extracts was measured 24 h later. As shown in Fig. 2B, R1881 treatment induced about 3.5-fold increase of the luciferase activity, which was abolished by bicalutamide addition (10 μM), consistent with the results as seen in zymography and Western blot (Fig. 2A). As expected, MMP-9 promoter activity was not affected by R1881 treatment but was strongly induced by a well-known MMP-9 stimulator PMA (25). In addition, PMA suppressed the basal activity of the MMP-2 promoter by almost 50% (Fig. 2B), which is in accordance with a previous report (26). When LAPC-4 cells were used for those luciferase assays, a very similar result was also observed (Fig. 2C). These results clearly suggest that androgen-stimulated pro-MMP-2 expression is mediated through AR transactivation.

MMP-2 is produced in a latent form, which is activated by the membrane type MMP, MT1-MMP, in participation with tissue inhibitor of MMP-2 (5). Because we observed an active form of MMP-2 in gelatinolytic zymography assays when LNCaP cells were treated with higher doses of R1881 (Fig. 1A), we checked the MT1-MMP expression to rule out the possibility that MT1-MMP is activated after R1881 treatment. As expected, MT1-MMP was expressed at a relatively low level in LNCaP cells (Fig. 3), which is consistent with a previous report (27). After R1881 treatment, however, there was no significant alteration to the protein levels of either the pro-form (65 kDa) or the active form (63 kDa) of MT1-MMP (28). These results indicate that MT1-MMP is constitutively expressed in LNCaP cells, and its activity or expression level is not regulated by AR signaling. Appearance of the MMP-2 active form after R1881 treatment at higher doses might be due to increased pro-MMP-2 expression, which in turn leads to subsequent accumulation of the active form in the media.

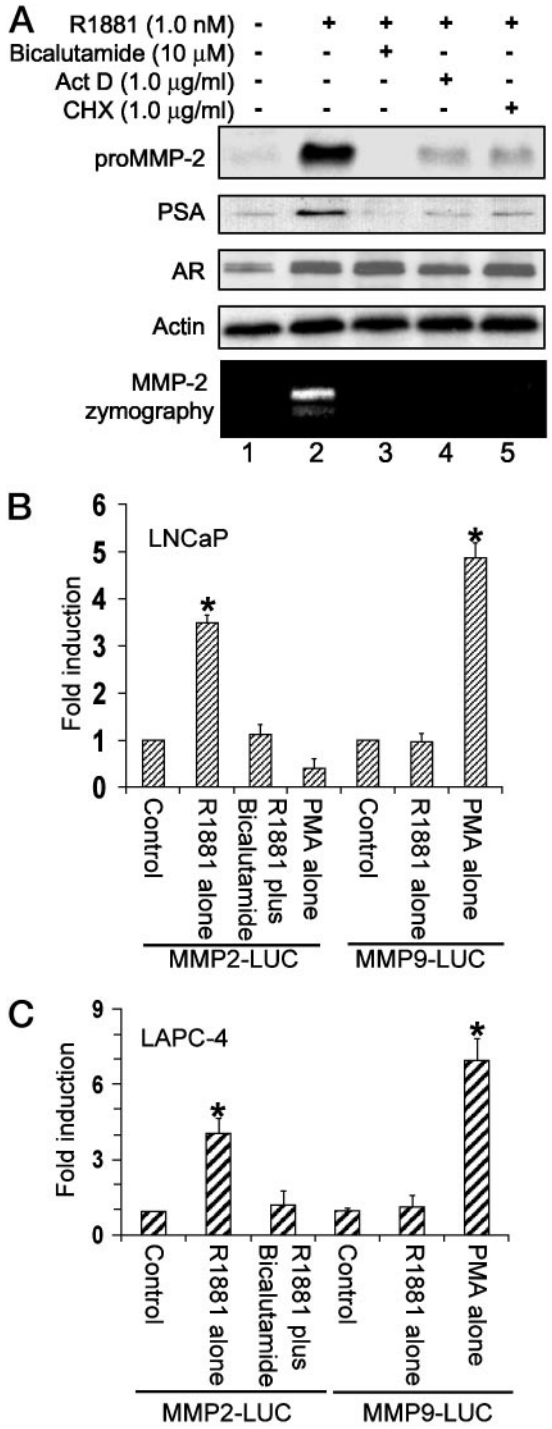

FIG. 2. A, R1881-stimulated pro-MMP-2 expression is mediated through AR transactivation. Following serum starvation for 24 h, LNCaP cells were left untreated (lane 1) or pretreated with bicalutamide (lane 3), actinomycin D (Act D, lane 4), and cycloheximide (CHX, lane 6) for 30 min followed by addition of R1881 for another 24 h in serum-free media. Expression of pro-MMP-2, PSA, AR, and actin (loading control) were determined by Western blot in whole-cell lysates. The MMP gelatinolytic activities secreted into media were examined by zymography. B and C, R1881 induced MMP-2 but not MMP-9 promoter activity. LNCaP (B) or LAPC-4 (C) cells were co-transfected with luciferase reporter constructs MMP2-LUC or MMP9-LUC together with pCMV-SEAP reporter construct overnight by using the Cytofectene reagent (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocol and then serum starved for 24 h. The solvent ethanol (control), R1881 (1.0 nM), or PMA (50 μM) was added once in the culture media containing 2% cFBS for another 24 h. Luciferase or SEAP activity was measured as described in *Materials and Methods.* The luciferase activity was presented as fold induction against control sample after normalized with protein content and SEAP activity. The *asterisk* indicates a significant difference ($P < 0.05$) between R1881 or PMA stimulation *vs.* the solvent control. Data represent three independent experiments.

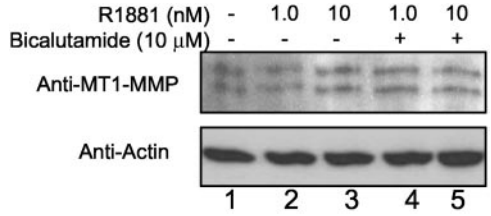

FIG. 3. MT1-MMP remains unchanged after R1881 treatment. Serum-starved LNCaP cells were either left untreated (lane 1) or pretreated with bicalutamide for 30 min (lanes 4 and 5) followed by R1881 addition (lanes 2–5) for another 24 h. MT1-MMP expression was assessed by Western blot in whole-cell lysates. Actin immunoblot served as loading control. Data represent three independent experiments.

AR-mediated pro-MMP-2 expression involves PI3K activity

To gain insights into the signal molecules involved in AR-mediated pro-MMP-2 expression in LNCaP cells, some commonly used specific inhibitors of protein kinase, including PD98059 for MEK-1, LY294002 and wortmannin for phosphatidylinositol 3′-kinase (PI3K), rapamycin for mammalian target of rapamycin, and PP2 for Src kinase, were used to block androgen induction of pro-MMP-2. The concentrations yielding maximum pharmacological effect without cytotoxicity were verified first in a cell-based survival assay (data not shown) and then used in the next experiments. Following serum starvation for 24 h, LNCaP cells were left untreated or pretreated with different kinase inhibitors for 30 min followed by addition of R1881 (1.0 nM) for another 24 h in serum-free media. The MMP gelatinolytic activities secreted into the media were examined by zymography. As shown in Fig. 4A, among the inhibitors used in this study, only the PI3K inhibitor LY294002 completely abolished the androgen induction of MMP-2 in LNCaP cells. To examine whether the inhibitory effect of LY294002 on androgen-induced MMP-2 gelatinolytic activity is in parallel with the protein expression but not caused by inhibition of MMP-2 secretion, protein levels of pro-MMP-2 from equal amounts of whole-cell lysates were determined by Western blot. As shown in Fig. 4B, two commonly used PI3K inhibitors (LY294002 and wortmannin) totally abolished pro-MMP-2 and PSA expression. MEK1 inhibitor PD98059 had no effect on pro-MMP-2 expression, which was consistent with the zymography data. In addition, the inhibitors alone did not cause any change, as shown in the *right panel* of Fig. 4B. These results suggest that PI3K is involved in AR-mediated pro-MMP-2 expression, which is consistent with previous reports that PI3K activity is required for the AR transactivation (29–31).

FGF-2 is an activator for both MEK1 and PI3K kinase cascades (32, 33) and was reported to induce MMP-2 expression in different cell types (34). Therefore, we asked whether

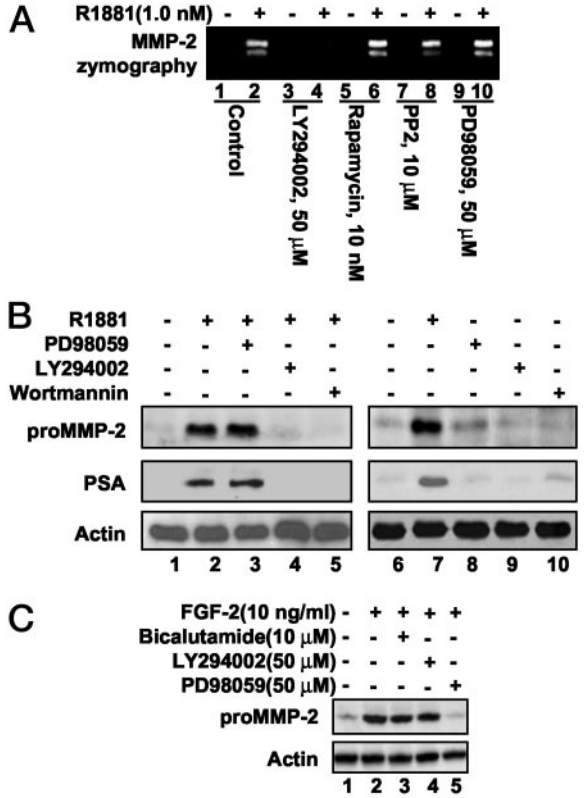

FIG. 4. AR-mediated pro-MMP-2 expression involves PI3K activity. Following serum starvation for 24 h, LNCaP cells were either left untreated (control) or pretreated with different kinase inhibitors as indicated for 30 min followed by R1881 (A and B) or FGF-2 (C) addition for another 24 h in serum-free media. The MMP gelatinolytic activities secreted into media were examined by gelatin zymography (A). Protein levels for pro-MMP-2, PSA, and actin from whole cell lysates were determined by Western blot (B and C). Data represent two separate experiments.

the PI3K signaling pathway is also required for FGF-2 to induce MMP-2 gene expression. To this end, we stimulated serum-starved LNCaP cells with FGF-2 (10 ng/ml) and pro-MMP-2 expression was evaluated 24 h later by Western blot (Fig. 4C) and gelatin zymography (data not shown). As expected, FGF-2 stimulation increased pro-MMP-2 expression dramatically. In contrast to androgen stimulation, pro-MMP-2 induction after FGF-2 stimulation was not suppressed by either the androgen antagonist bicalutamide or PI3K inhibitor LY294002 but by MEK1 inhibitor PD98059 (Fig. 4C). A similar result was also observed when LAPC-4 cells were used (data not shown). These results indicate that the regulatory mechanism for *MMP-2* gene expression is stimulus specific in cells.

Inhibition of MMP or PI3K suppresses LNCaP cell migration in response to R1881

Recently, MMPs have been shown to be correlated with tumor dissemination because of their proteolytic activity on ECM, and tumor cell migration is one of the most important events contributing to tumor dissemination (reviewed in Ref. 35). Because we observed androgen induction of pro-MMP-2 expression in LNCaP cells, we next asked whether MMP-2 inhibition could suppress cell migration in response to R1881 stimulation. Using a collagen-coated Transwell chamber assay, we assessed the ability of LNCaP cells to undergo unstimulated, steroid-depleted serum-stimulated, or R1881-stimulated migration. Without serum stimulation, almost no sign of migration was observed for LNCaP cells (data not shown), which is consistent with a previous report (36). However, addition of charcoal-stripped serum (steroids were depleted) stimulated cell migration. When R1881 (1.0 nM) was added in the culture, cell migration was significantly enhanced by almost 2-fold, compared with that incubated with charcoal-stripped serum alone (Fig. 5). To define the responsibility for MMP-2 in R1881-enhanced cell migration, we evaluated the effect of PI3K inhibitor LY294002 and a potent MMP-2 specific inhibitor OA-Hy (37) on LNCaP cell migration. Indeed, R1881-enhanced LNCaP cell migration was almost completely abolished by pretreatment with either OA-Hy (5.0 μM) or LY294002 (10 μM). In addition, we noticed that LY294002 alone or LY294002 plus R1881 resulted in a slight decrease (not statistical significant) of cell migration, compared with charcoal-stripped serum control, which is consistent with a previous report that PI3K activity is fundamental for serum-stimulated cell migration (38).

Discussion

The present study was undertaken to investigate whether androgen regulates MMP-2 or MMP-9 expression in prostate cancer cells. We found that: 1) androgen stimulated pro-MMP-2 but not MMP-9 expression, and androgen-stimulated pro-MMP-2 expression occurred at the gene transcription level via AR transactivation; 2) AR-mediated pro-MMP-2 expression is dependent on PI3K activity, a known modu-

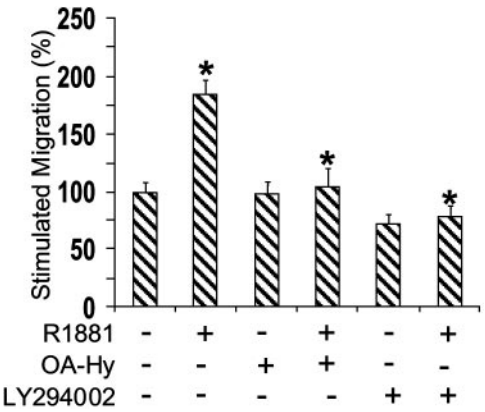

FIG. 5. Inhibition of MMP or PI3K suppresses LNCaP cell migration in response to R1881. After serum starvation, LNCaP cells were resuspended in culture media supplied with 5% cFBS. Then the cells were left untreated (control) or pretreated with OA-Hy (5.0 μM) or LY294002 (10 μM) for 30 min. A total of 1.0×10^5 cells were seeded on collagen-coated (50 μg/ml) Transwell chambers (8 μM pore) containing culture media and 5% cFBS. Cells were incubated in six different conditions: solvent only, R1881 alone, OA-Hy alone, R1881 plus OA-Hy, LY294002 alone, or R1881 plus LY294002. After 48-h incubation, cells on the upper surface were removed, and the filter was stained. The number of migrating cells in the absence of any treatment (solvent only) is assigned a relative value of 100%. The *asterisk* indicates a significant difference ($P < 0.05$) between R1881 stimulation *vs.* solvent only or R1881 alone *vs.* R1881 plus OA-Hy or LY294002. Data represent three independent experiments.

lator of the AR transactivation; and 3) androgen enhanced LNCaP cell migration via MMP-2 induction. These results indicate that androgen regulates MMP-2 expression via PI3K-dependent AR transactivation in prostate cancer cells.

Historically, *MMP-2* has been considered a constitutive gene because of a lack of well-characterized regulatory elements in the *MMP-2* promoter region (reviewed in Ref. 39). However, recent studies of the *MMP-2* promoter sequence analyses have revealed a number of potential *cis*-acting regulatory elements including p53, activator protein-1, Ets-1, CCAAT/enhancer-binding protein, cAMP response element-binding protein, polymavirus enhancer activator 3, surfactant protein 1, and activator protein-2 that could be involved in regulation of MMP-2 expression (13, 14, 40). Several factors, such as TGFβ1 (7), UVB/IL-8 (41), concanavalin A (42), short-term exposure to α- and γ-interferons (43), and transfection of *c-Ha-ras* (44) or Akt1 (45), have been previously reported to induce MMP-2 expression. In contrast, others including retinoic acid (46), PMA (26), long-term exposure to α- and γ-interferons (43), and the calcium influx inhibitor carboxy amidotriazole (47) were shown to suppress MMP-2 expression. We demonstrated for the first time in this study that MMP-2 expression was elevated when prostate cancer LNCaP and LAPC-4 cells were stimulated with androgen, adding androgen as a new member to the list of MMP-2 modulators. Our results are consistent with previous reports that long-term treatment with androgen increased prostate MMP-2 content in rats (48). However, another sex hormone, estrogen, was reported to inhibit MMP-2 expression in human fibroblast-derived cells (49), further suggesting that MMP-2 regulation is cell and stimulus specific.

Androgens such as R1881 produce most cellular responses through their cognate nuclear receptor, AR. On binding to the hormone, the AR forms homodimer. The dimerized protein then interacts with specific DNA sequences directly through an androgen-responsive element (ARE; reviewed in Ref. 50) or indirectly through other transcription factors that bind DNA in the regulatory region of the target gene promoters, such as Ets (51). The result is an alteration in protein synthesis and the generation of a cellular response. In this study, we demonstrated that R1881-stimulated pro-MMP-2 expression was abolished by the androgen antagonist bicalutamide that can block AR-mediated gene transcription (23) and by RNA transcription-specific inhibitor actinomycin D and protein translation inhibitor cycloheximide. Furthermore, in a luciferase reporter assay, human MMP-2 promoter activity was induced by R1881 stimulation that was also abolished by bicalutamide addition. These data all indicate that the expression of *MMP-2* gene is regulated by androgen via an AR transactivation mechanism, although the possibility of an indirect effect of AR on pro-MMP-2 expression could not be fully ruled out. Recently interaction of AR with Ets protein has been reported to negatively modulate MMP-1, 3, and 7 (51), and an Ets-1-binding site was found in the *MMP-2* promoter region (14). However, it might be unlikely that androgen-stimulated pro-MMP-2 expression is also via an interaction between AR and Ets-1 because androgen positively induces pro-MMP-2 expression.

The location, sequence, and number of AREs associated with a given androgen target gene varies, although androgen-responsive regions typically contain multiple nonconsensus AREs (5′-TGTTCT-3′; Ref. 52). By analyzing the published sequence of the *MMP-2* gene promoter (13), we noticed that there are two potential ARE-like motifs located in the promoter of the *MMP-2* gene (−1539-TGT-T*c*CT-1503, and −609-TGT*a*TCT-603) with one nucleotide mismatch (italicized). Further characterization of the *MMP-2* promoter for ARE elements is being carried out currently by our group.

It has been shown that overexpression of the *Akt1* gene induces MMP-2 activity in mouse mammary epithelial cells (45), but reintroduction of the *PTEN* gene reduces *MMP-2* gene expression in human glioma cells (53). In human prostate cancer LNCaP cells, PI3K-Akt activity is elevated because of an inactive mutant of the *PTEN* gene (54). Although the molecular basis in AR signaling is not fully understood, an involvement of PI3K-Akt and PTEN pathways was recently reported (29–31). Consistent with those reports, we also observed that AR-mediated pro-MMP-2 expression is PI3K dependent, further demonstrating that PI3K activity is required for AR transactivation. However, the detailed mechanism for PI3K-Akt's involvement needs further investigation.

Extensive work on the mechanisms of tumor invasion and metastasis has determined the MMP's function as key factors in the process of tumor dissemination. In this regard, the increased expression of various MMPs is strongly associated with tumor invasiveness. Specifically, MMP-2 expression is elevated in metastatic prostate cancers (reviewed in Ref. 8). Previous studies have pointed out that tumor cells achieve increased motility through mechanisms that circumvent the requirement for exogenous motogenic factors (*e.g.* androgen or other serum-derived factors). Among the signal cascades initiated by the factors, the PI3K-dependent pathway is essential in cell migration (38). The LNCaP cell line is the one among those cell lines that require serum stimulation to migrate (36). The failure of LNCaP cells to migrate was not due to their inability to adhere to or spread on ECM but to a requirement of extracellular factors to activate intracellular signals necessary for motility (36). In this study, we also observed androgen-enhanced LNCaP cell migration. Androgen-enhanced cell migration was totally blocked by the MMP-2 specific inhibitor OA-Hy, and the PI3K inhibitor LY294002. These results suggest that androgen-induced MMP-2 played a role in androgen-enhanced cell migration.

In conclusion, we have presented for the first time that pro-MMP-2 expression is induced by androgen-stimulated AR transactivation in human prostate cancer LNCaP and LAPC-4 cells. In addition, AR-mediated pro-MMP-2 expression, like PSA, is dependent on an active PI3K pathway, which is consistent with previous reports (29–31). Moreover, androgen-enhanced LNCaP cell migration is suppressed by the MMP-2 inhibitor OA-Hy or PI3K inhibitor LY294002. These findings suggest that AR-mediated pro-MMP-2 expression may participate in the process of prostate development or prostate cancer invasion/metastasis.

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Catalytic- and ecto-domains of membrane type 1-matrix metalloproteinase have similar inhibition profiles but distinct endopeptidase activities

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Membrane type 1-matrix metalloproteinase (MT1-MMP/MMP-14) is a major collagenolytic enzyme that plays a vital role in development and morphogenesis. To elucidate further the structure–function relationship between the human MT1-MMP active site and the influence of the haemopexin domain on catalysis, substrate specificity and inhibition kinetics of the cdMT1-MMP (catalytic domain of MT1-MMP) and the ecto domain ΔTM-MT1-MMP (transmembrane-domain-deleted MT1-MMP) were compared. For substrate **1** [Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, where Mca stands for (7-methoxycoumarin-4-yl)acetyl- and Dpa for *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl], the activation energy E_a was determined to be 11.2 and 12.2 kcal/mol (1 cal = 4.184 J) for cdMT1-MMP and ΔTM-MT1-MMP respectively, which is consistent with k_{cat}/K_M values of 7.37 and 1.46×10^4 $M^{-1} \cdot s^{-1}$. The k_{cat}/K_M values for a series of similar single-stranded peptide substrates were determined and found to correlate with a slope of 0.17 for the two enzyme forms. A triple-helical peptide substrate was predicted to have a k_{cat}/K_M of 0.87×10^4 $M^{-1} \cdot s^{-1}$ for ΔTM-MT1-MMP based on the value for cdMT1-MMP of 5.12×10^4 $M^{-1} \cdot s^{-1}$; however, the actual value was determined to be 2.5-fold higher, i.e. 2.18×10^4 $M^{-1} \cdot s^{-1}$. These results suggest that cdMT1-MMP is catalytically more efficient towards small peptide substrates than ΔTM-MT1-MMP and the haemopexin domain of MT1-MMP facilitates the hydrolysis of triple-helical substrates. Diastereomeric inhibitor pairs were utilized to probe further binding similarities at the active site. Ratios of K_i values for the inhibitor pairs were found to correlate between the enzyme forms with a slope of 1.03, suggesting that the haemopexin domain does not significantly modify the enzyme active-site structure.

Key words: activation energy, catalytic domain, diastereomeric MMP inhibitors, ecto-domain, membrane type 1-matrix metalloproteinase (MT1-MMP), substrate specificity.

INTRODUCTION

Matrix metalloproteinases (MMPs) are a family of related zinc endopeptidases known to play prominent roles during normal and pathological extracellular matrix remodelling events including cancer progression [1]. The first identified membrane type MMP, MT1-MMP, has been shown to play a key role in tumour cell invasion and metastasis by complex mechanisms, including activation of proMMP-2 and direct hydrolysis of interstitial collagens [2,3]. MT1-MMP is tethered to the cell membrane by a type 1 transmembrane region and is expressed as an active protease on the cell surface upon activation, primarily by proprotein convertases such as furin or furin-like serine proteinases [4]. In addition, consistent with other MMP family members, MT1-MMP has a propeptide, catalytic domain linker region and a haemopexin domain [5].

The haemopexin domain has been supposed to function mainly in protein-substrate recognition [6]. It is necessary for native type I collagen cleavage for reasons that are not completely understood [7,8]. Recently, new roles have been identified for the MT1-MMP haemopexin domain including enzymic regulation and subcellular localization [9,10]. Additionally, the linker region between the catalytic and haemopexin domains has been identified as having the ability to bind the collagen [11]. It is possible that this region is necessary for triple-helical substrate hydrolysis.

Although several groups have reported the cleavage of extracellular matrix constituents by MT1-MMP [12–14], a structural comparison of the active site of the catalytic domain with and without the haemopexin domain has not been accomplished. In the present study, a comparison of the substrate specificity for two truncated forms of MT1-MMP was performed using single-stranded peptide substrates. The haemopexin domain was then found to facilitate the hydrolysis of a triple-helical substrate. To demonstrate that the haemopexin domain does not influence the architecture of the active site, diastereomeric inhibitor pairs were utilized to show identical K_i ratios.

MATERIALS AND METHODS

Materials

Quenched fluorogenic peptide substrates (see Table 1) **1** and **3**–**5** [Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2, Mca-Pro-Lys-Pro-Leu-Ala-Leu-Dpa-Ala-Arg-NH_2, Mca-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys-Dpa-NH_2 and Mca-Pro-Leu-Ala-Gln-Ala-Val-Dpa-Arg-Ser-Ser-Arg-NH_2, where Mca stands for (7-methyloxycoumarin-4-yl)acetyl, Dpa stands for *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl and Nva for norvaline] were purchased from Bachem and **2** (Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-NH_2) was from Calbiochem (San Diego, CA, U.S.A.). The triple-helical peptide substrate **6** [$\{(GPP^*)_5$ GPK(Mca)GPQGLRGQK(Dnp)GVR$(GPP^*)_5$-$NH_2\}_3$, where Dnp stands for 2,4-dinitrophenyl and P^* for 4-hydroxy-L-proline] was synthesized as described previously [15]. The mercaptosulphide

Abbreviations used: MMP, matrix metalloproteinase; MT1, membrane type 1; cdMT1-MMP, catalytic domain of MT1-MMP; Dpa, *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; Mca, (7-methoxycoumarin-4-yl)acetyl; Nva, norvaline; RTTI, rat-tail tendon type I collagen; TIMP, tissue inhibitor of metalloproteinase; ΔTM-MT1-MMP, transmembrane-domain-deleted MT1-MMP.

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inhibitors were synthesized and characterized as described previously ([16,17]; Y. Jin and M. A. Schwartz, unpublished work). Hydroxamate inhibitors 444237 and 444238 were purchased from Calbiochem. The recombinant human cdMT1-MMP (catalytic domain of MT1-MMP; Ile^{114}–Ile^{318}) was expressed in *Escherichia coli* and was activated by autocatalysis [18] and ΔTM-MT1-MMP (transmembrane-domain-deleted MT1-MMP), the recombinant human ectodomain of MT1-MMP (Tyr^{112}–Glu^{523} consisting of the catalytic domain and the haemopexin domain) was expressed in *Pichia pastoris* and activated by yeast proteinases during maturation [19]. Human TIMP-2 (tissue inhibitor of metalloproteinase-2) was kindly provided by Professor Jack Windsor of Indiana University (Indianapolis, IN, U.S.A.). All standard chemicals were purchased from Fisher with the exception of 5,5′-dithio(bis-2-nitrobenzoate), which was from Sigma.

Enzyme kinetics

Kinetic assays were performed as described previously [20] in 50 mM Hepes buffer (pH 7.5), with 10 mM $CaCl_2$, 0.2 M NaCl and 0.05 % Brij-35 (polyoxyethylene lauryl ether) at 25 °C. The final substrate concentration was 1 μM (1 % DMSO), which is at least 10-fold lower than K_M. Hence k_{cat}/K_M is calculated using the equation:

$$\frac{k_{cat}}{K_M} = \frac{v}{[E][S]}$$

where v is the initial rate and [E] and [S] are the enzyme and substrate concentrations respectively. The activation energy E_a was determined by the Arrhenius equation:

$$\ln v = -\frac{E_a}{RT} + \ln A[ES]$$

where R is the universal/ideal gas constant, T the absolute temperature in Kelvin, A the collision constant/Arrhenius A factor/pre-exponential factor and [ES] the concentration of the enzyme–substrate complex. The initial rates were determined at 283 K and then at increments of 5 K up to 313 K. The fluorescence intensity was corrected for temperature using the standard Mca-Pro-Leu-OH (Bachem). For the inhibition assays, the inhibitors were incubated with enzyme for 15–30 min before adding substrate **1** to ensure equilibrium conditions. The inhibitor dissociation constant K_i was determined as described previously [21] using the Morrison equation [22]:

$$\frac{v_i}{v_o} = \frac{[E]_o + [I]_o - K_i^{app} + \sqrt{\left([I]_o + K_i^{app} - [E]_o\right)^2 + 4[E]_o K_i^{app}}}{2[E]_o}$$

where v_i and v_o are the initial rates with and without the inhibitor respectively, $[E]_o$ and $[I]_o$ are the initial enzyme and inhibitor concentrations respectively and K_i^{app} is the apparent K_i that is equal to the true K_i under the conditions of $[S] \ll K_M$ for competitive inhibition according to the equation:

$$K_i^{app} = K_i\left(1 + \frac{[S]}{K_M}\right)$$

The active mercaptosulphide inhibitor concentration was quantified with Ellman's reagent 5,5′-dithiobis-(2-nitrobenzoate) [23,24]. Active cdMT1-MMP and ΔTM-MT1-MMP concentrations were normalized by titration with standardized preparations of recombinant TIMP-2. The titration was performed as described above for determination of K_i with an $[E]/K_i$ ratio > 100 to ensure proper titrating conditions.

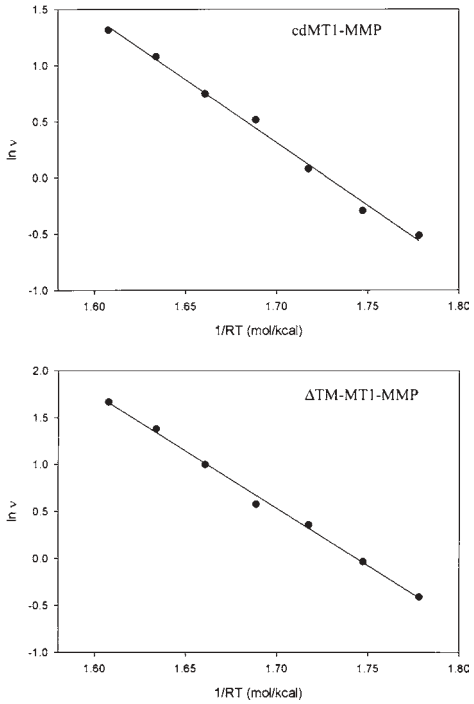

Figure 1 Arrhenius plot

E_a values were determined for the hydrolysis of substrate **1** with (**A**) cdMT1-MMP and (**B**) ΔTM-MT1-MMP as described in the Materials and methods section. Initial rates are expressed in nM/min. Fluorescence intensity was temperature-corrected. The slope of the line $-E_a$ (kcal/mol) was 11.2 and 12.2 for (**A**) and (**B**) respectively.

Collagen hydrolysis and electrophoresis

Samples containing 280 μg/ml purified RTTI (rat-tail tendon type I collagen) [20] were incubated with 80 nM enzyme at 37 °C in 50 mM Hepes (pH 7.5), 10 mM $CaCl_2$, 0.2 M NaCl and 0.05 % Brij-35. The reactions were stopped with 50 mM EDTA, 100 mM dithiothreitol and boiled for 5 min. The samples were analysed by SDS/PAGE (7 % gel) with silver staining. The cdMT1-MMP and ΔTM-MT1-MMP samples were visualized by a Western blot (12 % SDS/polyacrylamide gel) using a previously characterized antibody [3].

RESULTS

Substrate specificity

The catalytic domain alone is predicted to hydrolyse small peptide substrates more efficiently than ΔTM-MT1-MMP if the active sites are identical, possibly due to flexibility, rotational freedom or substrate exosite binding. The activation energy for enzyme hydrolysis of peptides should therefore be lower for cdMT1-MMP when compared with ΔTM-MT1-MMP. The E_a values for substrate **1** (see Table 1) with cdMT1-MMP and ΔTM-MT1-MMP were determined to be 11.2 and 12.2 kcal/mol respectively (Figure 1). Consistently, the k_{cat}/K_M values were 7.37 and 1.46×10^4 $M^{-1} \cdot s^{-1}$ respectively (Table 1). If the peptide binds to a haemopexin exosite region, the effective substrate concentration would be reduced, subsequently lowering the apparent k_{cat}/K_M.

Table 1 Comparison of substrate specificity

Fluorogenic peptide substrates	k_{cat}/K_M ($\times 10^4$ $s^{-1} \cdot M^{-1}$) cdMT1	k_{cat}/K_M ($\times 10^4$ $s^{-1} \cdot M^{-1}$) ΔTM
1 Mca-Pro-Leu-Gly ↓ Leu-Dpa-Ala-Arg-NH_2	7.37	1.46
2 Mca-Pro-Leu-Ala ↓ Nva-Dpa-Ala-Arg-NH_2	2.27	0.25
3 Mca-Pro-Lys-Pro-Leu-Ala ↓ Leu-Dpa-Ala-Arg-NH_2	7.82	1.04
4 Mca-Arg-Pro-Lys-Pro-Tyr-Ala ↓ Nva-Trp-Met-Lys-Dpa-NH_2	1.81	0.15
5 Mca-Pro-Leu-Ala ↓ Gln-Ala-Val-Dpa-Arg-Ser-Ser-Arg-NH_2	0.18	0.02
6 {(GPP*)$_5$GPK(Mca)GPQG ↓ LRGQK(Dnp)GVR(GPP*)$_5$-NH_2}$_3$*	5.12	2.18

* Single-letter amino acid abbreviations are used here. P* stands for 4-hydroxy-L-proline. The arrow indicates the putative cleavage site.

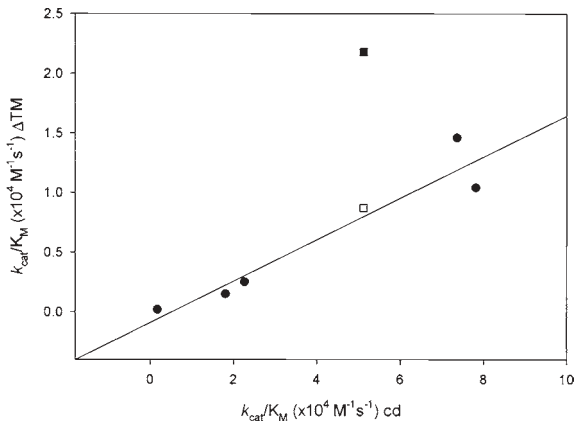

Figure 2 Correlation of specificity constants

The k_{cat}/K_M values from Table 1 for the single-stranded peptides **1**–**5** were plotted (●) and found to have a slope of 0.17. The triple-helical substrate **6** was predicted to have a k_{cat}/K_M value of 0.87×10^4 $M^{-1} \cdot s^{-1}$ for ΔTM-MT1-MMP (□) based on the value of 5.12×10^4 $M^{-1} \cdot s^{-1}$ for cdMT1-MMP. The actual value was determined to be 2.5-fold higher, i.e. 2.18×10^4 $M^{-1} \cdot S^{-1}$ (■).

Since the E_a and k_{cat}/K_M values correlate, exosite binding is probably not a valid reason for differences in hydrolytic efficiency. These results suggest that cdMT1-MMP is slightly more efficient than ΔTM-MT1-MMP for hydrolysing single-stranded peptides as predicted.

This trend was also noted with other similar peptides and a correlation curve was constructed with the k_{cat}/K_M values giving a slope of 0.17 (Table 1 and Figure 2). A prediction of k_{cat}/K_M values for ΔTM-MT1-MMP may then be found by multiplying the k_{cat}/K_M values for cdMT1-MMP by 0.17. The fluorescently labelled triple-helical peptide that was characterized previously [15] was tested with cdMT1-MMP and was found to have a k_{cat}/K_M value of 5.12×10^4 $M^{-1} \cdot s^{-1}$. Assuming this substrate is hydrolysed similarly to the single-stranded peptides, the predicted k_{cat}/K_M value would be 0.87×10^4 $M^{-1} \cdot s^{-1}$ for ΔTM-MT1-MMP (Figure 2, open square); however, the actual value was determined to be 2.18×10^4 $M^{-1} \cdot s^{-1}$, a value 2.5-fold higher (Figure 2, closed square).

Probing the active site

The difference in the predicted and actual values for triple-helical substrate hydrolysis by ΔTM-MT1-MMP may be due to structural differences at the active site. To probe the active-site structure, the inhibition profiles for cdMT1-MMP and ΔTM-MT1-MMP were compared with diastereomeric inhibitor pairs (Figure 3 and Table 2). The correct stereochemistry of these inhibitors is

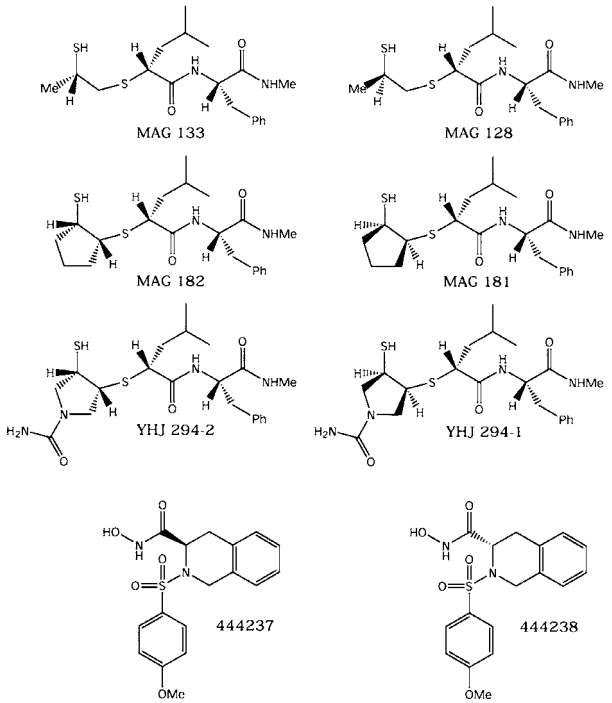

Figure 3 Inhibitor structures

The inhibitors are shown as pairs of diastereomers with the more potent inhibitor on the left. K_i values are shown in Table 2.

Table 2 K_i values of the inhibitor

Inhibitor	cdMT1 K_i (nM)	cdMT1 K_i ratio of the isomer	ΔTM-MT1 K_i (nM)	ΔTM-MT1 K_i ratio of the isomer
MAG 133	19	4	26	6
MAG 128	70		150	
MAG 182	24	11	29	19
MAG 181	259		540	
YHJ 294-2	13	231	24	242
YHJ 294-1	3000		5800	
444237	0.5	90	0.5	110
444238	45		55	

important for potency towards MT1-MMP as noted by the large differences in K_i values between inhibitor pairs. The K_i values were generally lower for cdMT1-MMP when compared with those for ΔTM-MT1-MMP, which is consistent with the peptide studies. Interestingly, the ratios of K_i values for diastereomers correlated with a slope of 1.03 between cdMT1-MMP and ΔTM-MT1-MMP (Figure 4). These results demonstrate that the haemopexin domain has no direct influence on the structure of the active site of the enzyme.

To ensure that ΔTM-MT1-MMP is a fully functional enzyme (i.e. ability to cleave collagen), RTTI was utilized as a substrate. Figure 5(B) shows the ability of this enzyme form to degrade native RTTI at 37 °C (three-quarter and one-quarter fragments not shown). The RTTI was not hydrolysed by cdMT1-MMP under the same conditions (reaction was also followed overnight with the same results; not shown). The same assay was performed with RTTI preparations that were first boiled for 5 min. Both the enzymes rapidly degraded the denatured RTTI (gelatin) samples. These results demonstrate that both enzyme forms maintain hydrolytic characteristics consistent with published reports.

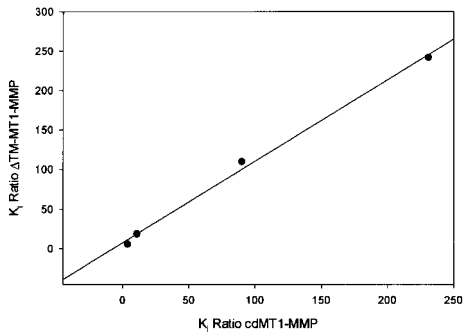

Figure 4 Correlation of diastereomer K_i ratio

The ratios from Table 2 were plotted and found to have a correlation of 1.03 ($r^2 = 0.9961$).

DISCUSSION

The haemopexin domain in MMPs is necessary for the cleavage of native interstitial type I–III collagens by mechanisms that are not completely understood [7,8]. In this study, the substrate specificity and inhibition of cdMT1-MMP was compared with ΔTM-MT1-MMP. The enzyme concentrations were normalized by titration with TIMP-2. Both forms of the enzyme were found to be fully active, as demonstrated by hydrolysis of either native or denatured RTTI, in addition to showing a single band on a Western blot. Although collagenolytic assays are typically performed at 25 °C, no cleavage was detected after incubation for up to 2 days at room temperature (23 °C). This is consistent with an earlier study that showed MT1-MMP does not hydrolyse type I collagen at 25 °C [13], although other groups have demonstrated cleavage [12,14]. The assay temperature was therefore increased to 37 °C to allow more flexibility in the collagen strands that are more readily hydrolysed. ΔTM-MT1-MMP alone is not stable after prolonged incubation at 37 °C; however, in the presence of a substrate (or an inhibitor), the stability is significantly improved (results not shown).

It may be expected that if the active sites were the same, the catalytic domain alone would be a more efficient enzyme to hydrolyse single-stranded peptides due to hindrance by the haemopexin domain. This domain may limit enzyme flexibility, diffusion of peptide into the enzyme-active site or alternatively decrease effective substrate concentration by exosite binding. The activation energies for substrate **1** are consistent with k_{cat}/K_M values which may suggest enzyme flexibility or diffusion as possible reasons for the difference. The k_{cat}/K_M values for five similar single-stranded peptides were correlated between both forms of the enzyme. The k_{cat}/K_M value for triple-helical substrates will correspond to this fit if the enzyme hydrolyses these substrates in a manner similar to the single-stranded substrates. Although the absolute k_{cat}/K_M values were slightly higher for cdMT1-MMP, the values did not correlate with the single-stranded substrates. Therefore the haemopexin domain of MT1-MMP facilitates the hydrolysis of triple-helical peptide substrates, consistent with previous studies, indicating that the haemopexin domain of

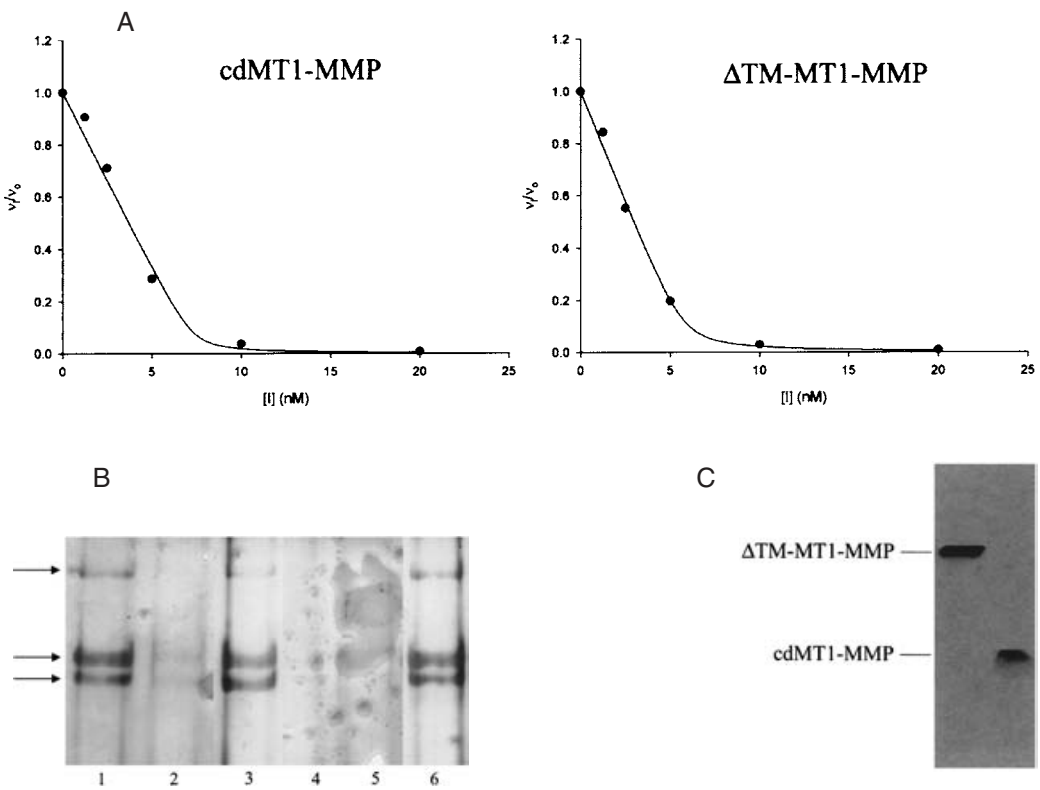

Figure 5 Enzyme titration and collagen cleavage

(**A**) The concentrations of active cdMT1-MMP and ΔTM-MT1-MMP were normalized by titration with TIMP-2 as described in the Materials and methods section: [E] = [I] at the *x*-intercept. (**B**) Collagen cleavage was verified for ΔTM-MT1-MMP with RTTI. Either ΔTM-MT1-MMP (lanes 2 and 4) or cdMT1-MMP (lanes 3 and 5) at 80 nM was incubated with 280 μg/ml RTTI (lanes 1–3 native; lanes 4–6 denatured by first boiling for 5 min) at 37 °C for 8 h. The reactions were stopped with 50 mM EDTA, 100 mM dithiothreitol and boiling for 5 min. The samples were analysed by SDS/PAGE (7 % gel) with silver staining. Lane 1 is native RTTI only and lane 6 is denatured RTTI only. (**C**) Western blot (SDS/PAGE, 12 % gel) of the purified enzymes shows one band for the ΔTM-MT1-MMP sample.

MT1-MMP modulates activity towards proteinaceous substrates [25].

Diastereomeric inhibitor pairs were utilized to probe the architecture of the enzyme-active site. Structural differences between the enzyme forms would be expected to be shown by different K_i ratios for inhibition by the pairs of stereoisomeric compounds tested. A correlation of 1 between the enzymes demonstrates that no significant differences were found at the active site. Although several groups have published the requirement of the haemopexin domain to hydrolyse native type I–III collagens, no previous report has addressed an active-site comparison for MT1-MMP with and without the haemopexin domain.

The triple-helical peptidase activities of collagenolytic enzymes (MMP-1, MMP-13 and MT1-MMP) exhibit noteworthy differences. MT1-MMP shows triple-helical peptidase activity much greater than either MMP-1 or MMP-13 [15]. The haemopexin domain of MMP-1 appears to have virtually no influence on triple-helical peptidase activity [15,26], unlike MT1-MMP. MMP-13 shows a far greater difference between single-stranded and triple-helical substrate hydrolysis compared with either MMP-1 or MT1-MMP [15,27]. Thus, although a number of MMP family members are classified as 'collagenolytic', their precise mechanisms for binding, distorting and hydrolysing triple-helical structures are almost certainly different in subtle, but as yet undefined, ways.

One important question remaining is how does the haemopexin domain influence the catalytic activity of MT1-MMP? It has been proposed that triple-helical peptidase and collagenase activity are distinguishable, as the catalytic domains of collagenases have the ability to cleave triple-helical peptides but not collagens [26]. The haemopexin domain may therefore be required for proper orientation and distortion of the collagen. Overall and co-workers [11] recently found that it is the linker region between the catalytic and haemopexin domain that is necessary for native type I collagen binding. The linker region may influence triple-helicase activity. Without knowing the three-dimensional structural organization of the linker and haemopexin domain, it is difficult to address specifically how the activities of MMPs are modulated by their structural motifs and domains.

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Endometase/Matrilysin-2 in Human Breast Ductal Carcinoma *in Situ* and Its Inhibition by Tissue Inhibitors of Metalloproteinases-2 and -4: A Putative Role in the Initiation of Breast Cancer Invasion

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ABSTRACT

Local disruption of the integrity of both the myoepithelial cell layer and the basement membrane is an indispensable prerequisite for the initiation of invasion and the conversion of human breast ductal carcinoma *in situ* (DCIS) to infiltrating ductal carcinoma (IDC). We previously reported that human endometase/matrilysin-2/matrix metalloproteinase (MMP) 26-mediated pro-gelatinase B (MMP-9) activation promoted invasion of human prostate carcinoma cells by dissolving basement membrane proteins (Y. G. Zhao *et al.*, J. Biol. Chem., *278:* 15056–15064, 2003). Here we report that tissue inhibitor of metalloproteinases (TIMP)-2 and TIMP-4 are potent inhibitors of MMP-26, with apparent K_i values of 1.6 and 0.62 nM, respectively. TIMP-2 and TIMP-4 also inhibited the activation of pro-MMP-9 by MMP-26 *in vitro*. The expression levels of MMP-26, MMP-9, TIMP-2, and TIMP-4 proteins in DCIS were significantly higher than those in IDC, atypical intraductal hyperplasia, and normal breast epithelia adjacent to DCIS and IDC by immunohistochemistry and integrated morphometry analysis. Double immunofluorescence labeling and confocal laser scanning microscopy revealed that MMP-26 was colocalized with MMP-9, TIMP-2, and TIMP-4 in DCIS cells. Higher levels of MMP-26 mRNA were also detected in DCIS cells by *in situ* hybridization.

INTRODUCTION

Matrix metalloproteinases (MMPs) are known to be associated with cancer cell invasion, growth, angiogenesis, inflammation, and metastasis (1, 2). MMP-26 is a novel enzyme that was recently cloned and characterized by our group (3) and others (4–6). It has several structural features characteristic of MMPs, including a signal peptide, a propeptide domain, and a catalytic domain with a conserved zinc-binding motif, but it lacks the hemopexin-like domain (3–6). A unique "cysteine switch" sequence in the prodomain, PHCGVPD as opposed to the conserved PRCG*XX*D sequence found in many other MMPs, keeps the enzyme latent.

MMP-26 mRNA is primarily expressed in cancers of epithelial origin, such as endometrial carcinomas (3, 7), prostate carcinomas (7), lung carcinomas (7), and their corresponding cell lines (3–6), and in a small number of normal adult tissues, such as the uterus (3, 5), placenta (4, 5), and kidney (6). Some parallels exist with MMP-7, which is also expressed epithelially and also lacks the hemopexin-like domain. We have also reported that the levels of *MMP-26 gene* and protein expression are higher in a malignant choriocarcinoma cell line (JEG-3) than in normal human cytotrophoblast cells (8). Recently, we found that the levels of MMP-26 protein in human prostate carcinomas from multiple patients were significantly higher than those in prostatitis, benign prostate hyperplasia, and normal prostate tissues (9). MMP-26 is capable of activating pro-MMP-9 by cleavage at the Ala^{93}-Met^{94} site of the pro-enzyme, and this activation facilitates the efficient cleavage of fibronectin (FN), promoting the invasion of highly invasive and metastatic androgen-repressed prostate cancer cells through FN or type IV collagen (9). The activation is prolonged but persistent, which is consistent with the process of tumor cell invasion. These findings indicate that MMP-26-mediated pro-MMP-9 activation may be one biochemical mechanism contributing to human carcinoma cell invasion *in vivo*.

MMP activities are inhibited by endogenous tissue inhibitors of metalloproteinases (TIMPs). Four mammalian TIMPs have been identified: (*a*) TIMP-1 (10); (*b*) TIMP-2 (11); (*c*) TIMP-3 (12); and (*d*) TIMP-4 (13). The hydrolytic activity of MMP-26 against synthetic peptides is blocked by TIMP-1, TIMP-2, and TIMP-4 (5, 6, 8), but the inhibitory potential of TIMP-1 is lower than that of TIMP-2 and TIMP-4 (5). TIMP-1 and TIMP-2 also inhibit the cleavage of denatured type I collagen (gelatin) by MMP-26 (6). TIMPs are expressed in human breast cancer cells (14–16). Here, we continue to explore the possible roles of MMP-26 and the coordination of MMP-26 with MMP-9, TIMP-2, and TIMP-4 in human breast carcinoma invasion.

In the present study, we showed that TIMP-2 and TIMP-4 completely inhibited the activation of pro-MMP-9 by MMP-26. The expressions of MMP-26, MMP-9, TIMP-2, and TIMP-4 proteins in human breast ductal carcinomas *in situ* (DCIS) were significantly higher than those in infiltrating ductal carcinoma (IDC), atypical intraductal hyperplasia (AIDH), and normal breast epithelia around the DCIS and IDC. Furthermore, MMP-26 was colocalized with MMP-9, TIMP-2, and TIMP-4 in human breast DCIS.

MATERIALS AND METHODS

Inhibition Assays of MMP-26 by TIMP-2 and TIMP-4. The quenched fluorescence peptide substrates, *Mca-Pro-Leu-Ala-Nva-Dpa-Ala-Arg-*NH_2 and *Mca-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(Dnp)-*NH_2 were purchased from Calbiochem. The MMP-26 used in this experiment is recombinant and partially active. Briefly, MMP-26 was expressed in the form of inclusion bodies from transformed *Escherichia coli* cells as described previously (3). The inclusion bodies were isolated and purified using B-PER bacterial protein extraction reagent according to the manufacturer's instructions. The insoluble protein was dissolved in 8 M urea to ~5 mg/ml. The protein solution was diluted to ~100 μg/ml in 8 M urea and 10 mM DTT for 1 h; dialyzed in 4 M urea, 1 mM DTT, and 50 mM HEPES (pH 7.5) for at least 1 h; and then folded by dialysis in 1× HEPES buffer [50 mM HEPES, 0.2 M NaCl, 10 mM $CaCl_2$, and 0.01% Brij-35 (pH 7.5)] with 20 μM $ZnSO_4$ for 16 h. To enhance the activity of MMP-26, the folded enzyme was dialyzed twice for 24 h at 4°C in the folding buffer without Zn^{2+} ions. The total enzyme concentration was measured by UV absorption using ϵ_{280} = 57,130 M^{-1} cm^{-1}, which was

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calculated using Genetics Computer Group software. The concentration of active MMP-26 was determined by active site titration with the tight-binding inhibitor GM-6001 as described previously (17). GM-6001 was the most potent inhibitor of MMP-26 tested, with a K_i^{app} of 0.36 nM (17). Because TIMPs are tight-binding and slow-binding inhibitors of MMPs, MMP-26 was incubated for 4 h with human TIMP-2 and TIMP-4 before the measurement of substrate hydrolysis to allow the enzyme and inhibitor to reach their binding equilibrium. Human fibroblast TIMP-2 was provided by Dr. L. Jack Windsor (Indiana University, Indianapolis, IN). Recombinant human TIMP-4 was purchased from R&D Systems (Minneapolis, MN). The concentrations of TIMPs ranged from 0.2 to 60 nM. The assay was initiated by the addition of a substrate stock solution (4 μl) prepared in 1:1 water and DMSO to an enzyme-inhibitor assay buffer (196 μl) for a final concentration of 1 μM. The release of the fluorogenic cleavage product was monitored by measuring fluorescence (excitation and emission wavelengths at 328 and 393 nm, respectively) with a Perkin-Elmer Luminescence Spectrophotometer LS 50B connected to a water bath with a temperature control. All kinetic experiments were conducted in 1× HEPES buffer. Fluorogenic peptide substrate assays were performed following the procedures we reported previously (17). To assess inhibition potency, the apparent inhibition constants (apparent K_i values) were determined by fitting the two trial data sets to the Morrison equation below (18) with nonlinear regression. In this equation, ν_i is the initial rate of MMP-26 catalysis in the presence of inhibitor, and ν_o is the initial rate without inhibitor. [E] and [I] are the initial enzyme and inhibitor concentrations, respectively, and K_i^{app} is the apparent inhibition constant.

$$\frac{\nu_i}{\nu_o} = 1 - \frac{([E] + [I] + K_i^{app}) - \sqrt{([E] + [I] + K_i^{app})^2 - 4[E][I]}}{2[E]}$$

Pro-MMP-9 Activation by MMP-26 and Inhibition of the Activation by TIMP-2 and TIMP-4. Zymography and silver staining were performed as reported previously (3, 7, 19, 20). MMP-26, pro-MMP-9, and active MMP-9 were purified in our laboratory (3, 21). The molar concentration ratios of TIMPs, MMP-26, and pro-MMP-9 were 10:1:4. Two metal chelators/metalloproteinase inhibitors, 1, 10-phenanthroline and EDTA, were used as controls. Briefly, MMP-26 was incubated in the presence or absence of different inhibitors (TIMPs, 1,10-phenanthroline, and EDTA) in 30 μl of 1× HEPES buffer at room temperature (25°C) for 4 h. Pro-MMP-9 was then added and incubated at 37°C for 20 h. For zymography, aliquots of the reaction solution were removed and treated with a nonreducing sample buffer. MMP-9 activity was analyzed by zymography on 9% SDS-polyacrylamide gels containing 1% gelatin (22). For silver staining, aliquots were removed and treated with a reducing sample buffer and boiled for 5 min. After electrophoresis on 9% SDS-polyacrylamide gels, the protein bands were visualized by silver staining (19).

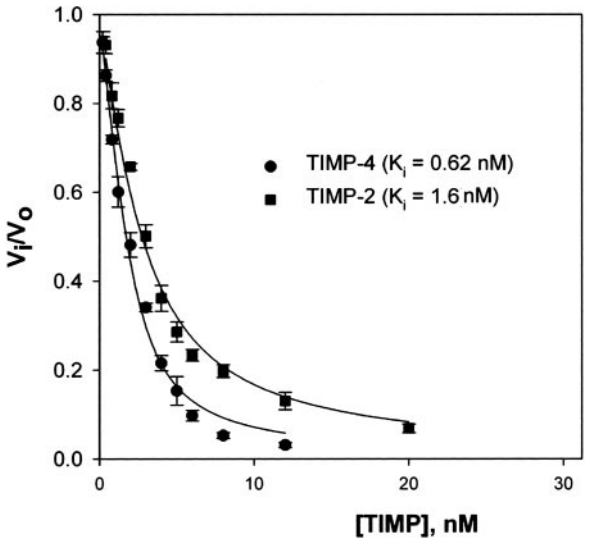

Fig. 1. Inhibition kinetics of matrix metalloproteinase 26 by tissue inhibitor of metalloproteinases (TIMP)-2 and TIMP-4. Matrix metalloproteinase 26 (2 nM) was incubated for 4 h in the presence of TIMP-2 or TIMP-4 at concentrations of 0.2–60 nM. The substrate hydrolysis assay was initiated by the addition of substrate stock solution. The data were fitted to the Morrison equation to calculate the apparent K_i values.

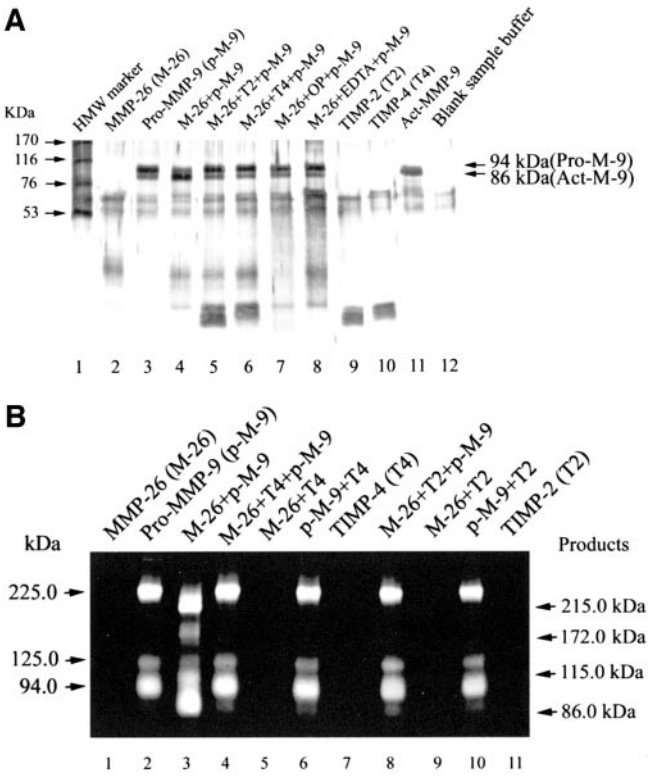

Fig. 2. Tissue inhibitor of metalloproteinases (TIMP)-2 and TIMP-4 inhibit pro-matrix metalloproteinase (MMP)-9 activation by MMP-26. *A*, pro-MMP-9 activation by MMP-26 was inhibited by both TIMP-2 and TIMP-4 (electrophoresis samples were treated under reducing conditions). MMP-26 activates pro-MMP-9, enhancing the 86-kDa band (*Lane 4*). This activation was completely blocked by adding TIMP-2 (*Lane 5*) or TIMP-4 (*Lane 6*). The inhibition by TIMP-2 and TIMP-4 is comparable with that of two broad-spectrum metal chelators/metalloproteinase inhibitors, 1,10-phenanthroline (*OP*) and EDTA (*Lanes 7* and *8*). *B*, zymogram assay of MMP-9 activation by MMP26 and inhibition by TIMP-2 and TIMP-4 (electrophoresis samples under nonreducing conditions). The 225-kDa band is a homodimer of pro-MMP-9, the 125-kDa band is a heterodimer of pro-MMP-9 and neutrophil gelatinase-associated lipocalin, and the 94-kDa band is a monomer of pro-MMP-9. MMP-26 activates pro-MMP-9 to generate new 215-, 172-, 115-, and 86-kDa active fragments (*Lane 3*). This activation was completely blocked by adding TIMP-4 (*Lane 4*) or TIMP-2 (*Lane 8*).

FN Cleavage Assay. MMP-26, pro-MMP-9, MMP-26-activated MMP-9, and TIMPs were prepared as described above. Active MMP-9, purified from human neutrophils (21), was used as a positive control. FN was incubated with MMP-26, pro-MMP-9, active MMP-9, or MMP-26 plus pro-MMP-9 in the presence or absence of TIMP-2 or TIMP-4 in 1× HEPES buffer at 37°C for 18 h. The molar concentration ratio of MMP-26:pro-MMP-9:FN:TIMP was approximately 1:4:10:10. Aliquots were removed and treated with a reducing sample buffer and boiled for 5 min. Samples were then loaded onto 9% polyacrylamide gels in the presence of SDS, electrophoresed, and subjected to silver staining (19).

***In Situ* Hybridization.** The DCIS samples were classified according to our previous reports (23, 24). Briefly, the formalin-fixed, paraffin-embedded samples were sectioned to 5-μm thickness and fixed onto slides. The full-length MMP-26 sense cDNA and antisense cDNA were amplified in pCR 3.1 and purified as described in our previous report (9). The sense and antisense plasmids were linearized with *Xho*I and *Xba*I, respectively. The sense and antisense digoxigenin-labeled RNA probes were generated by *in vitro* transcription with T7 polymerase. *In situ* hybridization was performed as per our previous report (22). Briefly, the paraffin-embedded sections (5 μm) were deparaffinized with xylene and treated with proteinase K solution [50 μg/ml in 0.2 M Tris-HCl (pH 7.5), 2 mM $MgCl_2$] for 15 min at room temperature. After prehybridization, the sections were hybridized to digoxigenin-labeled MMP-26 antisense cRNA probes for 18 h at 45°C and 100% humidity. The

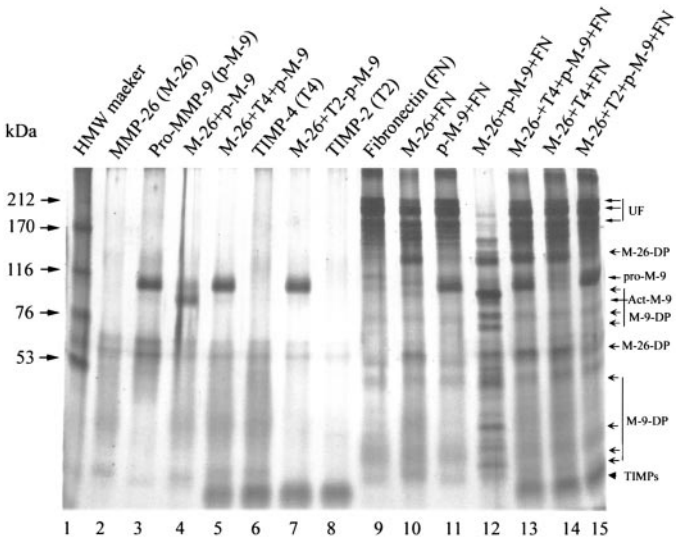

Fig. 3. Cleavage of fibronectin (FN) by matrix metalloproteinase (MMP)-9 and MMP-26. MMP-26 cleaved FN weakly, generating 125- and 58-kDa bands (*Lane 10*), pro-MMP-9 did not cleave FN (*Lane 11*), and MMP-26-activated MMP-9 (*Lane 4*) cleaved FN efficiently (*Lane 12*). Tissue inhibitor of metalloproteinases (TIMP)-4 weakly blocked FN cleavage by MMP-26 (*Lane 14*). Both TIMP-4 and TIMP-2 blocked pro-MMP-9 activation by MMP-26 (*Lanes 5* and *7*), which significantly diminished FN cleavage (*Lanes 13* and *15*). *UF*, uncleaved fibronectin; *M-26-DP*, MMP-26-degraded FN products; *M-9-DP*, MMP-9-degraded FN products; *pro-M-9*, pro-MMP-9; *Act-M-9*, activated MMP-9.

MMP-26 sense RNA probe was used under the same hybridization conditions as the control. After hybridization, the slides were washed with saline sodium citrate buffer and blocked [1% blocking reagents in Tris-buffered saline (pH 7.5)] for 30 min. The slides were then covered with anti-digoxigenin-alkaline phosphatase Fab fragments (1:400) for 2 h. Then the slides were stained with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science, Mannheim, Germany). The expression signals were photographed under a microscope.

Immunohistochemistry. The human breast DCIS, IDC, and hyperplasia tissue samples were classified according to our reports (23–26). Immunohistochemistry was performed on consecutive sections according to our previous report (9). Briefly, the formalin-fixed, paraffin-embedded samples were sectioned to 5-μm thickness and fixed onto slides. After dewaxing and rehydrating, the slides were blocked with 3% BSA/Tris-buffered saline for 1 h at room temperature before incubation with affinity-purified, polyclonal rabbit antihuman MMP-26, MMP-9, TIMP-2, and TIMP-4 antibodies (all 25 μg/ml) at room temperature for 90 min. Sections were then incubated with alkaline phosphatase-conjugated secondary antirabbit antibody (1:500; Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. The signals were detected with Fast-red (Sigma, St. Louis, MO). Purified preimmune IgGs from the same animal were used as negative controls.

Double Immunofluorescence and Confocal Laser Scanning Microscopy. Double immunofluorescence staining was performed as per our previous description (9). Briefly, the slides were incubated with a rabbit antihuman MMP-26 IgG (25 μg/ml) and a goat antihuman MMP-9 antibody (1:200 dilution; R&D Systems) or a mouse antihuman MMP-26 IgG (25 μg/ml; R&D Systems) and a rabbit antihuman TIMP-4 (25 μg/ml) or a rabbit antihuman TIMP-2 antibody (30 μg/ml) overnight at 4°C. The slides were then incubated with a goat antimouse-IgG IgG for 30 min at room temperature. Secondary Rhodamine Red-X-conjugated donkey antirabbit IgG and FITC-conjugated donkey antigoat IgG (Jackson ImmunoResearch) were subsequently applied at a 1:50 dilution for 30 min at room temperature. Slow Fade mounting medium was added to the slides, and fluorescence was analyzed using a Zeiss LSM510 Laser Scanning Confocal Microscope (Carl Zeiss, Heidelberg, Germany) equipped with a multi-photon laser. Images were processed for reproduction using Photoshop software version 6.0 (Adobe Systems, Mountainview, CA). Purified preimmune IgG and normal goat serum were used as negative controls.

Densitometric and Statistical Analysis. Four to 16 pictures were taken from the glandular epithelia after immunostaining by each of the four antibodies and the two preimmune IgGs in the DCIS, IDC, AIDH, and normal glands around the DCIS and IDC. Quantification of the immunostaining signals was performed using the Metamorph System (version 4.6r8; Universal Imaging Corp., Inc., West Chester, PA) according to our previous description (9). Briefly, an appropriate color threshold was determined (color model, HSI; hue, 230–255; saturation and intensity, full spectrum). The glandular epithelia

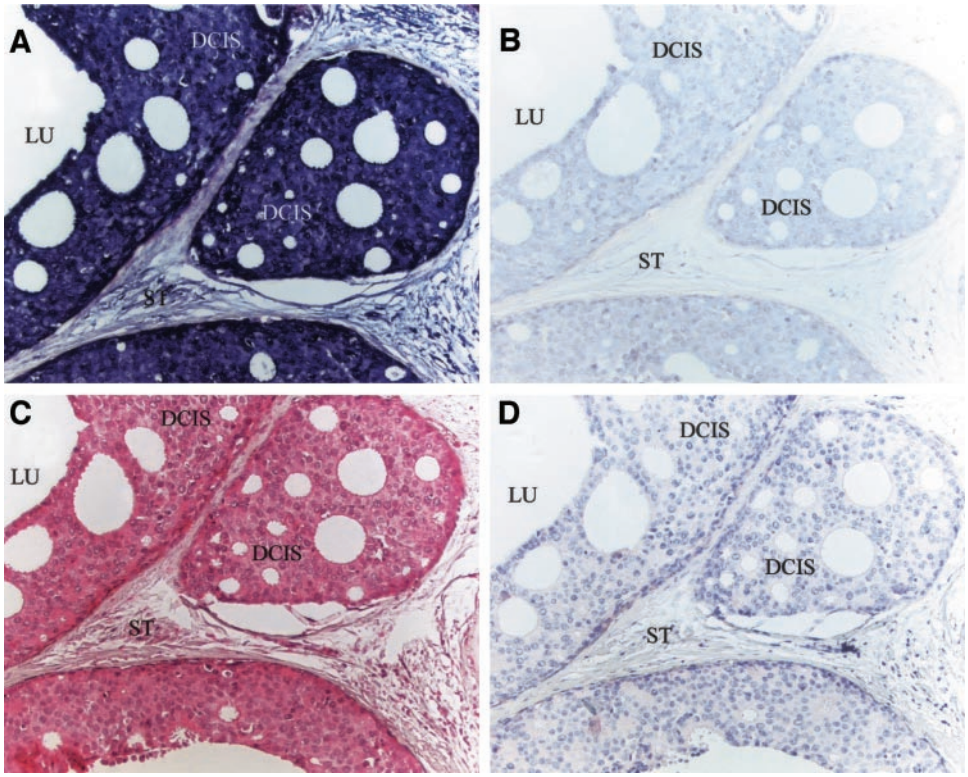

Fig. 4. Matrix metalloproteinase (MMP)-26 mRNA and protein expression in human mammary tissues. *A* and *B* are *in situ* hybridization to detect MMP-26 mRNA expression, and *C* and *D* are immunohistochemical staining to detect MMP-26 protein expression. *A*, MMP-26 antisense probe; *B*, MMP-26 sense probe; *C*, rabbit antihuman MMP-26 metallo domain IgG; *D*, pre-immune IgG. *Blue* indicates MMP-26 mRNA signals, and *red* indicates MMP-26 protein expression. Cells were counterstained lightly with hematoxylin for viewing of negatively stained epithelial and stromal cells in *C* and *D*. *DCIS*, ductal carcinoma *in situ*; *ST*, stroma; *LU*, lumen.

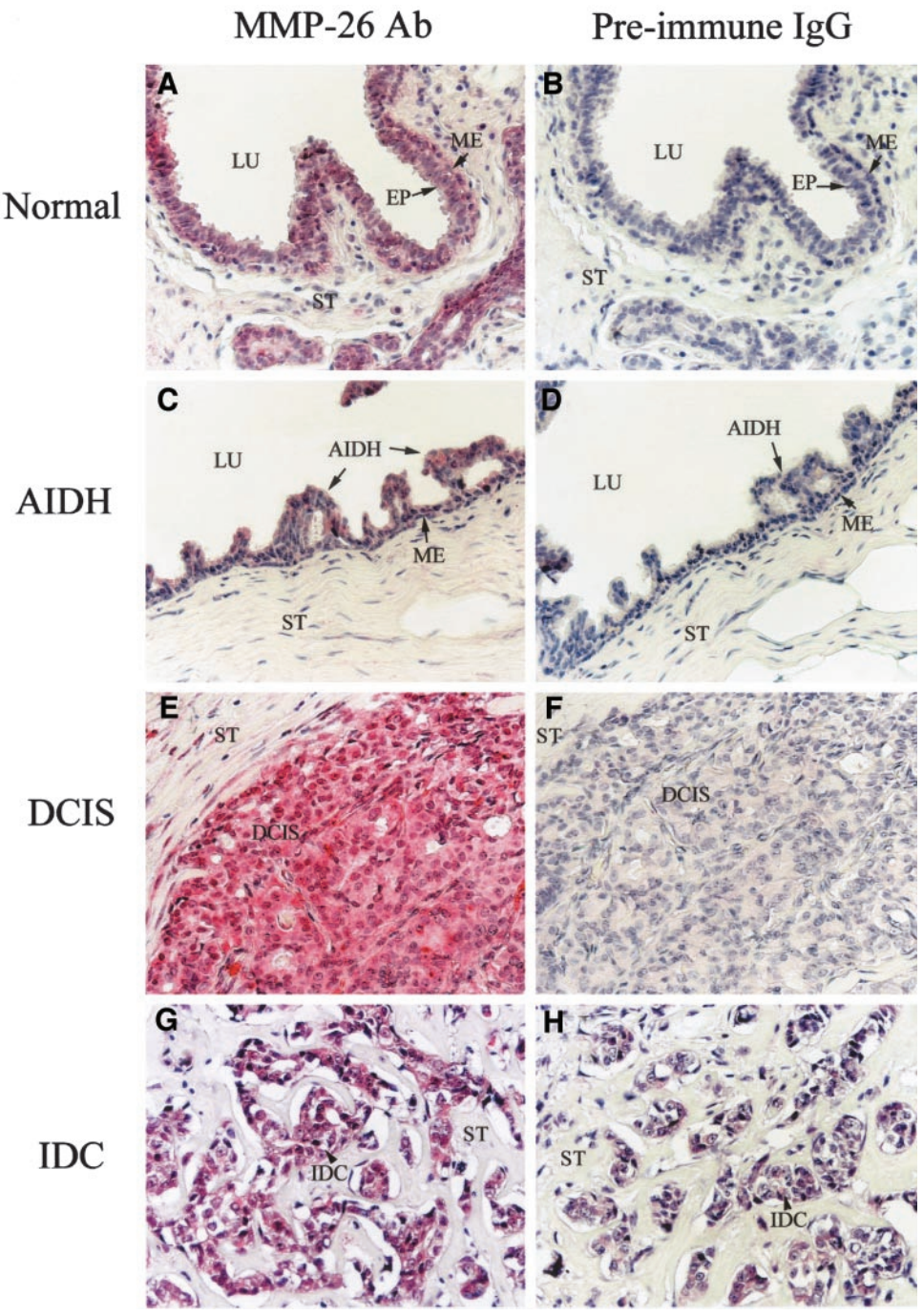

Fig. 5. Expression of matrix metalloproteinase (MMP)-26 protein in human mammary gland. Cells stained *red* indicate MMP-26 protein expression. All sections were counterstained lightly with hematoxylin for viewing negatively stained epithelial and stromal cells. *A*, *C*, *E*, and *G* are stained with anti-MMP-26 antibody, and *B*, *D*, *F*, and *H* are stained with pre-immune IgG. *A* and *B*, normal breast tissues; *C* and *D*, atypical intraductal hyperplasia (*AIDH*); *E* and *F*, breast ductal carcinoma *in situ* (*DCIS*); *G* and *H*, breast infiltrating ductal carcinoma (*IDC*). All figures are ×200 magnifications. *LU*, lumen; *EP*, epithelial cells; *ST*, stroma; *ME*, myoepithelial cells.

from each image were isolated into closed regions, and the signal areas and intensities of staining in compliance with the chosen parameters were measured by integrated morphometry analysis. The selected epithelial area was obtained by region measurement. The signal intensities were expressed as integrated absorbance (IOD, the sum of the optical densities of all pixels that make up the object). The ratio of the IOD to the selected epithelial area was determined, and the average ratios from each case were then calculated and used for statistical analysis. Statistical analysis of all samples was performed with the least significant difference correction of ANOVA for multiple comparisons. Data represent the mean ± SE, and $P < 0.05$ was considered significant.

RESULTS

Determination of the Apparent Inhibition Constants of TIMP-2 and TIMP-4 against MMP-26. The apparent K_i values of MMP-26 were measured and calculated to be 1.6 and 0.62 nM for TIMP-2 and TIMP-4, respectively (Fig. 1), using the Morrison equation (18). The apparent K_i values show that TIMP-4 is a slightly more potent inhibitor of MMP-26 than TIMP-2.

Activation of Pro-MMP-9 by MMP-26 and Inhibition of the Activation by TIMP-2 and TIMP-4. To explore the inhibition of MMP-26-mediated MMP-9 activation by TIMP-2 and TIMP-4, purified pro-MMP-9 and MMP-26 were incubated with these TIMPs, and the samples were subsequently analyzed by SDS-PAGE. MMP-26 cleaved pro-MMP-9 (94 kDa) to yield an enhanced active form 86-kDa band on a silver-stained gel under reducing conditions as per our recent report (Ref. 9; Fig. 2*A*, *Lane 4*). Zymography revealed that pro-MMP-9 presented as 225-, 125-, and 94-kDa gelatinolytic bands under nonreducing conditions. The 225-kDa band is a homodimer of pro-MMP-9, the 125-kDa band is a heterodimer of pro-MMP-9 and neutrophil gelatinase-associated lipocalin, and the 94-kDa band is a

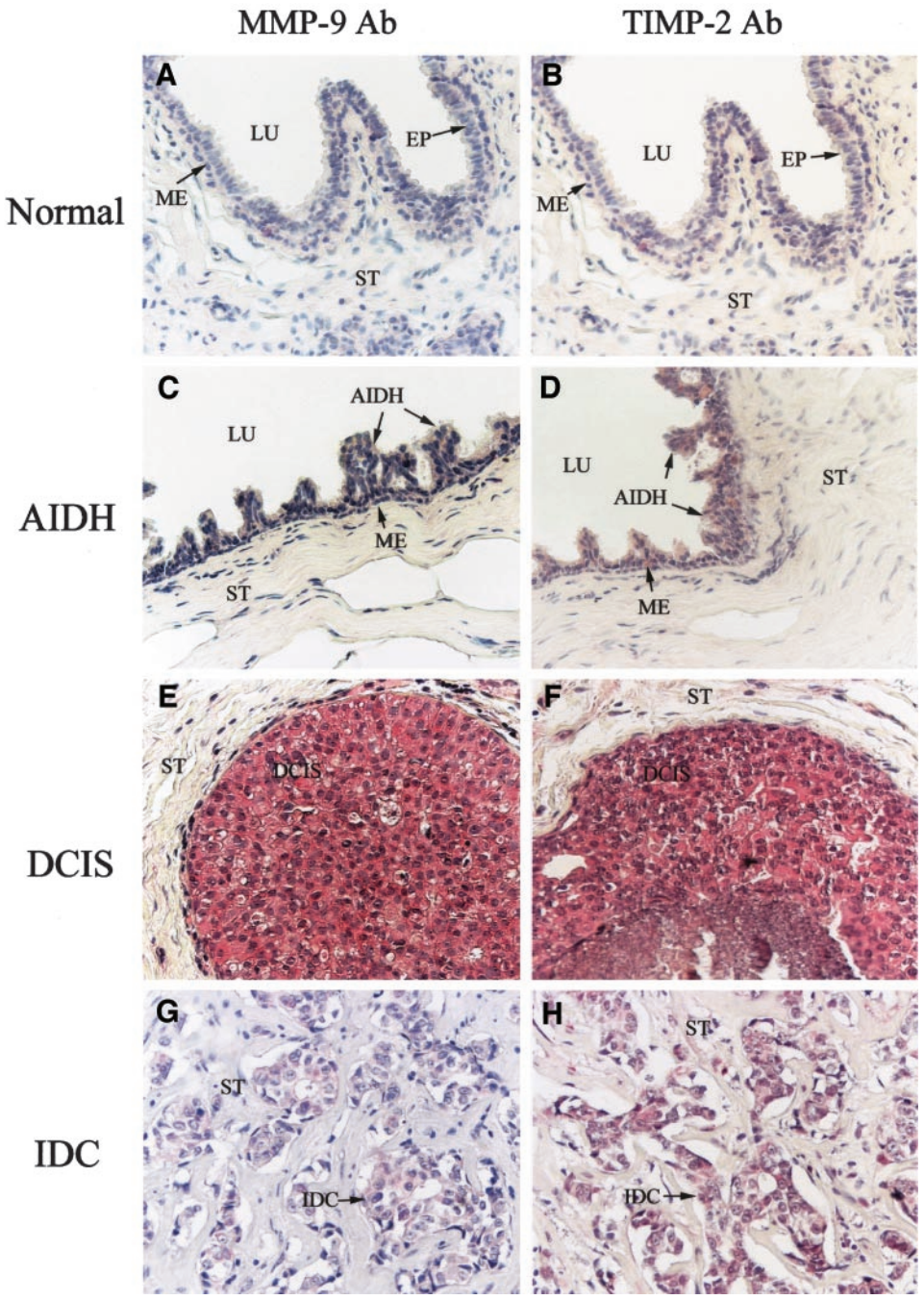

Fig. 6. Expression of matrix metalloproteinase (MMP)-9 and tissue inhibitor of metalloproteinases (TIMP)-2 proteins in human mammary gland. Cells stained *red* indicate MMP-9 or TIMP-2 protein expression. All sections were counterstained lightly with hematoxylin for viewing negatively stained epithelial and stromal cells. *A*, *C*, *E*, and *G* are stained with anti-MMP-9 antibody. *B*, *D*, *F*, and *H* are stained with TIMP-2 antibody. *A* and *B*, normal breast tissues; *C* and *D*, atypical intraductal hyperplasia (*AIDH*); *E* and *F*, ductal carcinoma *in situ* (*DCIS*); *G* and *H*, infiltrating ductal carcinoma (*IDC*). All figures are ×200 magnifications. *LU*, lumen; *EP*, epithelial cells; *ST*, stroma; *ME*, myoepithelial cells.

monomer of pro-MMP-9 (21, 27, 28). The new active 215-, 172-, 115-, and 86-kDa bands were generated after incubation with MMP-26 (Fig. 2*B*, *Lane 3*). The 215-, 115-, and 86-kDa bands are the active forms of the 225-, 125-, and 94-kDa forms, respectively. The 172-kDa band is a dimer of the 86-kDa forms. The activation of pro-MMP-9 by MMP-26 was completely inhibited by recombinant TIMP-2 and TIMP-4 (Fig. 2*A*, *Lanes 5* and *6*; Fig. 2*B*, *Lanes 4* and *8*). The blocking efficiencies of TIMP-2 and TIMP-4 were comparable with those of two broad-spectrum metal chelators/metalloproteinase inhibitors, 1,10-phenanthroline and EDTA (Fig. 2*A*, *Lanes 7* and *8*).

To further confirm the inhibition of MMP-26-mediated pro-MMP-9 activation by TIMP-2 and TIMP-4, *in vitro* FN cleavage assays were performed. MMP-26 slowly cleaved FN to generate 125- and 58-kDa bands (Fig. 3, *Lane 10*), whereas pro-MMP-9 did not cleave FN (Fig. 3, *Lane 11*). However, MMP-26-activated MMP-9 cleaved FN completely, generating at least seven new bands (Fig. 3, *Lane 12*). Both TIMP-2 and TIMP-4 completely blocked the activation of pro-MMP-9 by MMP-26, which subsequently resulted in inhibition of FN cleavage (Fig. 3, *Lanes 13* and *15*).

Expression of MMP-26 mRNA in Human Breast Tissues. *In situ* hybridization showed that MMP-26 mRNA was localized in human breast DCIS (Fig. 4*A*). On a serial section of the same tissue, MMP-26 protein was also detected in human breast DCIS (Fig. 4*C*). The MMP-26 sense probe and pre-immune IgG from the same animal as the MMP-26 antibody were used as controls (Fig. 4, *B* and *D*).

Expressions of MMP-26, MMP-9, TIMP-2, and TIMP-4 Proteins in Human Breast Tissues. MMP-26, MMP-9, TIMP-2, and TIMP-4 proteins were detected in human breast epithelia (Figs. 5–7). The expressions of the four proteins were extremely high in human breast DCIS (20 cases) cells (Fig. 5*E*; Fig. 6, *E* and *F*; Fig. 7*E*) but

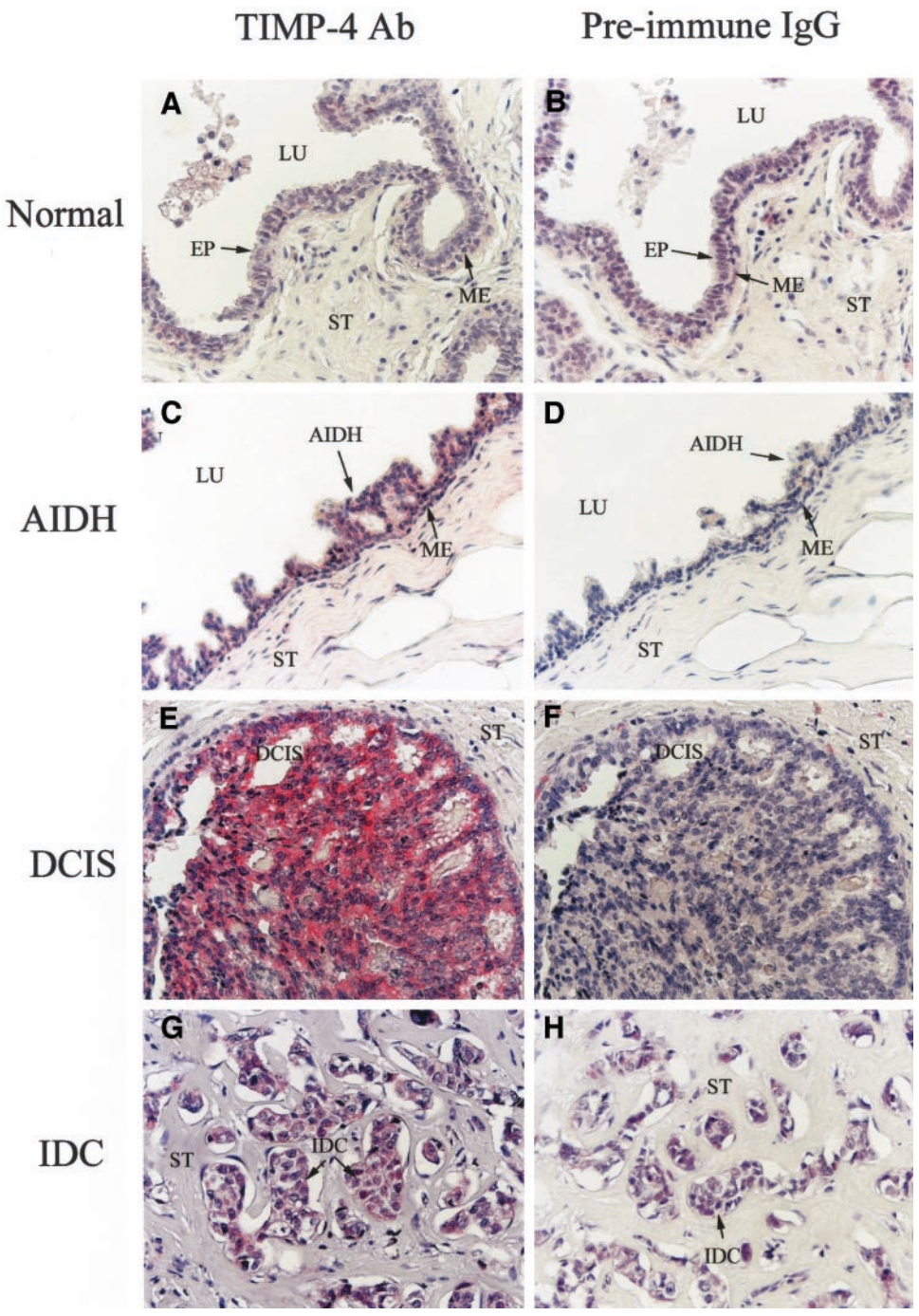

Fig. 7. Expression of tissue inhibitor of metalloproteinases (TIMP)-4 protein in human mammary gland. Cells stained *red* indicate TIMP-4 protein expression. All sections were counterstained lightly with hematoxylin for viewing negatively stained epithelial and stromal cells. *A*, *C*, *E*, and *G* are stained with antihuman TIMP-4 antibody. *B*, *D*, *F*, and *H* are stained with pre-immune IgG. *A* and *B*, normal breast tissues; *C* and *D*, atypical intraductal hyperplasia (*AIDH*); *E* and *F*, ductal carcinoma *in situ* (*DCIS*); *G* and *H*, infiltrating ductal carcinoma (*IDC*). All figures are ×200 magnifications. *LU*, lumen; *EP*, epithelial cells; *ST*, stroma; *ME*, myoepithelial cells.

very low in the normal glandular epithelial cells (25 cases) around DCIS and IDC (Fig. 5*A*; Fig. 6, *A* and *B*; Fig. 7*A*) and also in AIDH [15 cases (Fig. 5*C*; Fig. 6, *C* and *D*; Fig. 7*C*)]. Their expressions were substantially decreased in IDC [23 cases (Fig. 5*G*; Fig. 6, *G* and *H*; Fig. 7*G*)]. Statistical analysis revealed that the signal intensities of MMP-26, MMP-9, TIMP-2, and TIMP-4 proteins in DCIS were significantly higher than those in IDC, AIDH, and normal epithelia around the DCIS and IDC ($P < 0.05$ or $P < 0.01$; Fig. 8). There was no significant difference for the signals of MMP-26, MMP-9, TIMP-2, and TIMP-4 proteins among the normal epithelia around the DCIS and IDC, or in the AIDH and IDC samples ($P > 0.05$; Fig. 8). Pre-immune IgGs from the same animals as the MMP-26 or TIMP-4 antibodies were used as controls (Fig. 5, *B*, *D*, *F*, and *H*; Fig. 7, *B*, *D*, *F*, and *H*). There was no significant difference ($P > 0.05$) for the pre-immune IgG signals among the normal, AIDH, DCIS, and IDC samples.

Coexpression of MMP-26 and MMP-9 in Human Breast Carcinoma. To confirm the distributions of MMP-26, MMP-9, TIMP-2, and TIMP-4 within carcinoma cells, double immunofluorescence staining assays were performed in human breast DCIS samples. MMP-26 protein was localized mainly in the cytoplasm of the cancerous cells (Fig. 9*A*, *red*; Fig. 9*B*, *green*), which is consistent with our previous report (9). MMP-9 was localized both in the cytoplasm of the cancerous cells and at the cell surface, mainly on the cell membrane (Fig. 9*C*, *green*). The merged picture shows that MMP-26 and MMP-9 were coexpressed in the cytoplasm of the cancerous cells (Fig. 9*E*, *yellow*). The high magnification pictures in Fig. 9, *A*, *C*, and *E*, clearly demonstrate MMP-26 and MMP-9 colocalization (indicated by *ar-*

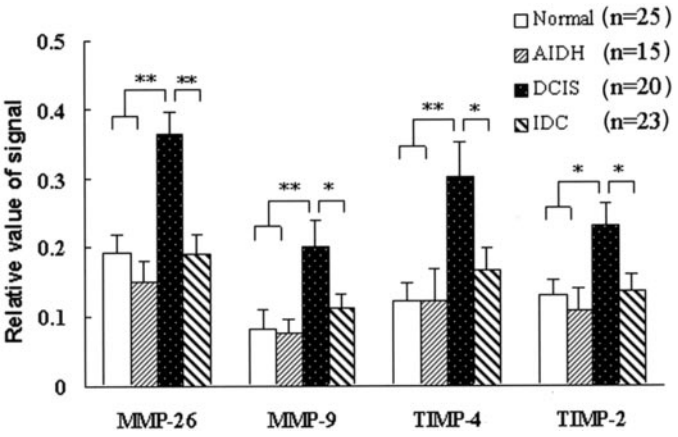

Fig. 8. Densitometric quantification analyses of matrix metalloproteinase 26, matrix metalloproteinase 9, tissue inhibitor of metalloproteinases 2, and tissue inhibitor of metalloproteinases 4 protein expression. The quantification analysis was described in "Materials and Methods." The epithelial regions were isolated, and the staining area and total selected area were obtained by Integrated Morphometry Analysis (IMA) and analyzed by one-way ANOVA with least significant difference (LSD) correction. Each value represents the mean ± SE. *, $P < 0.05$; **, $P < 0.01$. *Normal*, normal breast tissue; *AIDH*, atypical intraductal hyperplasia; *DCIS*, ductal carcinoma *in situ*; *IDC*, infiltrating ductal carcinoma; *n*, the number of cases.

rowheads). TIMP-4 is shown in Fig. 9*D* (*red*). MMP-26 and TIMP-4 were also coexpressed in the cytoplasm of the cancerous cells (Fig. 9*F*, *yellow*). MMP-26 and TIMP-2 were also colocalized in cancer cells, and minimal signals were detected in control tissues using purified pre-immune rabbit IgG and nonimmune goat sera (data not shown).

DISCUSSION

The epithelial component of the normal and noninvasive human breast is physically separated from the stroma by myoepithelial cells and the basement membrane, which is composed of a group of fibrous proteins, including type IV collagen, FN, laminin, and proteoglycans. The disruption of both the myoepithelial cell layer and the basement membrane is an essential prerequisite for the invasion of DCIS. In this present investigation, we found that the levels of MMP-26 and MMP-9 proteins in human breast DCIS were significantly higher than those in human breast IDC, normal mammary glands, and AIDH. MMP-26 and MMP-9 were colocalized in human breast DCIS cells. MMP-9 is a powerful enzyme associated with human breast cancer development and invasion (29, 30). Scorilas *et al.* (29) and Soini *et al.* (31) demonstrated that *MMP-9 mRNA* and protein were highly expressed in human breast carcinoma cells. MMP-9 protein is also expressed in the breast carcinoma cell lines MCF-7 (32, 33), SKBR-3 (34), MDA-MB-231 (35, 36), and MCF10A (36) and in normal breast epithelial cell lines HMT-3522 and T4-2 (32, 37). Our recent study (9) demonstrated that the level of MMP-26 protein in human prostate carcinomas is also significantly higher than those in prostatitis, benign prostate hyperplasia, and normal prostate tissues. MMP-26 and MMP-9 are not only coexpressed in human prostate carcinomas and in androgen-repressed prostate cancer cells, but they also promoted the invasion of androgen-repressed prostate cancer cells across FN or type IV collagen via MMP-26-mediated pro-MMP-9 activation.

Nguyen *et al.* (38) showed that active MMP-9 accumulates in the cytosol of human endothelial cells, where it is eventually used by invading pseudopodia, and it is possible that endogenous, self-activated MMP-26 serves as an activator for intracellular pro-MMP-9. The active form of MMP-9 may then be stored inside the cell, ready for rapid release when it is required to initiate invasion of human breast DCIS cells. Although DCIS is not invasive cancer, it may have the potential to develop into IDC, given time. The localization of *MMP-26 mRNA* and protein in carcinomas was confirmed by *in situ* hybridization and immunohistochemistry, providing evidence that MMP-26 is an epithelial cell-derived enzyme (3–6).

We demonstrated that the hydrolysis of synthetic peptides by MMP-26 is inhibited by TIMP-2 and TIMP-4, which is consistent with previous reports (5, 6, 8). TIMP-2 and TIMP-4 were also able to completely block MMP-9 activation by MMP-26, as well as the cleavage of FN by MMP-26-activated MMP-9. Therefore, one consequential function of TIMP-2 and TIMP-4 may be their inhibition of MMP-9 activation by MMP-26. TIMP-2 and TIMP-4 possess several distinct cellular functions, but their most widely appreciated biological functions are their roles in the inhibition of cell invasion *in vitro* (39–42) and their *in vivo* contributions to tumorigenesis (43, 44) and growth and metastasis (44–47). The underlying molecular mechanism for the tumor-suppressing activities of the TIMPs is thought to be dependent on their anti-MMP activities.

In our experiments, the expressions of TIMP-2 and TIMP-4 were all increased significantly in human breast DCIS but decreased in IDC, mimicking the expression of MMP-26 and MMP-9 in DCIS and IDC, which indicates that these four proteins are highly coordinated during human breast carcinoma development and progression. It may also suggest that remodeling of the extracellular matrix by MMP-26 and MMP-9 stimulates the expression of TIMP-2 and TIMP-4, implying self-regulation through a negative feedback loop. The consistently high expression of TIMP-2 and TIMP-4 proteins in human breast DCIS is in agreement with reports that the expression of TIMP-2 and TIMP-4 in human breast carcinomas is increased (14, 16,

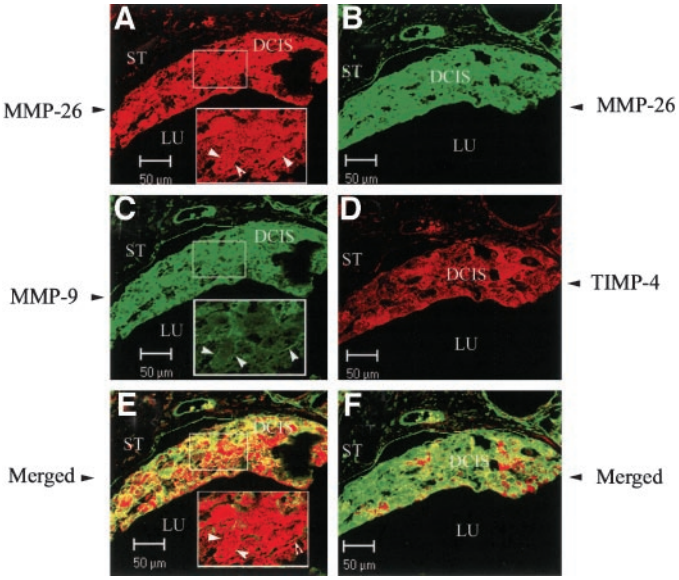

Fig. 9. Coexpression of matrix metalloproteinase (MMP)-26 with MMP-9 or tissue inhibitor of metalloproteinases (TIMP)-4 in human breast ductal carcinoma *in situ* (DCIS). In *A*, *C*, and *E*, the primary antibodies are rabbit antihuman MMP-26 IgG and goat antihuman MMP-9 IgG, the secondary antibodies are Rhodamine Red-X-conjugated donkey antirabbit IgG and FITC-conjugated donkey antigoat IgG. *A*, *red* indicates MMP-26 protein staining, which is localized mainly in the cytoplasm of DCIS cells. *C*, *green* indicates MMP-9 protein staining, which is localized mainly on the cell surface of the cancerous cells. *E*, merged images show a color shift to *orange-yellow*, indicating colocalization between MMP-26 and MMP-9 proteins in DCIS. In *B*, *D*, and *F*, the primary antibodies are mouse antihuman MMP-26 IgG and rabbit antihuman TIMP-4 IgG. After incubation with primary antibodies, the slides were incubated with goat antimouse-IgG IgG. The secondary antibodies are the same as those described in *A*, *C*, and *E*. *B*, *green* indicates MMP-26 protein staining, which is localized mainly in the cytoplasm of DCIS cells. *D*, *red* indicates TIMP-4 protein staining. *F*, merged images show a color shift to *orange-yellow*, indicating colocalization between MMP-26 and TIMP-4 proteins in DCIS. *Scale bars*, 50 μm. *ST*, stroma; *LU*, lumen.

39, 48). This may represent a compensatory response to the increased MMP-26- and MMP-9-mediated remodeling stimuli in DCIS in an attempt to reach a new balance between MMP-26/MMP-9 and TIMP-2/TIMP-4 to regulate degradation of the extracellular matrix and to suppress tumor progression by impeding MMP-26, MMP-9, and MMP-26-mediated MMP-9 activation. TIMP-2 and TIMP-4 may play multiple roles in human breast cancer in addition to inhibiting MMPs, including antiapoptotic activity and tumor-stimulating effects when administrated systemically (16). The new paradigms concerning the potential roles of TIMPs in suppressing or promoting tumor progression have been discussed previously (49).

The decreased MMP-26 and MMP-9 expression levels in IDC suggest that these two enzymes may play a role in the very early stages of DCIS invasion, but once the basement membrane has been breached, cancer cells become less dependent on the activities of MMP-26 and MMP-9. Nielsen *et al.* (50) demonstrated that MMP-9 immunostaining or *in situ* hybridization signals were not detected in human breast ductal carcinoma cells but were seen in tumor-infiltrating stromal cells including macrophages, neutrophils, and vascular pericytes. This indicates that MMP-9 may be transiently expressed in cells during the early stages of human breast carcinoma (DCIS), but not in the later stages of breast carcinoma (IDC). The controversy surrounding the expression of MMP-9 found in the literature (29, 31, 50), inclusive of our current findings, might also suggest that these different expression patterns arise from DCIS and IDC representing genetically distinct disease forms, raising the possibility that DCIS does not simply designate a transitory disease state that invariably leads to IDC.

Down-regulation of TIMPs contributed significantly to the tumorigenic and invasive potentials of cancer cells (43, 44). Decreased production of TIMP-2 resulted in increased MMP activity, leading to increased invasiveness by cancer cells (39). Therefore, maintenance of the balance between MMPs and TIMPs appears critical for the suppression of cancer cell invasion and metastasis. Once the balance between MMP-26/MMP-9 and TIMP-2/TIMP-4 in DCIS is destroyed, the inhibition by TIMP-2 and TIMP-4 may be inadequate to block the degradation of extracellular matrix components by MMP-26 and MMP-9 in DCIS, resulting in degradation of basement membrane components and initiation of the invasive processes of DCIS cells. MMP-26, MMP-9, TIMP-2, and TIMP-4 may be spatially and temporally expressed in the very early stages of DCIS invasion, whereas other enzymes/inhibitors are responsible for the late-stage invasion of IDC cells. Further investigations will provide a more complete understanding of the functions of MMP-26, MMP-9, and TIMPs in human breast cancer progression.

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Evidence for disulfide involvement in the regulation of intramolecular autolytic processing by human adamalysin19/ADAM19☆

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Abstract

Human adamalysin 19 (a disintegrin and metalloproteinase 19, hADAM19) is activated by furin-mediated cleavage of the prodomain followed by an autolytic processing within the cysteine-rich domain at Glu^{586}–Ser^{587}, which occurs intramolecularly, producing an NH_2 terminal fragment (N-fragment) associated with its COOH-terminal fragment (C-fragment), most likely through disulfide bonds. When stable Madin-Darby canine kidney (MDCK) transfectants overexpressing soluble hADAM19 were treated with dithiothreitol (DTT) or with media at pH 6.5, 7.5, or 8.5, the secretion and folding of the enzyme were not affected. Autolytic processing was blocked by DTT and pH 6.5 media, which favor disulfide reduction, but was increased by pH 8.5 media, which promotes disulfide formation. Cys^{605}, Cys^{633}, Cys^{639}, and Cys^{643} of the C-fragment appear to be partially responsible for the covalent association between the C-fragment and the N-fragment. A new autolytic processing site at Lys^{543}–Val^{544} was identified in soluble mutants when these cysteine residues were individually mutated to serine residues. Shed fragments were also detectable in the media from MDCK cells stably expressing the full-length Cys633Ser mutant. Ilomastat/GM6001 inhibited hADAM19 with an IC_{50} of 447 nM, but scarcely affected the shedding process. The cysteine-rich domain likely forms disulfide bonds to regulate the autolytic processing and shedding of hADAM19.

Keywords: A disintegrin and metalloproteinase 19; Meltrin beta; Ectodomain shedding; Proteolytic processing; Enzyme activity regulation; Disulfide bonds; Site-directed mutagenesis; Structure–function analyses; Reducing agent; pH effect

Introduction

Ectodomain shedding, a process by which transmembrane proteins proteolytically release their extracellular domains, plays a critical role in many physiological and pathological conditions [1–3]. Numerous studies have shown that a disintegrin and metalloprotease (ADAMs), also called adamalysin/metalloprotease, disintegrin, cysteine-rich (MDC), such as tumor necrosis factor-alpha converting enzyme (TACE)/ADAM17, ADAM10/Kuzbanian (KUZ), ADAM9, ADAM19/MDC beta, and ADAM12/MDC alpha, are the predominant sheddases responsible for the shedding

Abbreviations: ADAM, a disintegrin and metalloproteinase; ADAMTS, a disintegrin and metalloproteinase with thrombospondin-like motifs; alpha2-M, alpha-2-macroglobulin; APP, amyloid precursor protein; CaM, calmodulin; D-CR, soluble ADAM19 containing the metalloproteinase and disintegrin domains; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; ECM, extracellular matrix; FBS, fetal bovine serum; IL-1R-II, interleukin 1 receptor II; MDC, Metalloprotease/Disintegrin/Cysteine-rich; MDCK, Madin-Darby canine kidney; NRG, neuregulin; pAb, polyclonal antibody; PBS, phosphate buffered saline; PKC, protein kinase C; PMA, phorbol-12 myristate 13-acetate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TACE, tumor necrosis factor alpha convertase; TIMPs, tissue inhibitors of matrix metalloproteinases; TGF-alpha, transforming growth factor-alpha; TGN, *trans*-Golgi networks; TNF-alpha, tumor necrosis factor-alpha; W7, N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

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of most molecules identified thus far, such as tumor necrosis factor-alpha (TNF-alpha), transforming growth factor-alpha (TGF-alpha), interleukin 1 receptor II (IL-1R-II), Notch and its ligand Delta, amyloid precursor protein (APP), heparin-binding epidermal growth factor (HB-EGF), mucin MUC1, and neuregulin-beta1(NRG-beta1) [3–5].

The activities of ADAMs are regulated at multiple levels, including transcription, translation, zymogen activation, and inhibition by tissue inhibitors of matrix metalloproteinases (TIMPs) [2–6]. The principle regulatory step is zymogen activation, with the predominant focus on the regulation of prodomain removal [6–18]. Synthesized as zymogens, many ADAMs undergo prodomain cleavage by either furin or furin-like proprotein convertases before they display any proteolytic activities, as in the cases of ADAM1, 9, 12, 15, 17, 19, a disintegrin and metalloproteinase with thrombospondin 1-like motifs (ADAM-TS) 1, 4, 12, [6–16], or by autolysis, as seen in ADAM8 [17] and ADAM28 [18]. However, in some ADAMs, the prodomains are involved in their catalytic activities. For example, deletion of the prodomain destroys the proteolytic activity of ADAM10 [19,20]. In the cases of ADAM12 and 17, the prodomains are not only inhibitors of the catalytic domain, but also appear to act as chaperones, facilitating secretion and folding of the ADAM proteins [12,21]. In addition, many mutations interfere with the folding or processing of ADAMs, resulting in a loss of proteolytic processing. For instance, replacement of the lone cysteine residue in the prodomain of ADAM9 with alanine abolished prodomain removal [12]; both His^{346} and His^{350} substitutions with alanines in the metalloproteinase domain of mouse ADAM19 abrogated the processing of its prodomain [22]; the Leu73Pro mutant of ADAM12 resulted in complete retention of ADAM12 in the endoplasmic reticulum (ER) and inhibition of its processing [14]; the soluble form of ADAM13 has never been converted into its mature form, indicating that the transmembrane domain, cytoplasmic domain, or both, are indispensable for the processing of the prodomain in ADAM13 [23]; and the removal of the disintegrin and cysteine-rich domains of TACE/ADAM17 resulted in secretion of the mature catalytic domain in association with the precursor (pro) domain [21].

Recently, there is increasing evidence that C-terminal truncation plays a critical role in the regulation of the proteolytic activity of ADAMs, as illustrated by ADAM8, 13, 19, and ADAM-TS4 [16,17,23–25]. This truncation produces active enzymes or functional fragments that may then act as sheddases, bind with integrins on the cell surface, or digest components of the extracellular matrix (ECM) [16,17,23–25]. However, the regulation of this processing remains to be uncovered. Smith et al. [26] showed that the cysteine-rich domain affects the proteolytic activity of ADAM13 in vivo, probably by an intramolecular interaction with its metalloproteinase domain, and that this model might be applied to other ADAMs. This prompted us to explore how this intramolecular interaction occurs in ADAMs, as we have previously demonstrated that an autolytic processing of hADAM19 at E^{586}-S^{587} within the cysteine-rich domain takes place in the secretory pathway [24]. Based on the fact that the E^{586}-S^{587} processed active N-fragment containing the metalloproteinase and disintegrin domains and a segment of the cysteine-rich domain is not released from the C-fragment containing the C-terminal segment of the cysteine-rich domain and epidermal growth factor (EGF)-like domain under non-reducing conditions [24], we hypothesized that interfragment disulfide bond(s) may play a crucial role in the processing of hADAM19. This report provides evidence that disulfide bonds most likely formed between cysteine residues in the N-fragment and those in the segment of the cysteine-rich domain of the processed C-fragment may be involved in the regulation of intracellular processing of hADAM19.

Materials and methods

Chemicals, cell lines, cell culture, and immunological reagents

All common laboratory chemicals, proteinase inhibitors, phorbol-12 myristate 13-acetate (PMA), N-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide (W7), and the anti-clusterin-alpha/beta (H-330) polyclonal antibody and its agarose conjugates were purchased from Sigma (St. Louis, MO). The anti-FLAG-M2 monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The matrix metalloproteinase inhibitor Ilomastat (GM6001) was purchased from BACHEM (Philadephia, PA). Restriction enzymes were purchased from Promega (Madison, WI) or Invitrogen (Gaithersburg, MD). COS1, Madin Darby canine kidney (MDCK) cells and its derivatives were maintained as described [13,24]. Dulbecco's modified Eagle's medium (DMEM) was purchased from Invitrogen Gibco BRL (Gaithersburg, MD). Fetal bovine serum (FBS), penicillin G, and streptomycin were purchased from Life Technologies (Rockville, MD). Endoglycosidase H (Endo H) and alpha2-macroglobulin (alpha2-M) were purchased from Roche Molecular Biochemicals (Indianapolis, IN). The rabbit polyclonal hADAM19 antibody (anti-disintegrin domain, anti-Dis) was generated by our laboratory as reported [27].

Polymerase chain reaction (PCR) primers, mutagenesis, and expression constructs

All inserts tagged with FLAG at their C-terminus were cloned into pCR3.1uni, including wild type hADAM19 (F46), soluble hADAM19 (D52), D-E346A, D-E586D, D-CR (described in Ref. [24]), and all mutants used in this study. For D586 deleted from S587 to the end of the C-terminal: forward primer, 5′-ACC ATG CCA GGG GGC GCA GGC GCC-3′; reverse primer, 5′-CTC CAG GGG CCG GGC CTC

AGA G-3′. For the soluble C^{605} to S mutants (D-C605S): forward primer, 5′-CAG ATC CAG **TCC** CGG GGC ACC CAC-3’; reverse primer, 5′-GGT GCC CCG **GGA** CTG GAT CTG CC-3′. For the full-length and soluble C^{633} to S mutants (F-C633S and D-C633S): forward primer, 5′-GGA ACC AAG **TCT** GGC TAC AAC C-3′; reverse primer, 5′-GTT GTA GCC **AGA** CTT GGT TCC AG-3′. For the soluble C^{639} to S mutant (D-C639S): forward primer, 5′-C AAC CAT ATT **TCC** TTT GAG GGG CAG-3′; reverse primer, 5′-CCC CTC AAA **GGA** AAT ATG GTT G-3′. For the soluble C^{643} to S mutants (D-C643S): forward primer, 5′-GAG GGG CAG **TCC** AGG AAC ACC TC-3′; reverse primer, 5′-GGT GTT CCT **GGA** CTG CCC CTC AAA G-3′. For the double mutants, including E^{586} to D and C^{633} to S (D-C633S/E586D), E^{586} to D and C^{639} to S (D-C639S/E586D), E^{346} to A and C^{633} to S (F-C633S/E346A and D-C633S/E346A), and E^{346} to A and C^{639} to S (D-C639S/E346A), E^{346} to A and C^{605} to S (D-C605S/E346A), and E^{346} to A and C^{643} to S (D-C643S/E346A), we used D-E586D or D-E346A as the template and the primers for C^{633} to S, C^{639} to S, C^{605} to S, C^{643} to S, or C^{639} to S, respectively. All constructs were confirmed by DNA sequencing.

DNA transfection and generation of stable cell lines

COS1 cells were seeded in 24-well plates for 16–24 h at 80% confluence and transfected or co-transfected with the indicated plasmids using LipofectAMINE2000 according to the instructions provided by Invitrogen (Gaithersburg, MD). After 6–10 h, serum-free DMEM media and the indicated reagents were added and incubated for another 24 h. The conditioned media and cell lysates were then analyzed by Western blotting [13,24]. The same transfection procedure was performed to generate stable MDCK cell lines, and the selection for hADAM19 was begun in the presence of G418 (400 μg/ml) after transfection for 24 h. The conditioned media and/or cell lysates of the clones were subjected to Western blotting to confirm the expression of hADAM19 [13,24]. For co-culture cells, the indicated MDCK stable lines were equally mixed in the same wells overnight, followed by serum-free media for 24 h, and the media were then analyzed by Western blotting.

Western blotting

The experiments were carried out as described previously [13,24]. Briefly, cells were grown to 80% confluence and were treated as indicated. After centrifugation for 15 min at 14,000 × *g* and 4°C to clear any debris, the serum-free media were collected, treated with endoglycosidase H (Endo H) overnight if indicated using the provided protocol and prepared for SDS-PAGE. The cells were lysed with RIPA buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.25% sodium deoxycholate, 0.1% NP-40, 1 mM phenyl-methyl-sulfonyl-fluoride, 1 mM of 1, 10-phenasrol, 10 μg/ml aprotinin, 10 μg/ml E64, and 10 μg/ml pepstatin A) for 15 min on ice. The supernatant was collected after centrifugation for 20 min at 14,000 × *g* and 4°C. After electrophoresis, the proteins were transferred onto nitrocellulose membranes, probed with anti-FLAG-M2 or anti-Dis, and developed as before [13,24].

Purification of soluble hADAM19 and co-incubation with purified proteins

D-CR and D-E346A were produced earlier in our laboratory; all proteins were purified on anti-FLAG-M2 affinity columns as described previously [24]. Briefly, cells from stable lines expressing soluble hADAM19, D586, or D-C633S/E586D, were grown to 100% confluence, washed twice with phosphate-buffered saline (PBS), and incubated for 48 h in serum-free media. The conditioned media were collected, centrifuged to clear any debris, and loaded onto an anti-M2 immunoaffinity column (1 ml of resuspended agarose) that had been prewashed with HEPES buffer. The bound materials were extensively washed with HEPES buffer, eluted with FLAG peptides, and collected in 500 μl fractions. The fractions were analyzed by Western blot using anti-hADAM19 antibodies or anti-FLAG-M2, and the protein concentration was quantified by absorbance at 280 nm. The fraction containing the highest concentration of the proteins of interest was characterized by protein N-terminal sequencing as described previously [13,24], and the fractions containing the most wild type or mutant hADAM19 proteins were used for the flourescamine assay or the co-incubation of purified proteins to test substrate cleavage. For the co-incubation of purified proteins, we mixed the purified D-E346A with D-CR or D586 equally at 100 ng/μl overnight, then the processing of D-E346A by D-CR or D586 was detected by Western blotting with anti-FLAG-M2 as above.

Results

Autolytic processing at E^{586}-S^{587} of hADAM19 is an intramolecular interaction

We recently developed a peptide substrate to determine the activity of hADAM19 by a fluorescamine assay, and reported that autolytic processing at E^{586}–S^{587} within the cysteine-rich domain is necessary for the proteolytic activities of hADAM19 against both this peptide substrate and α2-M [24]. To directly prove that the processed N-fragment containing the metalloproteinase and disintegrin domains and a part of the cysteine-rich domain is active, we generated a truncated form of hADAM19, from the N-terminal to E^{586}, tagged with a C-terminal FLAG tag (D586) (see Fig. 3), which was found to have the same activity as D-CR, containing the metalloproteinase and disintegrin domains of hADAM19, against the peptide substrate (24, data not shown). To determine if hADAM19 processing E^{586}-S^{587}

is an intermolecular or intramolecular interaction, we used several independent approaches. As shown in Fig. 1A, the inactive soluble form of hADAM19 (D-E346A) [24] was not processed by either of its active soluble forms, D586 and D-CR, when co-cultured with their stably expressing MDCK cells. Furthermore, both D586 and D-CR failed to process D-E346A when we performed a transient co-transfection using D-E346A with either D586 or D-CR in COS1 cells, as shown in Fig. 1B, or when we incubated the purified proteins together in vitro, using D-E346A with D586 or D-CR (data not shown). These results strongly argue that an intramolecular interaction is responsible for the processing of hADAM19 at E^{586}–S^{587}.

The formation of disulfide bonds affects the processing of hADAM19, but not its folding or its secretion

We have previously surmised that the N-fragment generated by the processing of hADAM19 at E^{586}–S^{587} may be associated with its processed C-fragment via one or more disulfide bonds [24]. To explore this possibility, we ran the conditioned media from MDCK cells stably expressing soluble hADAM19 (D52-5) side by side under either reducing or non-reducing conditions. As shown in Fig. 2A, the mature forms of hADAM19 were detected under both reducing and non-reducing conditions. However, the fragment at 26 kDa, representing the processed C-fragment at E^{586}–S^{587} of hADAM19, was detectable only under reducing conditions (Fig. 2A). Moreover, the processed N-fragments emerged together with the mature forms under non-reducing conditions (Fig. 2A). Therefore, it is highly possible that the processed C-fragment, containing a part of the cysteine-rich domain and EGF-like domain, forms disulfide bonds with the processed N-fragment, including the metalloproteinase and disintegrin domains and a segment of the cysteine-rich domain, in soluble hADAM19.

Given that the formation of disulfide bonds often plays an important role in enzyme folding, secretion, and activity, we incubated cells expressing D52 (D52-5) overnight in serum-free media containing DTT, a membrane permeable thiol reducing agent known to prevent disulfide bond formation in intact cells, including MDCK cells [28–30] and the conditioned media were then treated with endoglycosidase H (Endo H) for 16 h. As shown in Fig. 2B, DTT almost completely blocked the processing of soluble hADAM19, as the fragment at 26 kDa was indiscernable following DTT treatment (Fig. 2B). However, DTT had little or no effect on either the folding or secretion of soluble hADAM19, as the proteins in the media were comparable and resistant to Endo H (Fig. 2B), an enzyme that preferentially hydrolyzes N-glycanses of the high mannose type found predominantly in premature proteins.

To further verify the involvement of disulfide bond formation in the processing of soluble hADAM19, we incubated D52-5 overnight with serum-free media at pH 7.5, 8.5, or 6.5. As shown in Fig. 2C, the processing of soluble hADAM19 is increased at pH 8.5, which is more favorable for the formation of disulfide bonds than normal conditions (pH 7.5) (Fig. 2C) [31]. Conversely, there was little processing of soluble hADAM19 at pH 6.5, which significantly impairs the formation of disulfide bonds [31]. Once again, the protein levels are almost equal among the different treatments.

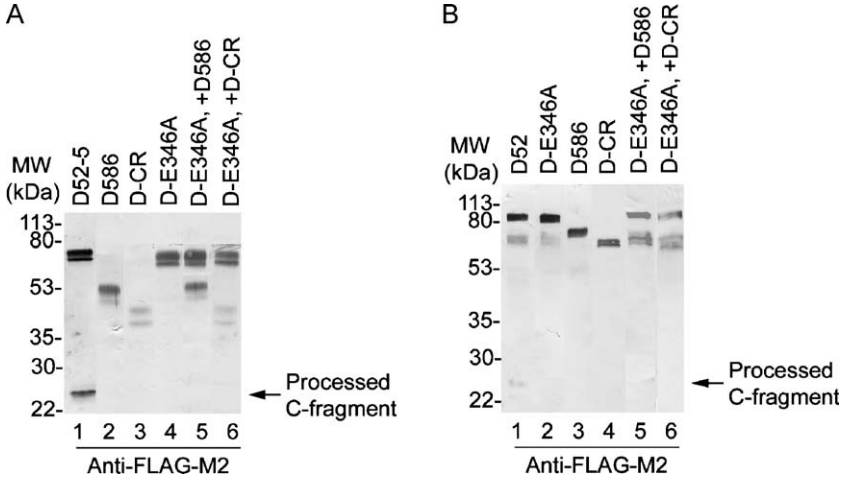

Fig. 1. Processing at E^{586}–S^{587} of hADAM19 occurs intramolecularly. (A) Active forms of hADAM19 fail to cleave its inactive form in the media using a co-culture system. MDCK cells stably expressing soluble inactive hADAM19 (D-E346A) were mixed equally in 24-well plates with MDCK cells transfected with the blank vector (lane 4), D586 (lane 5), or D-CR (lane 6) for 24 h in serum-free media, then the conditioned media were subjected to Western blotting with anti-FLAG-M2. The soluble stable lines, D52-5 (lanes 1), D586 (lane 2), or D-CR (lane 3) alone were used as controls. The processed C-fragments of soluble hADAM19 are indicated. Note that D-E346A is not processed by D586 or D-CR. (B) Active forms of hADAM19 lack the capacity to cleave its inactive form in the media using co-transfection into COS1 cells. COS1 cells were transfected with D52 (lane 1), D-E346A (lane 2), D586 (lane 3), D-CR (lane 4), D586 and D-E346A (lane 5), and D-CR and E346A (lane 6), followed by incubation for 24 h in serum-free media. The conditioned media were analyzed by Western blotting with anti-FLAG-M2. Note that D586 or D-CR is unable to process D-E346A, even they express in the same cell.

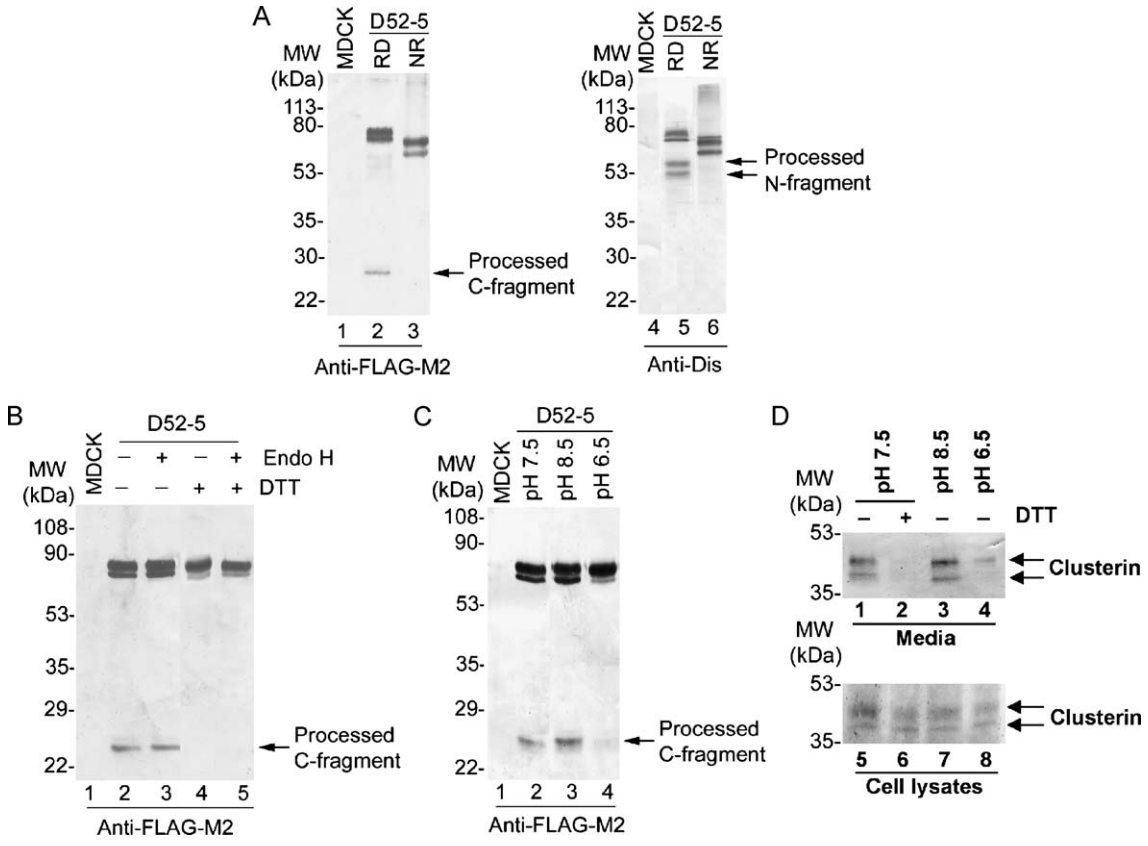

Fig. 2. Potential intramolecular disulfide bonds affect the activity of hADAM19, but not its folding or its secretion. (A) No detection of the processed forms under non-reducing conditions. MDCK cells stably expressing soluble hADAM19, D52-5, were incubated in serum-free media for 24 h, then the conditioned media was subjected to Western blotting by anti-FLAG-M2 (lanes 1–3) or a specific antibody against the disintegrin domain of hADAM19 (anti-Dis) (lanes 4–6) under reducing (lanes 1, 2, 4, 5) or non-reducing conditions (lanes 3, 6). The processed C-fragments or N-fragments of soluble hADAM19 are indicated. The medium from MDCK cells transfected with the blank vector was utilized as a control (lanes 1, 4). (B) The processing, but not the folding or secretion of hADAM19, is affected by DTT treatment. D52-5 were incubated in serum-free media with (lanes 4, 5) or without (lanes 1–3) 5 mM of DTT overnight, then the conditioned media was collected and treated with (lanes 3, 5) or without (lanes 1, 2, 4) 0.015 units of endoglycosidase H (Endo H) overnight before Western blotting by anti-FLAG-M2. The processed C-fragments of soluble hADAM19 are indicated. The media from MDCK cells transfected with the blank vector was utilized as a control (lane 1). (C) The pH of media affects the processing, but not the folding or secretion of hADAM19. D52-5 were incubated in serum-free media at pH 7.5 (lane 2), pH 8.5 (lane 3), or pH 6.5 (lane 4) overnight, then the conditioned media was subjected to Western blotting by anti-FLAG-M2. The processed C-fragments of soluble hADAM19 are indicated. The media from MDCK cells transfected with the blank vector was utilized as a control (lane 1). (D) DTT treatment and medium pH adjustment media affects the secretion of clusterin. D52-5 were incubated in serum-free media at pH 7.5 (lanes 1, 2, 5, 6), pH 8.5 (lanes 3, 7), or pH 6.5 (lanes 4, 8) with (lanes 2, 6) or without 5 mM of DTT for 16 h, then the conditioned media (lanes 1–4) and cell lysates (lanes 5–8) were subjected to Western blotting by anti-clusterin antibodies under reducing condition.

To confirm that DTT treatment or medium pH adjustment alters the rate of disulfide formation during progress through the secretory pathway, we examined clusterin protein levels in the media and cell lysates under these conditions, as clusterin is a highly expressed glycoprotein forming multiple disulfide bonds before its secretion in MDCK cells [32]. As shown in Fig. 2D, clusterin secretion is blocked by DTT treatment, which prevents the formation of intramolecular disulfide bonds by clusterin, consistent with results described previously by others [30]. The secretion of clusterin is dramatically impaired under the condition of pH 6.5, but not of pH 8.5, as medium at pH 6.5 likely impedes the formation of disulfide bonds in intact cells. On the other hand, there is no significant difference in total clusterin levels among the cell lysates under these conditions, as shown in Fig. 2D. Taken together, these data demonstrate that the formation of disulfide bonds is critical for the processing of soluble hADAM19, but are apparently nonessential to its secretion or its folding.

Interfragment disulfide bonds are responsible for the association of the N-fragment with the C-fragment of hADAM19 processed at E^{586}–S^{587}

The proteolytic activity of ADAM13 has recently been shown to be regulated by its cysteine-rich domain through intramolecular interactions with its metalloproteinase domain [26], and the processing of hADAM19 at E^{586}–S^{587} is also an intramolecular event (Fig. 1), after which the processed N-fragments associate with their C-fragments, most likely by disulfide bonds (Fig. 2A). We hypothesized that the disulfide bonds potentially formed between the

cysteine residues of the N-fragment and those within the fraction of the cysteine-rich domain retained by the processed C-fragment may play a critical role in the processing of hADAM19 at E^{586}-S^{587}. To evaluate this, we mutated the cysteine residues at C^{605}, C^{633}, C^{639}, and C^{643} to S within that region of soluble hADAM19 (Fig. 3), then transiently transfected these mutants into COS1 cells. The results shown in Fig. 4 reveal that all mutants produced a new C-fragment of approximately 32 kDa. The normal processing of the 26-kDa fragment at E^{586}–S^{587} remained unchanged in these mutants, although the protein levels in the conditioned media were different. When running these samples under non-reducing conditions, we found that the processed C-fragments at 26 and 32 kDa were not associated with their processed N-fragments, suggesting that the association of the N-fragment with the C-fragment of hADAM19 processed at E^{586}–S^{587} was destroyed by the disruption of one or more disulfide bonds that include C^{605}, C^{633}, C^{639}, or C^{643}.

The new processing at K^{543}–V^{544} is also autolytic, but independent of the processing at E586–S587

Since the processing at E^{586}–S^{587} of soluble hADAM19 is autolytic, we sought to determine if this new processing is also autolytic. Deactivating E^{346} to A mutations were generated in all C to S soluble mutants, then these double

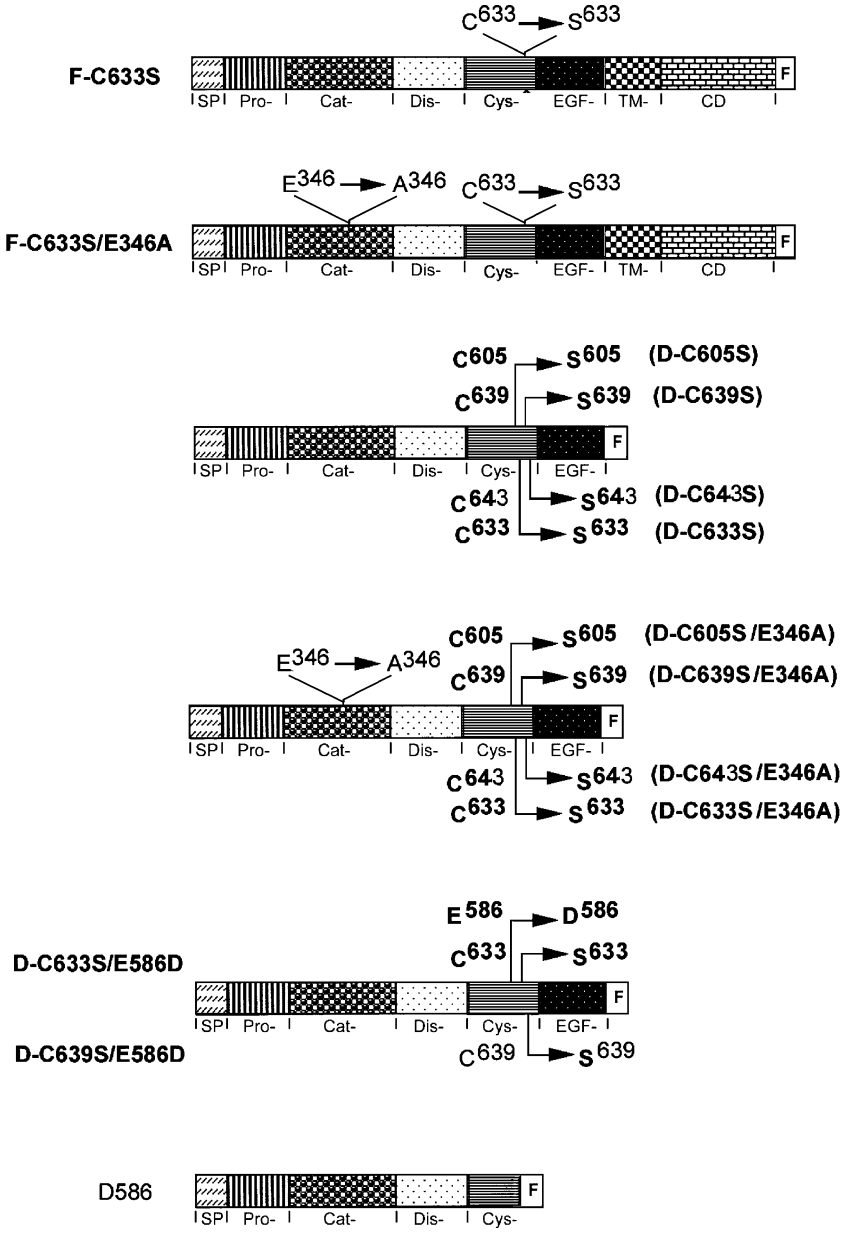

Fig. 3. A schematic illustration of the mutants of hADAM19 inserted in the expression vector pCR3.1. All of the constructs have a C-terminal FLAG tag. SP: Signal peptide; Pro-: Prodomain; Cat-: Catalytic domain; Dis-: Disintegrin domain; Cys-: Cysteine-rich domain; EGF-: EGF-like domain; TM: Transmembrane domain; CD: Cytoplasmic domain; F: FLAG tag.

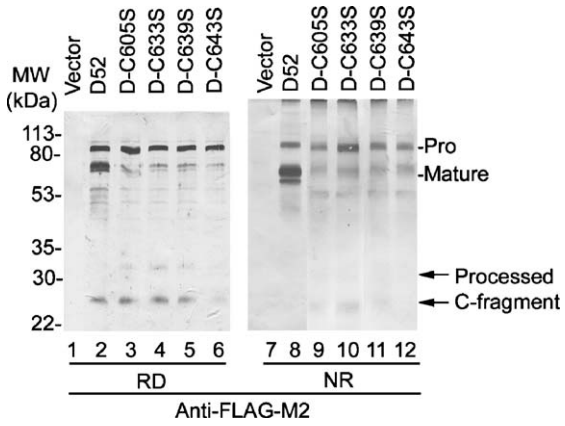

Fig. 4. Any one of the cysteine residues at C^{605}, C^{633}, C^{639}, or C^{643} is indispensable for the association of the processed N-fragment with its C-fragment in soluble hADAM19 in COS1 transfected cells. COS1 cells were transfected with blank vector (lanes 1, 7), D52 (lanes 2, 8), soluble mutants with C^{605} to S (D-C605S, lanes 3, 9), C^{633} to S (D-C633S, lanes 4, 10), C^{639} to S (D-C639S, lanes 5, 11), or C^{643} to S (D-C643S, lanes 6, 12), followed by incubation for 24 h in serum-free media. The conditioned media were analyzed by Western blotting with anti-FLAG-M2 under reducing (lanes 1–6) or non-reducing conditions (lanes 7–12). The pro- and mature forms, and the processed C-fragments of soluble hADAM19 are indicated. Note that any one of these Cys to Ser mutations induces two processing sites.

mutations were transiently transfected into COS1 cells. As shown in Fig. 5A, there is no processing detectable in any of the inactive forms of soluble hADAM19, demonstrating that the new processing induced by mutating these cysteine residues is also autocatalytic. Given that the autolytic processing at E^{586}–S^{587} of soluble hADAM19 is necessary for its proteolytic activity [24], and the new processing is also an autolysis (Fig. 5A), it was intriguing to determine if this new processing was dependent upon the processing at E^{586}-S^{587}. To address this issue, we made two soluble double mutants, C^{633} to S and E^{586} to D (D-C633S/E586D), and C^{639} to S and E^{586} to D (D-C639S/E586D) (Fig. 3) because there is rare processing at E^{586}-S^{587} when E^{586} is mutated to D^{586}, as described in our previous report [24]. We then performed transient transfection with these mutants into COS1 cells. As shown in Fig. 5B, the 32-kDa fragment was detectable in these double mutants, but the 26-kDa fragment, representing the processing at E^{586}-S^{587}, disappeared in these media, suggesting that the new processing is independent of, and not necessarily preceded or followed by, the processing at E^{586}–S^{587}.

To further confirm the results obtained from the COS1 cells, we chose C^{633} to S, C^{633} to S and E^{346} to A, and C^{633} to S and E^{586} to D as representative mutants to generate MDCK stable transfectants, designated D-C633S, D-C633S/E346A, and DC633S/E586D, respectively. As shown in Figs. 6A and B, the fragments at 26 and 32 kDa were clearly detectable by anti-FLAG-M2 in the media from D-C633S (Fig. 6A), but not from D-C633S/E346A (Fig. 6B). In striking contrast, the fragments at 32 kDa presented as the dominant forms in D-C633S/E586D (Fig. 6C). The protein levels in the conditioned media from these stable transfectants were nearly equal (Fig. 6). These results are consistent with those obtained in the COS1 transient transfection as shown in Figs. 4 and 5, confirming that disruption

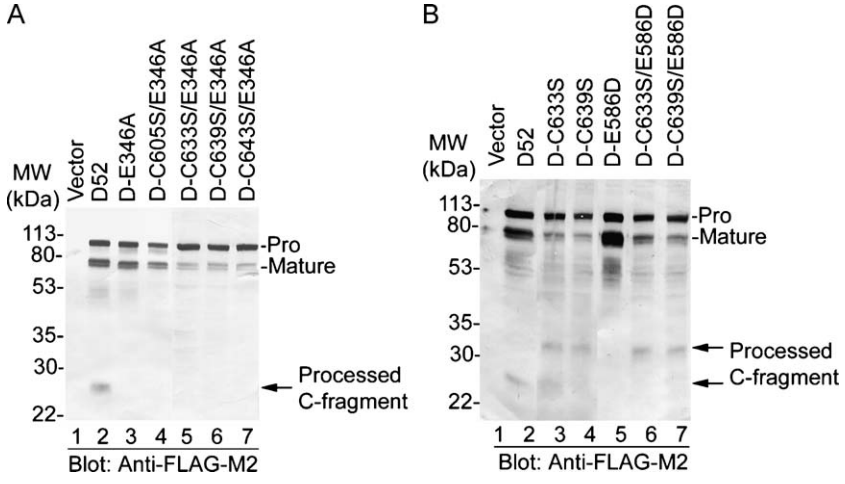

Fig. 5. The new processing induced by mutating the cysteines is autolytic, but independent of the normal processing at E^{586}–S^{587}. (A) The novel processing induced by cysteine residue mutation is autocatalytic. COS1 cells were transfected with blank vector (lane 1), D52 (lane 2), D-E346A (lane 3), soluble mutants with C^{605} to S and E^{346} to A (D-C605S/E346A, lane 4), C^{633} to S and E^{346} to A (D-C633S/E346A, lane 5), C^{639} to S and E^{346} to A (D-C639S/E346A, lane 6), or C^{643} to S and E^{346} to A (D-C643S/E346A, lane 7), followed by incubation for 24 h in serum-free media. The conditioned media were analyzed by Western blotting with anti-FLAG-M2. The pro- and mature forms, and the processed C-fragments of soluble hADAM19 are indicated. There is no processing whatsoever in the media from the COS1 cells transiently transfected with the double mutants. (B) The independence of the new processing in transfected COS1 cells. COS1 cells were transfected with the blank vector (lane 1), soluble hADAM19 (D52, lane 2), C^{633} to S (D-C633S, lane 3), C^{639} to S (D-C639S, lane 4), soluble mutants of hADAM19 with E^{586} to D (D-E586D, lane 5), C^{633} to S and E^{586} to D (D-C633S/E586D, lane 6), or C^{639} to S and E^{586} to D (D-C639S/E586D, lane 7). After incubation for 24 h in serum-free media, the conditioned media were analyzed by Western blotting with anti-FLAG-M2. The pro- and mature forms, and the processed C-fragments of soluble hADAM19 are indicated. Note that D-E586D has no processed forms, but both D-C633S/E586D and D-C639S/E586D execute the novel processing in the absence of normal processing.

of the formation of disulfide bonds by mutating cysteine residues, such as C^{605}, C^{633}, C^{639}, or C^{643}, may not disturb the folding and secretion, but do induce a new processing site in soluble hADAM19. To determine the new peptide bond cleavage site, we chose one representative stable MDCK transfectant, D-C633S/E586D-9, to purify from the media by anti-FLAG-M2 affinity column and electrophoresis. N-terminal sequencing revealed that the starting sequence of the purified 32 kDa protein was VNVAGDT, which is identical to the ^{544}VNVAGDT sequence within the cysteine-rich domain of hADAM19. This suggests that K^{543}–V^{544} is an alternative processing site within the cysteine-rich domain of hADAM19 when the primary site at E^{587}–S^{587} is impaired dramatically or its intramolecular disulfide bonds are destroyed.

Shedding is detectable in the full length of hADAM19 with C^{633} to S^{633}, and this shedding is regulated by protein kinase C (PKC), calmodulin (CaM), and calcium signals

Based upon the results shown in Figs. 2A, 4, 5B, and 6, the shedding ability of the full-length Cys to Ser mutants was further examined by choosing C^{633} to S (F-C633S) as a representative. As shown in Fig. 7A, shed fragments of 44–53 kDa were clearly detected in the media from the MDCK cells stably transfected with F-C633S, but not with the wild type (F46), in which the N-terminal is associated with its C-terminal as described previously [24]. Expression levels were comparable among these stable transfectants (Fig. 7A), consistent with the results obtained earlier with their soluble forms (Figs. 2A, 4, 5B, and 6). The double mutants F-C633S/E346A also failed to shed (data not shown), demonstrating that F-C633S undergoes autolytic shedding. Moreover, we revealed that phorbol-12-myristate 13-acetate (PMA), a PKC activator, apparently enhanced the shedding of full-length hADAM19 with C^{633} to S, and that W7, an inhibitor of CaM, and A23187, a calcium ionophore, inhibited this shedding, consistent with our recent report showing that PKC, CaM, and calcium signal pathways may be involved in the processing of hADAM19 at E^{586}–S^{587} [24]. A potent and broad-spectrum matrix metalloproteinase inhibitor, Ilomastat (GM6001), slightly inhibited the shedding of MDCK cells stably expressing F-C633S (Fig. 7B), although it impedes many shedding processes [3,33]. Interestingly, GM6001 was able to dramatically inhibit the activity of soluble hADAM19 against our peptide substrate, described previously [24], with an IC_{50} value of 447 nM, indicating that GM6001 was largely

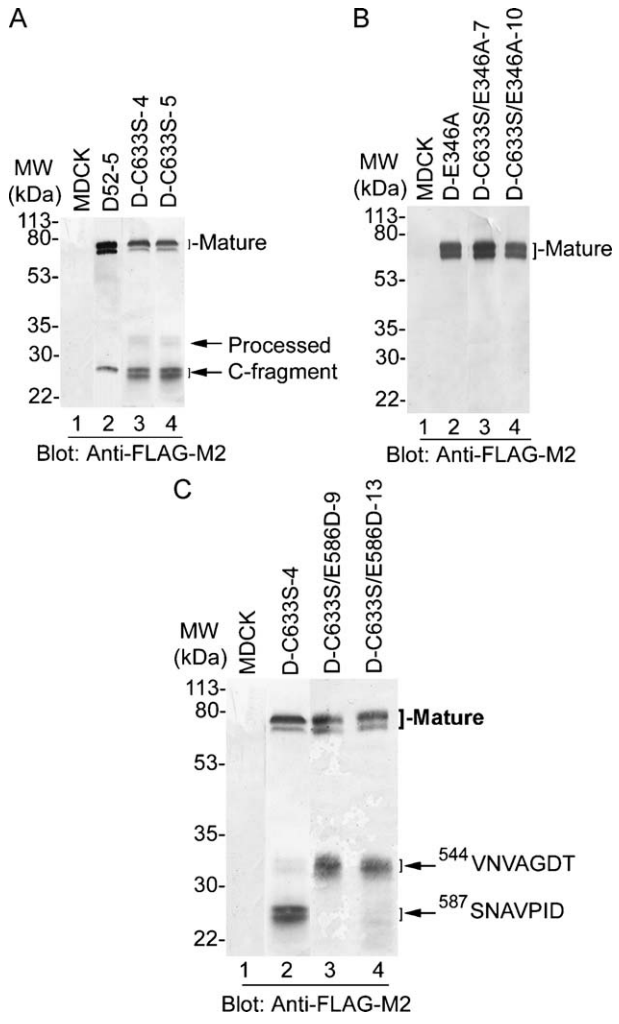

Fig. 6. The new autolytic and independent processing site induced by mutation of cysteines is K^{543}–V^{544}. (A) Soluble C to S mutants of hADAM19 in stable MDCK transfectants have multiple processing fragments. Representative MDCK cells stably expressing D52 (D52-5, lane 2), or D-C633S (D-C633S-4 and D-C633S-5, lanes 3, 4) were seeded in equal amounts into different wells of 24-well plates. After reaching 80% confluence, the cells were incubated in serum-free media for 24 h, and the conditioned media were analyzed by Western blotting using anti-FLAG-M2. The mature forms and the processed C-fragments of soluble hADAM19 are indicated. MDCK cells transfected with the blank vector were used as a control. The stable transfectants of D-C633S seem to exhibit more processing than D52-5. (B) The novel processing in the C^{633} to S mutant is an autolysis in its stable MDCK transfectants. Representative MDCK cells stably expressing soluble inactive hADAM19 (D-E346A, lane 2), C^{633} to S and E^{346} to A (D-C633S/E346A-7 and D-C633S/E346A-10, lanes 3, 4) were seeded in equal amounts and changed to serum-free media when the cells reached 80% confluence. The conditioned media were analyzed by Western blotting using anti-FLAG-M2. MDCK cells transfected with the blank vector were used as a control (lane 1). The complete lack of processing in the media from the MDCK stable lines of the double mutant, C^{633} to S and E^{346} to A, confirms that the new processing is autocatalytic. (C) The novel processing induced by the mutated cysteine residues occurs independently at $K^{543}\downarrow V^{544}$. Representative MDCK cells stably expressing soluble double mutants, C^{633} to S^{633} and E^{586} to D^{586} (D-C633S/E586D-9 and D-C633S/E586D-13, lanes 3, 4), were seeded in equal amounts and changed to serum-free media when the cells reached 80% confluence. The conditioned media were analyzed by Western blotting using anti-FLAG-M2. MDCK cells transfected with the blank vector (lane 1) and D-C633S-4 (lane 2) were used as the controls. The sequences for the processed C-terminal proteins are shown at the right. Note that the $K^{543}\downarrow V^{544}$ processing site is dominant in the media from the MDCK cells stably expressing the soluble double mutants of C633S/E586D, while the $E^{586}\downarrow S^{587}$ site is predominant in D-C633S.

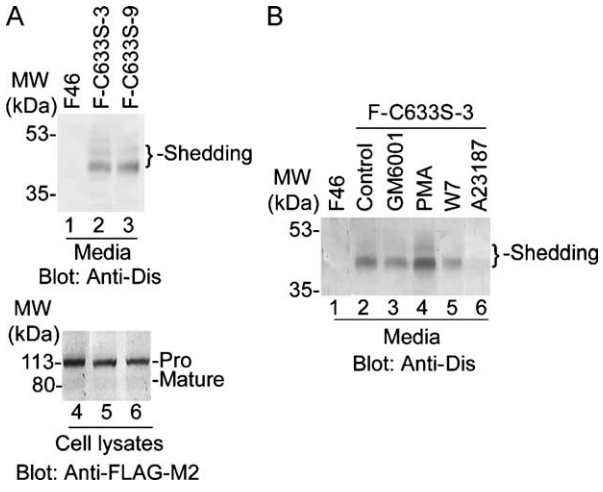

Fig. 7. Shedding of full-length hADAM19 with cysteine to serine mutations. (A) The shedding status of MDCK cells stably expressing full-length mutants of hADAM19. Representative MDCK cells stably expressing wild type hADAM19 (F46, lanes 1, 4), or mutants with C^{633} to S^{633} (F-C633S-3 and F-C633S-9, lanes 2, 5, 3, 6) were seeded at equal amounts into different wells of 24-well plates, then changed to serum-free media when the cells achieved 80% confluence. After 24 h incubation, the conditioned media (lanes 1–3) and the cell lysates (lanes 4–6) were subjected to Western blotting using anti-Dis (lanes 1–3) or anti-FLAG-M2 (lanes 4–6). The pro-, mature, and shed forms of hADAM19 are indicated. F-C633S shed significantly, but not F46. (B) The regulation of shedding in MDCK cells stably expressing F-C633S. MDCK cells stably expressing F-C633S (F-C633S-3) were seeded equally into 24-well plates, and the cells were treated with serum-free media alone (lane 2), or media containing GM6001 (2.5 μM, lane 3), PMA (50 ng/ml, lane 4), W7 (100 μM, lane 5) or A23187 (500 nM, lane 6) for 24 h. The conditioned media were analyzed by Western blotting using anti-Dis. The shed fragments of hADAM19 are indicated. MDCK cells transfected with wild type (F46) were used as a control (lane 1). The shedding of F-C633S was significantly inhibited by A23187 and W7, slightly inhibited by GM6001, and enhanced by PMA.

excluded from the intracellular environment during the shedding process. Our current results also support the concept that the processing of hADAM19 at E^{586}–S^{587} takes place intracellularly [24].

Discussion

In this report, we provide evidence that disulfide bonds, which are most likely intrafragment, play an important role in the processing of hADAM19, and that any one of the cysteine residues within the fraction of the cysteine-rich domain of the C-fragment of hADAM19 processed at E^{586}–S^{587} is indispensable for the association of the processed N-terminal with its C-terminal. This may be the first report that the cysteine-rich domain likely forms disulfide bonds to regulate the autolytic processing/shedding of hADAM19 at E^{586}–S^{587} or K^{543}–V^{544}, thereby modulating the proteolytic activity of this enzyme, as such processing/shedding is an indicator of hADAM19 activity [24]. This report lends in vitro support to the proposed model for the regulation of ADAMs, as was shown in ADAM13 in vivo [26].

Significance of autolytic processing or shedding in hADAM19

Among ADAMs, ADAM8, 13, 19, and ADAM-TS4 have been shown to be processed intracellularly by autolysis [16,17,23–25], producing an active enzyme or a functional fragment responsible for binding with alpha2-M or integrins, mediating cell adhesion, or digesting components of the ECM. Following our previous report [24], we have demonstrated in this report that under certain conditions, hADAM19 processing or shedding occurs at a different site within its cysteine-rich domain, K^{543}–V^{544}, which is before the normal processing site at E^{586}–S^{587}. Conditions leading to this alternative processing include the disruption of disulfide bonds by mutation or reduction. This also produces an active enzyme, as shown in Figs. 4, 5B, and 6, because the processing or shedding of hADAM19 depends on its own proteolytic activity (Figs. 5A and 6B, and Ref. [24]), but the processing at K^{543}–V^{544} occurs independent of the normal processing at E^{586}–S^{587} (Figs. 5B and 6C). Notably, the processing site of E^{586}–S^{587} is the predominant one, even with disruption of the intramolecular disulfide bonds (Figs. 4, 5B, and 6A). However, the site of K^{543}–V^{544} becomes dominant when the intramolecular disulfide bonds are destroyed and its primary site is mutated (Fig. 6B). Given that processing within the cysteine-rich domain is necessary for the proteolytic activities of hADAM19 [24], we speculate that the presence of an alternative processing site within cysteine-rich domain capable of producing fully activated hADAM19 is indicative of its very important roles in both physiological and pathological conditions.

Our results showed that the shorter truncated form D-CR (E^{204}–T^{504}) of hADAM19 had catalytic activity similar to the longer truncated form D586 (E^{204}–E^{586}) against a peptide substrate (data not shown). We presume that the intermediate form (E^{204}–K^{543}) produced by autolytic processing at K^{543}–V^{544} may have comparable proteolytic activity against a peptide substrate and alpha2-M. It is likely that the cleavage of a peptide substrate and alpha-2 macroglobulin by these three truncated forms of hADAM19 is due to an essential interaction between the protease and the substrate. Regarding the mechanism for these two cleavage events, we speculate that they may occur in a similar fashion, as shown by the results obtained by treatment with GM6001, PMA, W7, or A23187 (Fig. 7B and Ref. [24]).

The functions of the cysteine-rich domain in ADAMs

The ‘‘cysteine-rich domain’’ is sometimes referred to together with the disintegrin domain. In these cases, the ‘‘cysteine-rich domain’’ has been shown to be related to cell adhesion, such as in the cases of ADAM8, 12, 13, or to the proteolytic activity of TACE/ADAM17. For example, the recombinant disintegrin/cysteine-rich domain of ADAM8 mediates cell adhesion in cells expressing ADAM8 [17]; the ‘‘cysteine-rich domain’’ of ADAM12 promotes the

adhesion of fibroblasts and myoblasts [34]; the disintegrin and cysteine-rich domains of ADAM13 bind to both fibronectin and to beta1-containing integrin receptors, and the binding can be inhibited by antibodies against the cysteine-rich domain [23]; the "cysteine-rich domain" of TACE/ADAM17 is required for shedding of IL-1R-II while affecting the inhibitor sensitivity of TNF-alpha shedding [35].

Despite knowledge about the combined functions of both the disintegrin and cysteine-rich domains, very little information is available about cysteine-rich-domain-specific functions. Iba et al. [36,37] reported that the cysteine-rich domain of ADAM12 acts as a ligand for the cell-adhesion molecule syndecan. Dr. DeSimone's group [26] recently showed that the cysteine-rich domain of ADAM13 cooperates intramolecularly with the metalloproteinase domain of ADAM13 to regulate its function, providing the first evidence that a downstream extracellular adhesive domain plays an active role in the regulation of ADAM protease function in vivo. In this report, we have demonstrated that disulfide bonds likely play a crucial role in the regulation of hADAM19 processing, but not in its folding or secretion (Figs. 2B, C, 4, 5, and 6), as illustrated by DTT treatment or changes of pH (Figs. 2B and C). DTT is a reducing agent capable of penetrating into cells and preventing the formation of disulfide bonds, as with clusterin, a secreted glycoprotein that forms disulfides intracellularly [32]. As a result of DTT treatment, clusterin is retained in the endoplasmic reticulum and is undetectable in the cultured medium of MDCK cells (Fig. 2D and Ref. [30]). Decreased pH in culture media may also impair the formation of disulfides, interfering with the processing of hADAM19, as shown in Fig. 2C. Moreover, disulfide bonds that include the cysteine residues at C^{605}, C^{633}, C^{639}, and C^{643} within the fraction of the cysteine-rich domain of the C-fragment processed at E^{586}-S^{587} of hADAM19 are necessary for the association of the processed N-fragment with its C-fragment (Fig. 4). A new processing site at K^{543}–V^{544} is exposed when these cysteine residues are mutated individually, suggesting that intrafragment disulfide bonds likely contribute to the normal conformation of hADAM19 and conceal this alternative processing site in hADAM19. This site may become exposed when the conformation of hADAM19 is modified, either by C to S mutations (Figs. 4, 5B, 6A, C, and 7A), reduction with DTT (Fig. 2B), or alteration of pH (Fig. 2C). This may explain why GM6001 slightly inhibits the shedding of F-C633S hADAM19 (Fig. 7A), but has no effect on the processing of soluble hADAM19 [24]. In particular, all mutants with these specific cysteine residues exchanged for serines seem to exhibit increased processing, as the fragment at 26 kDa is more detectable in these mutants (Figs. 4, 5B, and 6). Therefore, we may speculate that the C-fragment, containing part of the cysteine-rich domain after the processing at E^{586}–S^{587}, might have an inhibitory effect on hADAM19 processing through the involvement of interfragment disulfide bonds with the N-fragment containing the metalloproteinase and disintegrin domains and the remainder of the cysteine-rich domain. This proposed model may also be applied to other ADAMs, such as ADAM8, 13, 19, and ADAM-TS4, as they must also be processed at their C-termini by autolysis to be functional after cleavage of their prodomains [16,17,23,25]. Our results may provide the first pieces of in vitro evidence to support the results of the in vivo ADAM13 studies discussed above [26], and show that the processing or shedding of hADAM19 occurs autolytically and intramolecularly within its cysteine-rich domain ([24]; Figs. 1 and 5A).

There are a total 43 cysteine residues available in the metalloproteinase, disintegrin, cysteine-rich, and EGF-like domains of hADAM19. Although we have not conclusively identified the precise disulfide pairs, it is possible that disulfide bonds are formed between the disintegrin domain of hADAM19 and the cysteine-rich domain of the C-fragment because the disintegrin domain of hADAM19 also plays a key role in the proteolytic activity of hADAM19. We have previously shown that our specific antibody against the disintegrin domain inhibited substrate cleavage in vitro [27], and a deletion mutant containing only the metalloproteinase domain of hADAM19 lacked proteolytic activity during alpha2-M and peptide substrate assays in vitro (data not shown). Any one of the four cysteine residues at C^{605}, C^{633}, C^{639}, and C^{643} in the C-fragment cysteine-rich domain is indispensable for the association between the N-fragment and C-fragment processed at E^{586}–S^{587}. We hypothesize three disulfide bonds; one formed between two cysteine residues of the C^{605}, C^{633}, C^{639}, and C^{643} group, and two formed by the remaining two cysteine residues paired with two other cysteine residues, with at least one occurring before the position of E^{586} in the N-fragment. Alternatively, four disulfide bonds may exist, formed between C^{605}, C^{633}, C^{639}, and C^{643} and four other cysteine residues, with at least one before E^{586}. These bonds have a strong coordination with each other, and when one of these disulfide bonds is disrupted, the others will be subsequently disturbed. Therefore, we propose that three or four disulfide bonds, likely linked by the four cysteine residues at C^{605}, C^{633}, C^{639}, and C^{643} with other cysteine residues in the N-fragment, are responsible for the association between the N-fragment and C-fragment processed at E^{586}–$0S^{587}$. In the near future, we would like to determine the identity of these disulfide bonds using multiple approaches including structural biology, analytical chemistry, and biochemical methods.

Acknowledgments

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Review

The significance of focal myoepithelial cell layer disruptions in human breast tumor invasion: a paradigm shift from the "protease-centered" hypothesis

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Abstract

Human breast epithelium and the stroma are separated by a layer of myoepithelial (ME) cells and basement membrane, whose disruption is a prerequisite for tumor invasion. The dissolution of the basement membrane is traditionally attributed primarily to an over-production of proteolytic enzymes by the tumor or the surrounding stromal cells. The results from matrix metalloproteinase inhibitor clinical trials, however, suggest that this "protease-centered" hypothesis is inadequate to completely reflect the molecular mechanisms of tumor invasion. The causes and signs of ME cell layer disruption are currently under-explored. Our studies revealed that a subset of pre- and micro-invasive tumors contained focal disruptions in the ME cell layers. These disruptions were associated with immunohistochemical and genetic alterations in the overlying tumor cells, including the loss of estrogen receptor expression, a higher frequency of loss of heterozygosity, and a higher expression of cell cycle, angiogenesis, and invasion-related genes. Focal ME layer disruptions were also associated with a higher rate of epithelial proliferation and leukocyte infiltration. We propose the novel hypothesis that a localized death of ME cells and immunoreactions that accompany an external environmental insult or internal genetic alterations are triggering factors for ME layer disruptions, basement membrane degradation, and subsequent tumor progression and invasion.

Keywords: Human breast tumor microinvasion; Ductal carcinoma in situ; Focal myoepithelial layer disruption; Basement membrane degradation; Leukocyte infiltration; Epithelial–stromal interactions; Cell proliferation; Loss of heterozygosity (LOH); "Protease-centered" hypothesis; Matrix metalloproteinase inhibitors (MMPIs)

Contents

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Introduction

The epithelium of normal human breasts and in situ breast tumors is physically separated from the stroma by both the myoepithelial (ME) cell layer and the basement membrane, whose degradation is an absolute prerequisite for tumor invasion and metastasis. Basement membrane degradation and tumor invasion have been attributed primarily, if not solely, to the over-production of proteolytic enzymes by the tumor or the surrounding stromal cells [1]. This hypothesis alone, though compelling, appears inadequate to completely reflect the intrinsic mechanisms of these events for three main reasons: first, the ME cell layer is a normal structural constituent that should not be a target of the host's own enzymes; second, neither the natural turnover nor the dynamic alterations of ME cells during tumor invasion have been elucidated; and third, although results from in vitro tests and animal models have clearly demonstrated that protease inhibitors could effectively inhibit or prevent tumor invasion or metastasis, results from protease inhibitor-based human oncology clinical trials have not been successful [2,3].

This review attempts to elucidate the biological and clinical profiles of ME cells based on previously published reports and our own recent studies, and to assess the potential significance of ME cells in tumor progression and invasion. In addition, this article presents several unique findings from our recent studies correlating the structural integrity of ME cell layers with the genetic and immunohistochemical profiles in adjacent tumor cells. These unconventional findings and other supportive data have led to the novel hypothesis that ME cell layer disruption and basement membrane degradation, and subsequent tumor invasion, are triggered by a localized ME cell death and the resultant leukocyte infiltration and immunoreactions that accompany an external environmental insult or internal genetic alterations. The rationale and postulated steps for our hypothesis are summarized and discussed.

The normal distribution, morphology, and functions of myoepithelial cells

The normal human breast consists of two major compartments, the epithelium and the stroma. The epithelium contains two cell types, ME cells and luminal cells, which form both the secretory lobules and branching ducts. ME cells are positioned between the luminal cells and basement membrane, attaching to luminal cells by desmosomes and the basement membrane by hemidesmosomes [4–6]. The basement membrane is composed laminin, type IV collagen, entactin, heparan sulfate proteoglycans, glycosaminoglycans, and other components, forming a continuous lining surrounding the ME cells [7,8]. ME cells are joined by intercellular junctions and adhesion molecules, forming a continuous layer that encircles the entire duct system, and a discontinuous layer or a basket-like structure that covers a vast majority of the cells at terminal duct-lobular units and lobules. The ME layer and the basement membrane are normal structures, separating the epithelium from the stroma (Fig. 1).

In clear-cut normal breast tissues, ME and epithelial cells are approximately equal in number, and ME cells are generally of a cuboid or spindle shape, with pale cytoplasm and nuclei. The number and morphology of ME cells, however, can vary substantially at different stages of tumor progression. In hyperplastic and in situ lesions, ME cell layers are often substantially attenuated, and the cells stretched out, with little or no distinct cytoplasm (Fig. 2), morphologically resembling the smooth muscle cells of small blood vessels and fibroblasts. In invasive lesions and a rare benign lesion, microglandular adenosis, ME cells are either not appreciable or are occasionally found in residual normal tissues trapped in these lesions. ME cells contain a large number of microfilaments, a unique structural component of smooth muscle cells, and intermediate filaments, a unique structural component of epithelial cells [9].

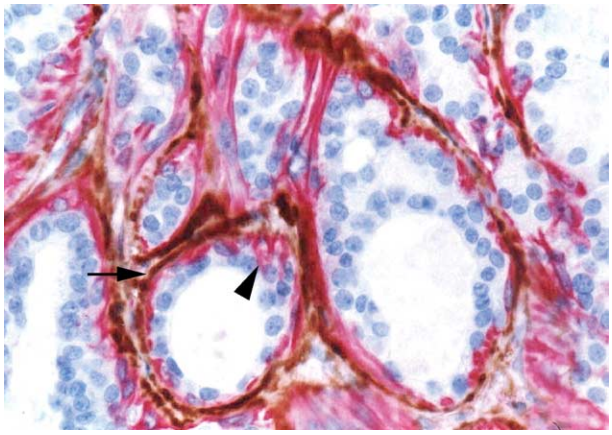

Fig. 1. The architectural relationship of the basement membrane, myoepithelial (ME) cells, and epithelial cells of ducts in a normal human mammary gland. A paraffin section double-immunostained for ME cells and basement membrane with anti-smooth muscle actin (red) and collagen IV (brown) antibodies, respectively. The arrow identifies the basement membrane (brown), the arrowhead designates a ME cell (red), and blue nuclei stained by hematoxylin are those of the luminal epithelial cells (400×).

This architecture confers upon the ME cells and basement membrane two essential biological and clinical functions. First, as normal epithelial components are devoid of blood vessels and lymphatic ducts, and are therefore totally dependent upon the stroma for their nutritional, metabolic, and survival needs, the ME cells and basement membrane serve as natural structural barriers that directly mediate communications between these two compartments. Second, the cells of in situ tumors must first pass through the ME cell layer, then the basement membrane, to physically reach the stroma. Thus, the degradation of both the ME cell layer and the basement membrane is an absolute prerequisite for tumor invasion (Fig. 3).

In addition to these passive functions, recent studies have revealed that ME cells might also possess the following active functions.

Production of tumor suppressor proteins

A number of tumor suppressor proteins, including maspin, Wilms' tumor 1, p63, p73, and 14-3-3Sigma have been detected exclusively or preferentially in ME cells [10–15]. In vitro and in vivo studies have demonstrated that these proteins have significant inhibitory functions against tumor growth and invasion [10,12–15].

Paracrine down-regulation of matrix metalloproteinase (MMP) expression

ME cells may modulate breast tumor invasion by controlling the expression of MMPs in both the tumor and the surrounding fibroblasts. To explore this possibility, a recent study quantitatively compared MMP expression levels in breast cancer cell lines and fibroblasts co-cultured with and without ME cells purified from normal breast tissues [16]. Results revealed that both cancer cells and fibroblasts co-cultured with ME cells had significantly lower expression of MMP-2, MMP-9 and MT1-MMP when compared to those cultured without ME cells, and this reduction of MMP expression was accompanied by reduced invasion [16].

Turnover of extracellular matrix (ECM)

While many ECM-degrading enzymes found in breast tissues are manufactured by the stromal cells, several such enzymes, including a recently described angiogenesis-related matrix metalloproteinase, MMP-19, are reported to be produced by normal ME cells [17], and are expected to participate in the turnover of ECM [17,18].

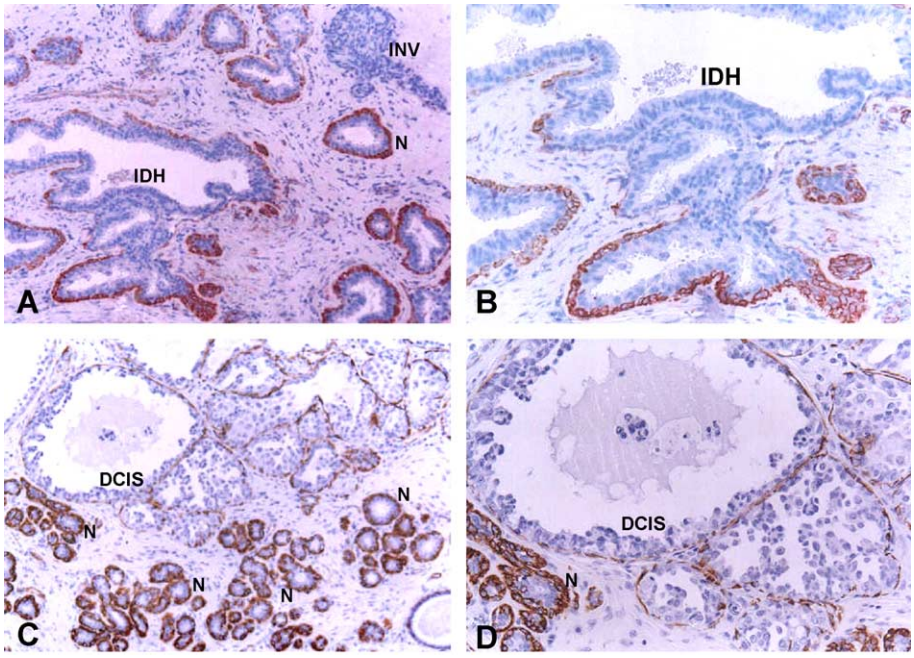

Fig. 2. The morphology of human breast tissues containing normal and hyperplastic epithelial cells, ductal carcinoma in situ, and invasive carcinoma. Two paraffin sections were immunostained for smooth muscle actin (red). The ratio of ME cells to epithelial cells is approximately 1:1 in the normal ducts (N). The ME cell layer is generally intact and distinct in the normal ducts, but attenuated in intraductal hyperplasia (IDH) and ductal carcinoma in situ (DCIS), and absent in the invasive lesion (INV). 2B and 2D (200×) are higher magnifications of 2A and 2C (100×), respectively.

Regulation of normal development

During normal development, a number of growth factors and their corresponding receptors, including the epidermal growth factor, basic fibroblast growth factor and its receptor, Tcf transcription factors, and transforming growth factors, are differentially expressed in the luminal and ME cells [19–22]. A perturbation in the expression of these and other related molecules in ME cells led to abnormal breast morphogenesis and development [20,21].

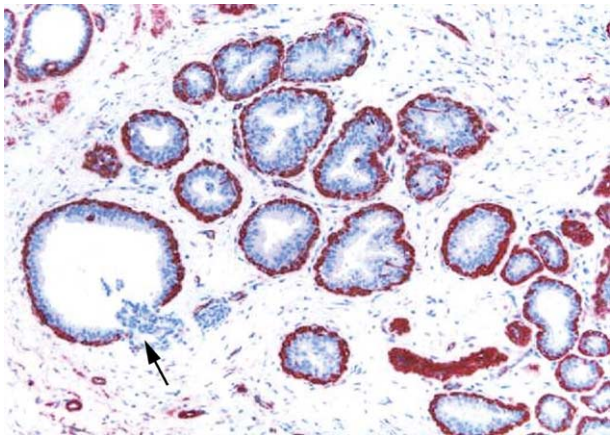

Fig. 3. Disruption of the ME cell layer and degradation of the basement membrane are prerequisites for invasion. A paraffin section was immunostained for both smooth muscle actin and collagen IV using the same chromogen (red). The ME cell layer and basement membrane in one duct is focally disrupted and the epithelial cells are in direct contact with the stroma (an arrow; 100×).

Establishment and stabilization of the polarity of the bilayer epithelial structure

In tissue cultures, luminal cells alone could form acinus-like structures with a reversed polarity [18]. The addition of ME cells from normal breast tissues could establish and stabilize a correct polarity, whereas ME cells from breast tumors failed to correct the reversed polarity, suggesting that normal ME cells play important roles in the establishment and stabilization of bilayer epithelial structures [18].

Steroid hormone metabolism

A recent study assessed the mRNA expression and enzymatic activity of steroid sulfatase (STS) in normal human breast ME and human adenocarcinoma (MCF-7) cells, and the effects of 17-beta estradiol on the activity of STS [23]. The study revealed that sulfatase activity was about 120 times greater in the ME cells than in the MCF-7 cells, and that exposure to 17-beta estradiol was associated with a 70% reduction of sulfatase activity in the MCF-7 cells, but a 9% increase in ME cells, suggesting a potential role for ME cells in the conversion of hormone precursors into active steroid hormones [23].

Exclusively or preferentially expressed bio-molecules in myoepithelial cells

Currently, there are more than a dozen commercially available antibodies that can be used for immunohistochemistry in formalin-fixed, paraffin-embedded sections to detect the molecules reported to be exclusively or preferentially present in ME cells. Based on their compositions, subcellular localizations, and potential functions, these molecules can be roughly classified into two groups.

Structure-specific proteins (related to microfilaments and intermediate filaments)

Smooth muscle actin (SMA)

SMA forms microfilamentous contractile polypeptides, and presents in cells with myogenous features such as smooth muscle cells, myoepithelial cells, myofibroblasts, and vascular pericytes. Each smooth muscle cell contains thick (myosin) and thin (actin) filaments that slide against each other to produce contraction of the cells. Smooth muscle cells are found in the walls of many hollow organs of the body, and their contraction reduces the size of these structures. In human breast tissues, SMA immunostaining is present in the cytoplasm of about 95% of the ME cells in normal ducts and lobules, as well as in non-invasive breast lesions [24,25]. Invasive lesions, and a very rare benign lesion, microglandular adenosis, are devoid of SMA immunoreactivity due to the lack of ME cells surrounding these lesions. A diagnosis of invasive malignancy may be confirmed when both type IV collagen and SMA are negative, and ruled out when both markers are positive. In some instances, false-positive SMA staining may represent the smooth muscle cells of small blood vessels or myofibroblast cells of the stroma that are often adjacent to the invasive components.

Smooth muscle myosin heavy chain (SMMHC)

SMMHC, a cytoplasmic structural protein, is a major component of the contractile apparatus of smooth muscle cells, and is specific for the early development of smooth muscle. It is also a myoepithelium-associated protein, presenting heavily in the ME cells of normal and benign breast tissues, and has been reported to be a very useful marker in differentiation between noninvasive and invasive breast lesions [26,27].

Calponin

Calponin, a calmodulin, binds tropomyosin and F-actin, and is thought to be involved in the regulation of smooth muscle contraction. The expression of calponin is largely restricted to smooth muscle cells, but has also been demonstrated in human breast ME cells. It is a useful marker in the differentiation between ME cells and spindle cells of the stroma, and also between non-invasive and invasive lesions [27,28]. However, calponin staining in ME cells may occasionally be discontinuous or absent in in situ ductal lesions [29].

H-caldesmon (HCD)

HCD is a cytoskeleton-associated actin-binding protein. In human breast tissues, HCD is predominantly expressed in ME cells, but a high level of HCD expression is also often seen in the smooth muscle cells of small blood vessels [30,31].

P-cadherin

P-cadherin, like E-cadherin, is a Ca^{2+}-dependent cell adhesion molecule with a fundamental role in the maintenance of the integrity of multicellular structures and the phenotype of epithelial cells [32]. In breast tissues, p-cadherin is preferentially expressed in the ME cells of normal ducts, lobules, and sclerotic lesions [33]. However, p-cadherin is also occasionally seen in hyperplastic tissues and in some tumor cells of in situ carcinomas [34].

Cytokeratins (CK) 5, 7, 14, and 17

Cytokeratins 5, 7, 14, and 17 are a group of cellular structural proteins found in epithelial cells and cells with epithelioid features [35]. In human breast tissues, these molecules are predominantly present in the myoepithelial cell layer [36,37]. CK-17 is also related to early skin development and wound repair [38].

Non-structural molecules

Maspin

Maspin, or mammary-specific serpin, is a tumor suppressor protein belonging to the serine proteinase inhibitor family [39,40]. It is present in both breast and prostate glands. In the breast, maspin is predominantly a soluble cytoplasmic protein that is consistently detectable in normal human ME cells and ME-derived tumors [39,41]. The expression of maspin decreases with increasing malignancy of breast tumors [39–41]. Both in vitro and in vivo studies have shown that maspin exhibits significant inhibition of tumor growth and invasion [39,40].

Wilms' tumor 1 (WT-1)

WT-1 is as a transcription factor regulating gene expression in a fashion similar to that of p53 [42]. The expression of WT-1 in the breast appears to correlate with the behavior and progression of breast tumors [43]. Our previous study showed that WT-1 expression was consistently detectable in ME cells, and that the level of WT-1 expression was inversely correlated with breast tumor progression [44].

p63 and p73

p63 is a nuclear protein that shares significant amino acid identity with the p53 protein in the transactivation domain, the DNA-binding domain, and the oligomerization domain [12,13]. The expression of p63 in the breast is exclusive to

the nuclei of ME cells, and neither stromal fibroblasts nor vascular smooth muscle cells show p63 immunostaining [45]. p63 is believed to be critical for maintenance of the progenitor cell populations that are necessary to sustain epithelial development and morphogenesis [12,13]. p73 is also a homologue of p53, and is believed to have a function and distribution similar to that of p63 [14].

14-3-3Sigma

This recently introduced ME cell marker is a candidate tumor suppressor gene transactivated by p53 in response to DNA damage [15]. It is consistently present in benign and pre-invasive breast lesions, and is preferentially expressed by myoepithelial cells. It might also serve as a novel prognostic factor for breast cancer patients [15].

Neuropilin-1 (NRP-1)

NRP-1 is a recently identified specific receptor for the vascular endothelial growth factor [46,47]. ME cells in both hyperplastic and neoplastic lesions displayed a higher level of NRP-1 expression than those in normal breast tissues. NRP-1 expression is also detectable in vascular smooth muscle and endothelial cells [46,47].

CD 10

CD10 is a 100-kDa cell surface metalloendopeptidase, also called neprilysin, which inactivates a variety of biologically active peptides. This protein was initially classified as a common acute lymphoblastic leukemia antigen. Subsequent studies have shown that CD10 was consistently positive in the ME cells of all normal breast tissues, whereas the number of ME-positive cells and the intensity of the immunostaining were substantially reduced in distended ducts and ductal adenomas [48–50].

S100

The S100 gene family comprises more than 20 members whose protein sequences encompass at least one EF-hand Ca^{2+}-binding motif. The expression of individual family members appears to be tissue-specific [51]. S100 proteins are consistently present in human breast ME cells, but are often expressed in luminal epithelial cells, thus, they are not specific markers for myoepithelial cells [52–54]. Molecular analysis of breast tumors has revealed that several S100s, including S100A2, S100A4, and S100A7, exhibit altered expression levels during breast tumorigenesis and progression [51]. S100A4 is considered a tumor promoter gene [55]. Elevated levels of S100A4 are associated with poor survival rates in breast cancer patients and induce metastasis in rodent models, as well as increased cell motility and invasion in vitro [56]. S100A9 expression in human invasive breast adenocarcinoma is closely associated with poor tumor differentiation [57]. Further studies are needed to better understand the important roles of S100s in human breast cancer.

Other important molecules

A number of growth factors, growth factor receptors, and other biologically important molecules have also been reported to be present in ME cells, but their clinical significance has yet to be determined [20–22,35].

Common myoepithelial markers used in distinguishing in situ from invasive tumors

Immunohistochemically, some of the above-mentioned ME cell markers have been routinely used in the clinic for the detection of ME cells in the differentiation between in situ and invasive tumors. Among these, SMA is the most frequently employed, and is considered to be very reliable. We and others, however, have repeatedly noticed that about 4–6% of morphologically distinct ME cells in hematoxylin and eosin (H&E) stained sections fail to show SMA immunostaining [24,25]. In an attempt to further characterize these SMA-negative cells, one of our recent studies assessed H&E- and SMA-immunostained sections from 175 breast cancer patients, and identified three cases harboring ducts that displayed morphologically distinct ME cell layers in H&E sections while showing no SMA immunostaining in $\geq$ 1/3 or the entire ME cell layer [11]. Eight additional consecutive sections from each of these cases were stained for SMA with a black chromogen, and the same ducts with SMA-negative ME cells in each of the eight sections were photographed. Then, each section was re-stained with a red chromogen for one of eight additional markers that are supposed to be exclusively or preferentially expressed in ME cells. The same duct with SMA-negative cells in each of the eight sections was then re-examined for the expression of the other markers. Results showed that SMA-negative ME cells in two cases also failed to display immunoreactivity for any of the other markers, including calponin, CD10, smooth muscle myosin-heavy chain (SMMHC), maspin, Wilms' tumor-1, and cytokeratins 5, 14, and 17 (CK5, 14, and 17). SMA-negative ME cells in one case, however, showed distinct immunoreactivities for maspin, as well as CK5, 14, and 17 [11].

Major myoepithelial cell lesions

Compared to epithelial lesions, ME cell lesions are very rare, which frustrates a detailed biological and clinical profiling of this entity. Morphologically, ME cell lesions can be classified into three primary categories [58,59].

Myoepitheliosis

This lesion is characterized by a mild proliferation of ME cells within and around ducts. The proliferating cells are of spindle or cuboid shape, often distending or occluding the involved ducts. These ME cells can be easily distinguished from the adjacent ductal epithelial cells by immunohisto-

chemical staining because antibodies against microfilament- and intermediate filament-related molecules will recognize ME cells, while antibodies against epithelial-specific antigen (ESA) will uniformly identify the epithelial cells.

Adenomyoepithelioma

This lesion is characterized by extensive proliferation of ME cells. Morphologically, these proliferating cells can be divided into spindle cells, tubular cells, and lobular variants. The common feature of this abnormality is the formation of solid masses that distend or occlude the ductal structures. Similar to myoepitheliosis, these hyperplastic lesions can be easily differentiated from epithelial cells by immunohistochemical staining with antibodies against microfilament- and intermediate filament-related molecules or epithelial-specific antigen (ESA).

Myoepithelial carcinoma

These lesions are rare, accounting for less than 1% of breast malignancies. Based on published reports, the most commonly seen type of this lesion is the spindle cell type. This type of lesion is not typically clinically aggressive, although examples of metastases from pure ME carcinomas have been reported [60–62]. Breast carcinomas composed in part or entirely of myoepithelial cells have been diagnosed in well-defined categories: benign tumors, tumors with low malignant potential, and malignant tumors, including adenomyoepithelioma, low-grade adenosquamous carcinoma, adenoid cystic carcinoma, and malignant myoepithelioma [58,59,63]. Pure myoepithelial carcinoma of the breast is rare, and little is known about its natural history or long-term outcome following treatment. A recent report showed that it adopts an aggressive clinical course, with an outcome comparable to poorly differentiated adenocarcinoma of the breast [64]. Comparative genomic hybridization analysis of myoepithelial carcinomas of the human breast compared to ductal carcinomas revealed that the mean number of genetic alterations was 2.1 for myoepithelioma and 8.6 for ductal carcinoma [65]. The relatively few genetic alterations found in otherwise aggressive neoplasms indicate that myoepithelial tumors may be useful models for the delineation of genes important in breast tumorigenesis.

Impacts of ME cell alterations on adjacent epithelial cells

While attempting to identify the early signs of ME layer disruptions and precursors of invasive breast lesions, we have recently carried out a number of studies focusing on the correlation between structural integrity in the ME cell layers and the immunohistochemical and genetic profiles in adjacent epithelial cells. Our studies have revealed, for the first time, several lines of evidence suggesting that the structural integrity of ME cells directly impacts the biological phenotypes of epithelial cells, tumor progression, and invasion processes.

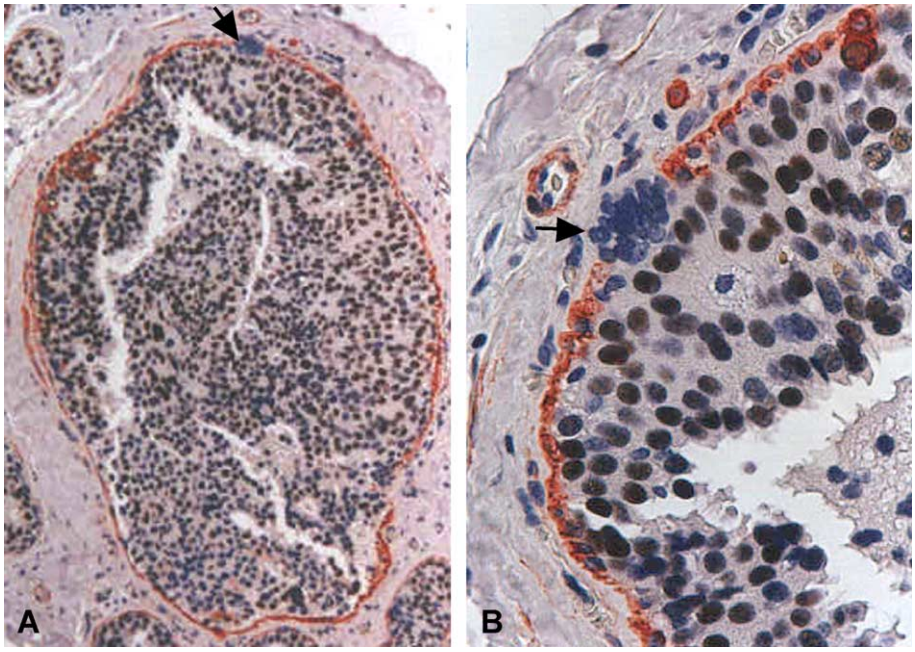

Fig. 4. Focal disruptions in ME cell layers and loss of ER expression in overlying tumor cells. A paraffin section was double-immunostained for ER (black or brown) and SMA (red). Arrows identify focal ME disruptions and overlying ER-negative cell clusters. (A) 100× magnification; (B) 400× magnification. (Adapted from Fig. 2, Ref. No. 44, copyright of Dr. Yan-Gao Man).

Focal ME layer disruptions and loss of ER expression in overlying epithelial cells are correlated events

In 5698 duct cross-sections from 220 patients with estrogen receptor (ER) positive, in situ breast tumors, we detected a total of 405 (7.1%) focal ME cell layer disruptions, defined as the absence of ME cells resulting in a gap equal to or greater than the combined size of 3 ME cells [44]. Of these, 350 (86.4%) were overlaid by clusters of epithelial cells with no or substantially reduced ER expression, in sharp contrast to adjacent epithelial cells within the same duct, which showed strong ER immunoreactivity and overlaid a non-disrupted ME cell layer (Fig. 4) [44].

Cells in ducts with and without focal ME disruptions have different proliferation rates

Compared to those in morphologically comparable ducts without focal ME layer disruptions, cells in ducts with focally disrupted ME layers with or without ER-negative clusters had a significantly higher ($P < 0.01$) proliferation rate, 19.05% with disruptions versus 4.0% without disruptions (Man et al., unpublished data [66]), as determined by immunohistochemical staining for Ki-67 (Fig. 5). From 200 patient cases, positive cells were counted under a microscope, and the proliferation rate was statistically analyzed in morphologically similar ducts (defined as the same histological type and grade, with similar size, shape, and architecture) with and without focal ME disruptions from 20 randomly selected cases (one section for each case) using Student's *t* test. A cell was considered Ki-67-positive if a distinct nuclear staining was seen, based on the instructions provided by the manufacturers (Vector and Dako). The expression of the human Ki-67 protein is strictly associated with cell proliferation. Ki-67 is present during all active phases of the cell cycle, including G(1), S, G(2), and mitosis, but is absent from resting cells, in G(0), making it an excellent marker for cell proliferation [67].

Loss of heterozygosity (LOH) in estrogen receptor (ER)-negative cells

ER-negative cells had a substantially higher frequency and different pattern of loss of heterozygosity (LOH) at multiple chromosomal loci when compared to ER-positive cells, including those harboring tumor suppressor genes fragile histidine triad and Wilms' tumor 1 [11,44] (Fig. 6).

Breast epithelial cell clusters at the site of ME layer disruptions have increased expression of genes related to proliferation, apoptosis, invasion, and metastasis

ER-negative and adjacent ER-positive cells within the same duct were microdissected for RNA extraction and amplification from consecutive sections of 30 frozen DCIS tissues with focally disrupted ME cell layers. Amplified RNA molecules were converted to biotin-labeled cDNA molecules and interrogated with "Cancer PathwayFinder"

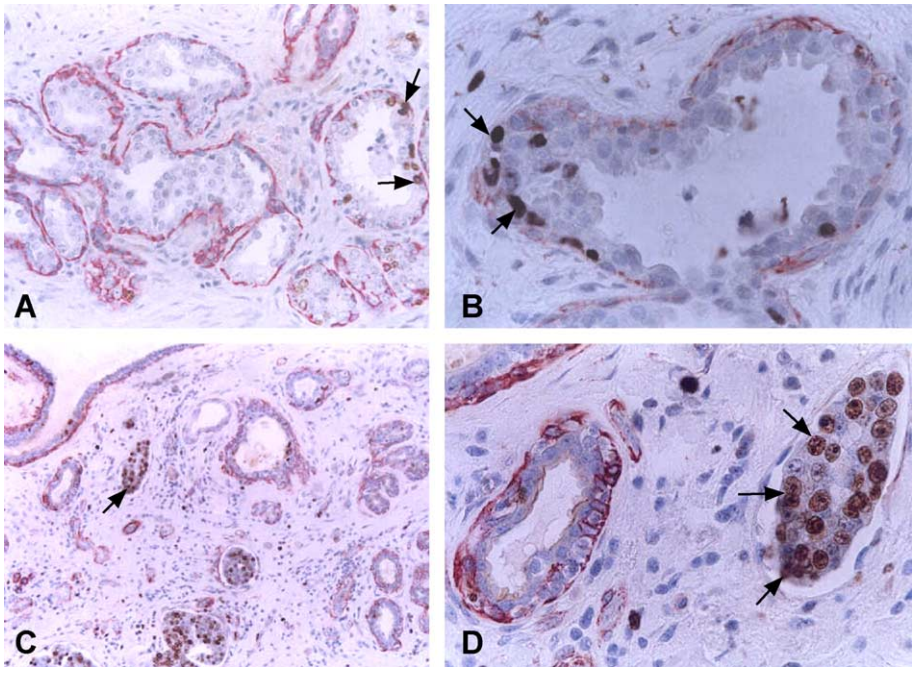

Fig. 5. Cells in ducts with and without focal ME cell layer disruptions show a different proliferation rate and localization of clusters of proliferating cells. Three paraffin sections were double-immunostained for Ki-67 (brown to black) and SMA (red). Note that more Ki-67-positive cells are seen in the ducts with a focally disrupted ME cell layer (arrows) compared to adjacent ducts without focal ME cell layer disruptions. Many Ki-67-positive cells are located near focal ME layer disruptions (A and B), or in the ducts without distinct SMA staining (C and D) (arrows). A (200×) and B (400×) are from two different slides, while C (100×) and D (400×) are from the same slide.

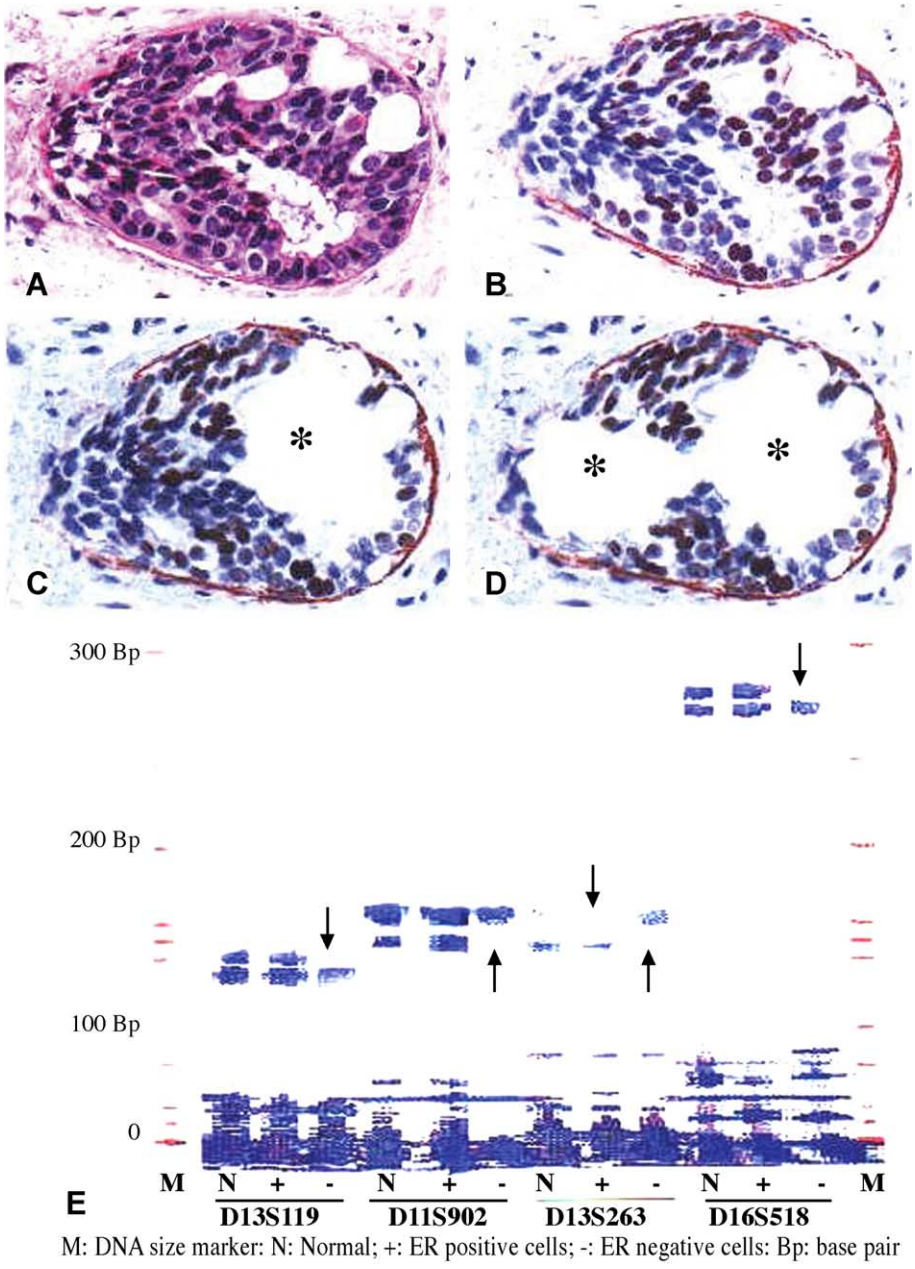

Fig. 6. Comparison of the loss of heterozygosity (LOH) pattern in ER-negative and ER-positive cells within the same duct. (A) H&E staining of a duct with atypical intraductal hyperplasia. (B) The adjacent section of A, immunostained for ER (brown) and SMA (red). (C) Microdissection of ER-positive cells. (D) Microdissection of ER-negative cells. (E) LOH at four selected DNA markers. Asterisks indicate the ER-positive and negative cells removed for LOH assessment; arrows identify LOH. (Adapted from Fig. 7, Ref. No. 44, copyright of Dr. Yan-Gao Man).

arrays. Epithelial cell clusters overlying focally disrupted ME layers had higher levels of mRNA expression in genes related to proliferation, apoptosis, invasion, and metastasis when compared to their adjacent counterparts within the same duct [68] (Fig. 7 and Table 1). Among the 11 up-regulated genes in ER-negative cell clusters overlying focally disrupted ME cell layers, 8 directly or indirectly promote proliferation and tumor progression, while 3 promote apoptosis. The intrinsic mechanism for a simultaneous elevation of both proliferation and apoptosis-related genes in the same cell cluster after a focal ME disruption is unknown and counterintuitive, but this may represent a defensive response resulting from leukocyte infiltration and immunoreactions.

Leukocyte infiltration is increased at focal ME disruptions

As macrophages and other leukocytes contain digestive enzymes capable of degrading the basement membrane and injuring host cells, our recent studies assessed the possible roles of these cells in ME cell layer disruptions and tumor invasion. A total of 23 DCIS samples containing ducts with

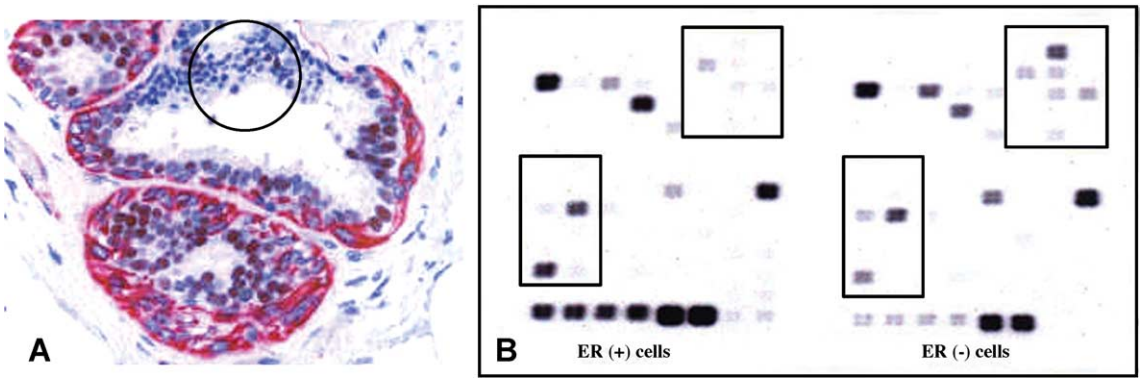

Fig. 7. Comparison of the frequency and level of mRNA expression in ER (−) and adjacent ER (+) cells within the same duct. Frozen sections from 30 selected cases were double-immunostained for ER (brown) and SMA (red). The ER-negative cells overlying focally disrupted ME cell layers and their adjacent ER-positive counterparts within the same duct were microdissected for RNA extraction, amplification, and analyses using a focused cDNA microarray containing 96 tumor progression and invasion related genes. Chemiluminescent array images were captured and digitized using the FluorChem 8800 Imaging System. The integrated density values in paired samples with distinct signals in both ER-negative and ER-positive cells were measured and compared. Of 15 differentially expressed genes, 11 were higher in ER (−), 2 were higher in ER (+), and 2 were equal in these two cell types ($P < 0.01$). (A) The microdissected ER (−) and ER (+) cells. (B) cDNA microarray images of ER (−) and ER (+) cells in a selected case.

focally disrupted ME cell layers were selected from 94 cases identified in our previous studies. Two consecutive sections from each case were double immunostained, one with leukocyte common antigen (LCA) plus smooth muscle actin (SMA), the other with Ki-67 plus SMA. Ducts lined by ≥50 epithelial (EP) cells and distinct ME cell layers were similarly examined. To the best of our knowledge, our studies have revealed several interesting phenomena that have not been reported previously:

Ductal tumors with and without ME disruptions have different rates of leukocyte infiltration

A total of 191 duct cross-sections were found to contain focal ME cell layer disruptions; of which, 186 (97.4%) were with and 5 (2.6%) were without leukocyte infiltration. Of 207 morphologically similar sections without ME disruptions, 46 (22.2%) were with and 161 (77.8%) were without leukocyte infiltration (Man et al., unpublished data [69]) (Fig. 8 and Table 2).

Table 1
Comparison of the frequency and level of mRNA expression in ER (+) and ER (−) cells

Gene group	Gene name	Higher in ER (−)	Higher in ER (+)	Equal in both	Case number
Adhesion	CD44	13	4	1	18
	CDH1	13	4	3	20
	MUC18L	5	5	2	12
Angiogenesis	EGFR	5	6	1	12
	IFNA1	10	5	2	17
	TNF	5	9	6	20
Apoptosis	BAX	9	4	0	13
	CASP9	9	5	5	19
	CFLAR	10	5	1	16
Cell cycle	CDC25A	9	1	7	17
	CDKN2A	10	4	2	16
	RAD53	9	1	2	12
Inv and metastasis	NME4	10	4	6	20
	TIMP1	6	6	3	15
Signal transduct	NFKB1	11	6	3	20
Total		134 (54.3%)	69 (27.9%)	44 (17.8%)	247
P	<0.01				

ME cells surrounded by or adjacent to leukocytes show distinct morphologic alterations

When compared to their counterparts farther away from the leukocytes, ME cells surrounded by or adjacent to leukocytes, where focal disruptions of the ME cell layer are observed, often show distinct morphologic alterations, including the loss of SMA immunostaining (Fig. 8 and Man et al., unpublished results]. The ME cells surrounding focally disrupted ME layers, in contrast to the epithelial cells, generally display a substantially lower proliferation rate when compared to those in non-disrupted layers (Man et al., unpublished results).

The vast majority of proliferating clusters of epithelial cells is located at or near focally disrupted ME layers

Ki-67-positive cells in ducts with focally disrupted ME cell layers were generally subjacent to disruptions, and over 30 clusters of proliferating cells were seen directly overlying or near focally disrupted ME cell layers (Fig. 5). In contrast, Ki-67-positive cells in ducts without ME disruptions were scattered randomly over the entire epithelial compartment [69].

Our hypothesized mechanism for ME cell layer disruption and tumor invasion

The mechanisms for focal ME layer disruptions and the formation of ER-negative cell clusters are unknown, but these are likely the result of localized ME cell death, leukocyte infiltration, and the resultant responses, based

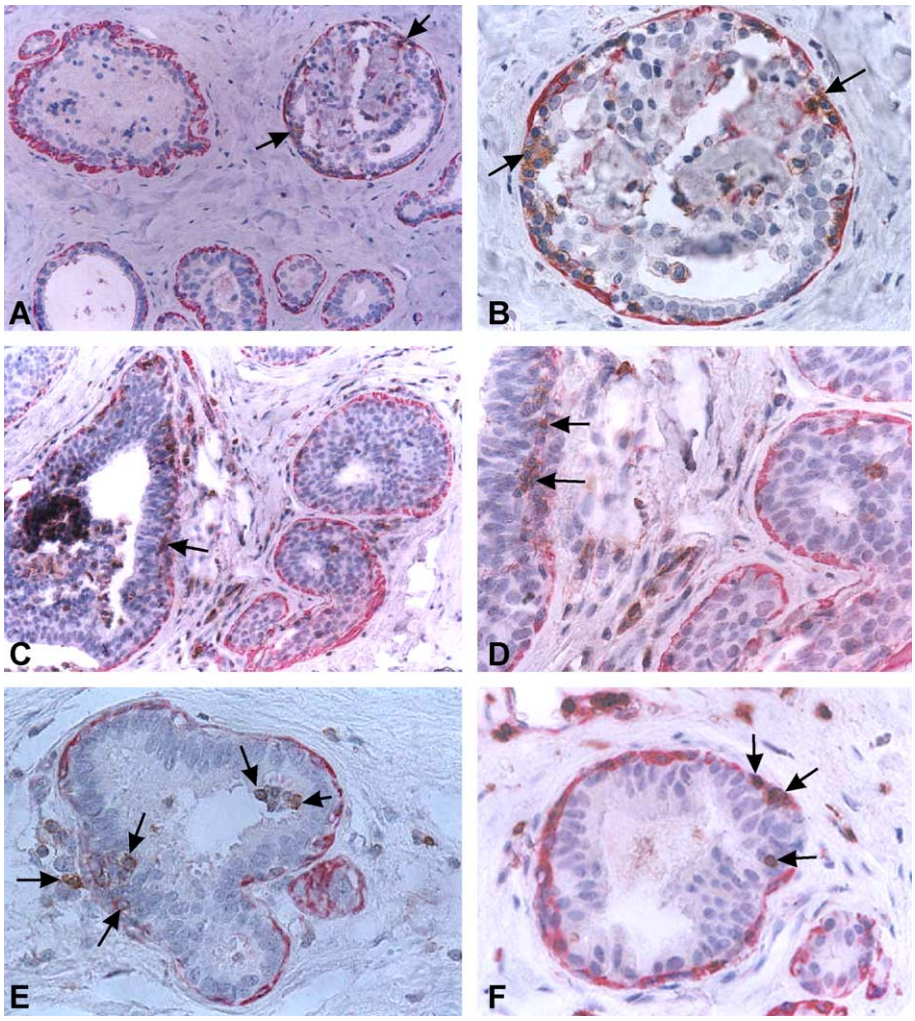

Fig. 8. The correlation between focal ME cell layer disruption and leukocyte infiltration. Four sections were double-immunostained for SMA (red) and leukocyte common antigen (brown). Many leukocytes (arrows) are located at or near the focal ME cell layer disruptions, suggesting a correlation between focal ME cell layer disruptions and leukocyte infiltration. Epithelial cells adjacent to leukocytes often show noticeable changes in size and nuclear shape compared to cells farther away from the leukocytes. A (200×) and B (400×) are from the same slide, C (200×) and D (400×) are from the same slide, and E (400×) and F (400×) are from two different slides. Human lymph nodes and blood smears were used as positive controls for leukocytes and normal mouse serum was used as a negative control for the monoclonal antibody directed against the leukocyte common antigen (data not shown).

upon the following observations. First, no morphologically distinct ME cells are detectable at the disruptions in multiple consecutive sections in all cases, indicating a physical absence of ME cells. Second, our previous studies have shown that the frequency of focal ME cell layer disruptions is independent of the size, length, and architecture of the ducts and acini [44], suggesting that it is unlikely the disruption is induced by mechanical forces, such as elevated pressure in the lumen or an increased cell number. Third, our previous studies have shown that 97.4% of the ducts with focal ME disruptions showed leukocyte infiltration, significantly increased when compared to 22.2% ($P < 0.01$) in morphologically similar ducts without focal ME cell layer disruptions [66,68]. Fourth, ME cells surrounded by or adjacent to leukocytes often showed distinct morphological alterations. Fifth, the ME cells near the focally disrupted layers, in contrast to the epithelial cells, seem to have a substantially lower proliferation rate compared to those with intact ME layers (Man et al. unpublished data [68]).

Table 2
Frequencies of white blood cell infiltration in mammary ducts with and without focal myoepithelial cell layer disruptions

	Section number	With WBC	Without WBC	*P*
Disrupted	191	186 (97.4%)	5 (2.6%)	<0.01
Non-disrupted	207	46 (22.2%)	161 (77.8%)	

Our speculation is further supported by several lines of evidence. More and more data have shown that a variety of external and internal insults could specifically affect the physical and functional status of ME cells. A wide variety of proteolytic enzymes produced by tumor or stromal cells could substantially change the physical integrity of the base-

ment membrane, which might subsequently affect the functions of ME cells [1,70]. A number of chemical compounds or biological reagents could also target ME cells. For example, exposure to lambda carrageenan, an anionic polymer, could result in filament disassembly and loss of the ME cells, while exposure to oxytocin could substantially enhance the rate of ME cell proliferation and differentiation [71,72].

The human immunosurveillance system could specifically or non-specifically target the ME cells. It has been well documented that leukocyte infiltration into tumor tissues is a common event, and that the number of leukocytes within tumor tissues is linearly increased with tumor progression [73,74]. This increase of leukocytes is particularly evident during the progression of DCIS to infiltrating ductal carcinoma, in which up to a four-fold increase of lymphoid infiltration has been reported. The extent of increased macrophage presence in tumor tissues has been found to correlate with a worse prognosis and a significantly higher mortality rate [73,74]. Furthermore, patients with lymphocyte infiltration at the tumor edge were found to have a noticeably poorer short-term prognosis compared to those with lymphocyte infiltration in other locations [75]. More importantly, leukocytes are capable of freely crossing both the basement membrane and ME cell layer, and leukocytes contain a number of digestive enzymes that can effectively degrade the basement membrane and alter host cells [76–79].

Based on these findings, we propose that a localized ME cell death and the resultant immunoreactions are the triggering factors for disruptions in ME cell layers, the formation of ER-negative cell clusters, and subsequent stromal and vascular invasion. These ER-negative cell clusters are most likely to be of epithelial origin, derived from mutated primitive stem cells or de-differentiated proliferating cells. We hypothesize that the major steps leading to the initiation and progression of tumor invasion are as follows:

(1) The ME cells constitute a self-renewing population, which normally undergoes proliferation to replace aged or injured ME cells.
(2) An external or internal insult, such as a localized trauma, inflammatory reactions of the stroma, or exposure to lambda-carrageenan, directly disrupts the structural integrity of the ME cell layers or impairs the normal replacement process, resulting in a cluster of damaged or dying ME cells.
(3) The damaged or dying ME cells lose their capacity to manufacture tumor-suppressor proteins, with subsequent loss of the paracrine regulation of adjacent tumor cells.
(4) The residual products of dying ME cells attract macrophages and other leukocytes, which migrate to the injured site and interact with altered ME cells.
(5) Macrophages and other leukocytes release digestive enzymes, leading to the physical destruction of altered ME cells and the local basement membrane, resulting in a focal disruption or gap that allows direct communication between the tumor and stromal cells.
(6) The focal disruption or gap results in a focally increased permeability for the supply of metabolism- and growth-related molecules to overlying and adjacent tumor cells.
(7) This altered micro-environment leads to variable consequences in overlying tumor and adjacent ME cells, depending on the nature of these cells: First, if the overlying and adjacent cells are fully differentiated, no substantial alteration may be detectable; second, if these cells are partially differentiated, a limited or mild increase in cell proliferation might be seen, which may lead to a localized stromal invasion; third, if they are primitive stem cells, extensive cell proliferation might be observed, which may lead to the formation of new structures with normal morphology and functions; and fourth, if they are mutated stem cells, a series of events that differ markedly from both primitive stem cell-mediated and differentiated progenitor-mediated proliferation may take place, possibly leading to the inauguration of cancer cell clones. Previous studies have shown that a mutated stem cell is the common cellular origin of teratocarcinomas and epithelial cancers [80,81].
(8) The newly formed cell clusters undergo differentiation, releasing invasion-associated molecules, which triggers angiogenesis, tissue remodeling, and increased production of growth factors in the stroma, providing a favorable environment for tumor cell growth [3,82–86].
(9) The interactions between tumor and stromal cells lead to further destruction of ME layers and degradation of the basement membrane, accompanied by deeper and wider migration and invasion of the newly formed cell clusters.
(10) In addition to in situ ductal carcinoma, the above processes could potentially occur in normal breast tissues and hyperplastic lesions, which may lead to direct invasion. These processes may also be triggering factors for the progression of tumor stages.

Implications of our hypothesis

At present time, it is unknown what factors initiate the localized ME cell death. It remains to be investigated whether myoepithelial cell death is triggered by the primary lesion during carcinoma development, inflammatory changes of the stroma, or other factors. This question would be resolved through the study of developing disruptions as opposed to existing disruptions, but as yet, the formation of these disruptions cannot be predicted. Thus, it is very difficult to solve this problem, and this review article

concentrates on the elucidation of the significance of ME cell layer disruptions in tumor progression and invasion rather than the causes of ME cell death. We submit that localized ME cell death and subsequent immunoreactions are the direct cause for ME cell layer degradations based upon five observations arising from our current studies and other reports.

(1) Our previous studies have shown that the form, size, or frequency of ME cell layer disruptions seemed to be independent of the size, length, architecture, and overall ER negativity of the ducts, and also of the histological grade of the lesions [44,84–86]. This observation indicates that it is less likely that the developing tumor cells initiated the ME cell death, as a fraction of ducts with focal ME disruptions maintain an appearance that is otherwise morphologically normal.
(2) Ducts with focal ME cell layer disruptions have a significantly higher leukocyte infiltration, and most leukocytes are located at or near the disruptions [69]. Similar correlated events of basal cell layer disruptions and leukocyte infiltration have been observed in human prostate tumors [87]. It appears that the leukocyte infiltration is in response to ME cell death, with inflammatory responses following ME cell death rather than preceding it.
(3) Proteolytic enzyme inhibitor-based human clinical trials have been disappointing, indicating that blocking basement membrane-degrading proteases alone is not sufficient to inhibit tumor invasion and metastasis [2,3]. These reports also suggest that additional factors are involved in the processes of tumor invasion and metastasis.
(4) Both mammary epithelial and ME cells belong to self-renewing populations, with a life span of about 300 days. Aged and damaged cells are constantly replaced by normal cellular replacement and regeneration processes. If the rate of ME renewal is slower than the rate of death due to genetic alternations or microenvironmental factors at a highly localized site, focal ME cell layer disruptions may occur.
(5) As described earlier in this review article, previous studies have documented that a number of chemicals and the host's own leukocytes could specifically and non-specifically impact the physical integrity and functions of ME cells. These observations suggest that microenvironmental factors may induce ME cell death and ME cell layer disruptions. Therefore, ME cell death and ME cell layer disruptions are highly intricate processes that may be caused by multiple factors, and they may represent the earliest signs of tumor invasion and progression.

Although our hypothesis is intriguing, it is not known to what extent it reflects the intrinsic mechanism of ME cell disruptions and the formation of ER-negative tumor cell clusters for two main reasons: first, our data are limited, as observations are extracted from a small sample size, which may not reflect the real status of the general population; and second, the underlying mechanisms and detailed processes for each of our hypothesized steps have not been elucidated. However, given the facts that; first, the disruption of the ME cell layer is an absolute prerequisite for tumor invasion; second, cells overlying focally disrupted ME cells are morphologically, immunohistochemically, and genetically distinct from their adjacent counterparts within the same duct; and third, focal ME cell layer disruptions and leukocyte infiltration appear to be correlated events, our hypothesis might have important scientific and clinical implications.

In the scientific research field, our hypothesis may open a new window for the exploration of the mechanism of ME cell layer disruptions and tumor invasion. Our hypothesis might also be useful in the reconciliation of conflicting reports regarding the immunohistochemical and genetic profiles of breast lesions, as our findings clearly indicate that those conflicts are likely the result of the presence or absence of ME cell layer disruptions, and the differences in the molecular nature and growth pattern of the cells overlying or adjacent to these disruptions. In the clinical field, our hypothesis might be beneficial for the early detection and treatment of breast tumors. As genetic alterations determine the scope and extent of, and frequently precede, biochemical and morphologic changes, microdissection of these ER-negative cell clusters and their adjacent ER-positive counterparts for genetic and biochemical comparisons could potentially lead to the identification of specific molecules associated with the early events of ME cell layer disruption, tumor invasion, and metastasis.

The development of antibodies or chemical reagents to target these ER-negative cells and potential markers for tumor invasion and progression might provide a more effective and less toxic means to block tumor invasion at the earliest stages. Furthermore, microdissection of these ER-negative cell clusters in frozen tissues for tissue culture may lead to the establishment of useful cell lines for the benefit of cancer and stem cell researchers. More importantly, as our studies suggest that leukocytes and other immunosurveillance-related cells might be direct triggering factors for breast tumor progression and invasion, and that invasion could potentially occur from normal or hyperplastic lesions, the development of new strategies and approaches to attack these problems may have direct impact on the diagnosis, treatment, and prognosis of breast cancer patients [88].

Conclusions

The ME cell layer and the basement membrane are positioned between the mammary epithelium and the stroma, normally permitting the exchange of only small

molecules between these two cellular compartments. Due to this structural characteristic, the disruption of both the ME cell layer and the basement membrane is absolutely required for the progression of ductal carcinoma in situ to invasive status. In addition to this passive function as a structural barrier, ME cells have been found to possess several active functions, including the production of tumor suppressors, paracrine down-regulation of MMP expression, and participation in steroid hormone metabolism.

ME cells exclusively or preferentially express a number of proteins, and are subject to a variety of pathological alterations. Focal disruptions in ME cell layers are associated with the emergence of unusual cell clusters overlying these disruptions exhibiting several unique features, including the loss of estrogen receptor expression, a significantly higher proliferation rate, a significantly higher and different pattern of LOH, and a significantly higher frequency and level of mRNA expression for cell cycle, apoptosis, and invasion-related genes when compared to their adjacent counterparts in the same duct. Focal ME layer disruptions are also correlated with significantly higher leukocyte infiltration, and a higher rate of epithelial cell proliferation at or near the disruptions. Based on these and other findings, we have proposed the novel hypothesis that focal ME cell layer disruptions, leukocyte infiltration, and the emergence of ER-negative cell clusters might be correlated events, representing an early sign of ME disruption and the formation of a biologically more aggressive cell clone(s) inaugurating the processes of invasion. This novel hypothesis appears to be useful in the reconciliation of conflicting reports regarding the heterogeneous genetic and immunohistochemical profiles of breast lesions, and it may lead to the development of more effective and specific approaches for breast cancer detection, treatment, and prevention.

Acknowledgments

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Focal prostate basal cell layer disruptions and leukocyte infiltration are correlated events: A potential mechanism for basal cell layer disruptions and tumor invasion☆

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Abstract

To assess the potential correlation between basal cell layer disruptions and leukocyte infiltration, consecutive sections of normal ($n = 5$) and tumor ($n = 50$) prostate tissues were double immunostained for cytokeratin 34βE12 (CK 34βE12) plus leukocyte common antigen, Ki-67, or proliferating cell nuclear antigen (PCNA). Of 2047 acini and ducts examined, 201 contained focal basal cell layer disruptions. Of those, 183 (91%) showed leukocyte infiltration, compared to 67 (33.3%) in 201 morphologically comparable structures with an intact basal cell layer ($P < 0.01$). Basal cell layers adjacent to or surrounded by leukocytes were often attenuated or fragmented, and leukocytes were generally located at or near disruptions. Disrupted basal cell layers showed a markedly reduced proliferation rate, compared to their non-disrupted counterparts. Cells overlying focal basal cell layer disruptions often displayed distinct changes in the size, nuclear shape, density, and polarity, compared to those away from disruptions. A vast majority of proliferating tumor cells were located at or near basal cell layer disruptions. These findings suggest that focal basal cell layer disruptions and leukocyte infiltration are correlated events, representing a potential trigger factor for prostate tumor invasion.

Keywords: Tumor progression and invasion; Focal basal cell layer disruption; Leukocyte infiltration; Epithelial–stromal interactions; Cell growth pattern

1. Introduction

The epithelium of normal and pre-invasive human prostate tissues is physically separated from the stroma by both the basement membrane and basal cells, which rest on the basement membrane and form a continuous layer that surrounds the epithelium [1–5]. This architectural feature confers the basal cell layer and basement membrane two important biological and clinical functions. First, as the epithelium is normally devoid of lymphatic and blood vessels and totally depends on the stroma for its metabolism and survival needed materials, the basement membrane and basal cell layer function as gatekeepers, directly mediating the communication between these two compartments. Second, due to the physical interposition of the basal cell layer and basement membrane, tumor cells have to first pass through the basal cell layer, followed by the basement membrane, to physically reach the stroma. In other words, a physical or functional disruption of both the basal cell layer and basement membrane is a pre-requisite for tumor progression from an in situ to invasive status.

A generally accepted hypothesis for the cause of basement membrane disruptions and subsequent tumor invasion has been attributed primarily, if not solely, to the over-production of proteolytic enzymes by tumor and/or

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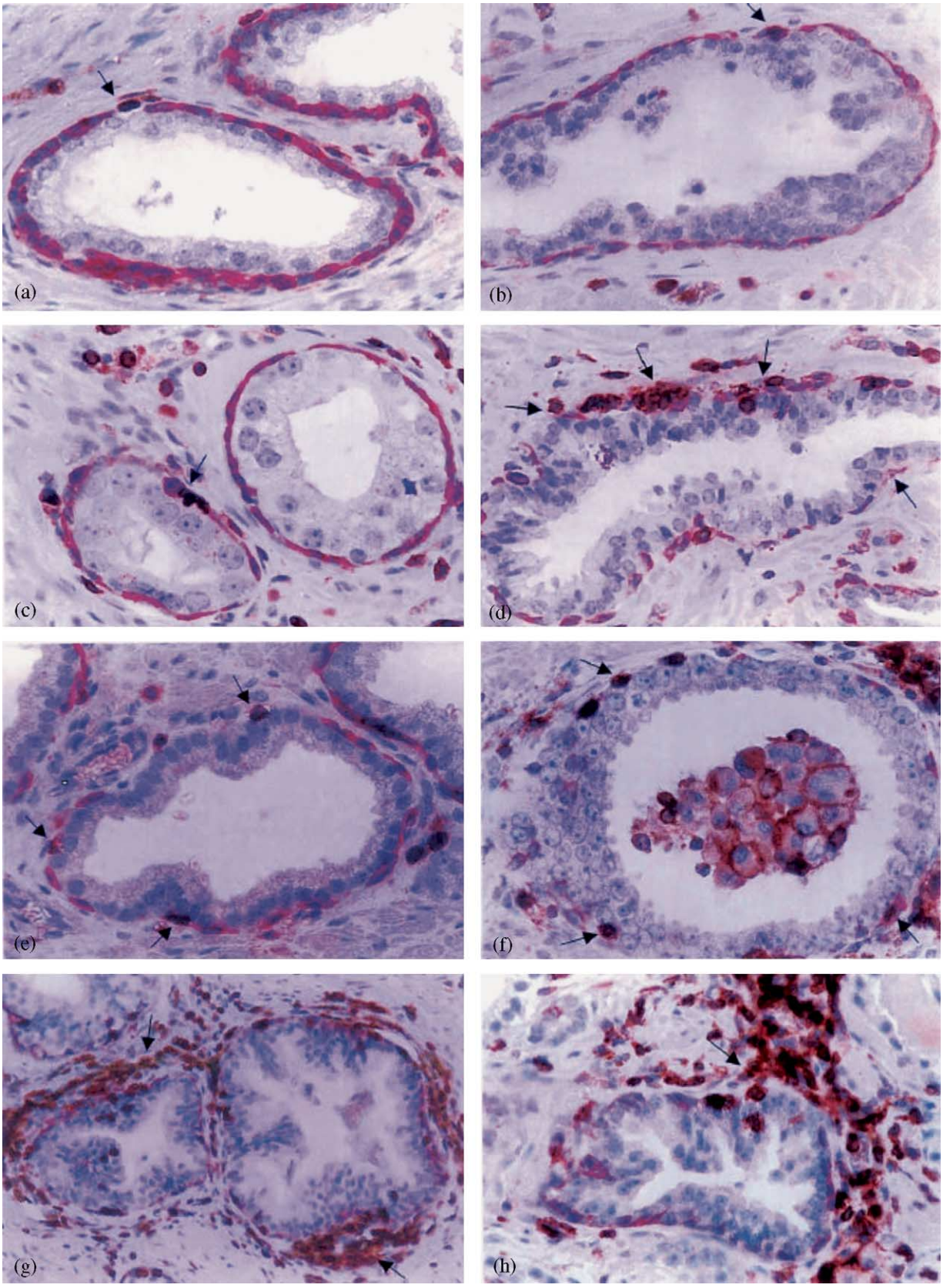

Fig. 1. Focal basal cell layer disruptions and leukocyte infiltration in non-invasive prostate tumors. Sections were double immunostained for CK 34βE12 (red) and LCA (brown). Arrows identify focal disruptions and leukocytes: 300×.

stromal cells [6]. A wide variety of enzymes, including glycanases and proteases, have been found to promote basement membrane degradations and tumor invasion, while the corresponding inhibitors to these enzymes specifically suppress or block these processes in both test tubes and animal models [7–10]. This hypothesis, however, might not reflect the intrinsic mechanism of basal cell degradations and tumor invasion for two reasons: (1) neither the normal cellular kinetics nor the dynamic alterations of basal cells during tumor invasion have been well elucidated; and (2) results from proteinase inhibitor-based human clinical trials have been disappointing [11,12].

While attempting to identify the intrinsic mechanism and early signs of tumor invasion, we have carried out a number of studies on mammary ductal carcinoma in situ (DCIS), focusing on the correlation between the structural integrity of the myoepithelial (ME) cell layer and the biologic presentations of the overlying tumor cells. In focally disrupted ME cell layers, 97.4% of the ducts showed leukocyte infiltration at or near the disruption at a higher cell proliferation rate than ducts without focal ME cell layer disruption. A majority of the proliferating cells were located at or near ME cell layer disruptions [13–15].

Because the prostate basal cell layer is apparently structurally and functionally comparable to the ME cell layer [1–4], this study attempted to assess: (1) whether focal basal cell layer disruptions and leukocyte infiltration are detectable in prostate tumors; (2) whether epithelial cells in ducts and acini with and without focal basal layer disruptions have a different rate and clusters of multiple proliferating cells; and (3) whether a focal basal cell layer disruption and leukocyte infiltration are triggered by localized basal cell death or degenerations.

2. Materials and methods

Consecutive sections at 4–5 μm thickness were made from formalin-fixed, paraffin-embedded human prostate tissues, and placed on positively charged microscope slides. Sections were stained with hematoxylin and eosin (H&E) for morphological classification based on published criteria [17]. Sections from 5 normal and 50 prostate tumors with co-existing normal, hyperplastic, prostatic intraepithelial neoplastic (PIN), and invasive components were selected for immunohistochemical assessment as previously described [18,19]. Briefly, two adjacent sections from each case were incubated at 70 °C for 30 min, deparaffinized with three changes of xylene, and washed in descending concentrations of ethanol and tap water. Deparaffinized sections were incubated at about 70 °C overnight in 1X antigen retrieval solution (BioMedcare, Foster City, CA, USA), washed in warm tap water, and incubated with H_2O_2 blocking solution and normal serum. Then, one section was sequentially immunostained with antibodies to leukocyte common antigen (LCA, clones PD7/26 and 2B11, which reacts with both nucleated cells of the haematopoietic origin and lymphocytes) plus cytokeratin (CK) 34βE12, and the other with antibodies to Ki-67 plus CK 34βE12 (Dako Corporation, Carpinteria, CA, USA).

Each immunostained section was examined with attention to the cross section profiles of ducts and acini with ≥40 epithelial cells and morphologically distinct basal cell layers on H&E and immunostained sections. A focal basal cell layer disruption was defined as the absence of basal cells in at least three adjacent sections, resulting in a gap equal to or greater than the combined size of at least three basal cells, or multiple such gaps in a given duct or acinus. Leukocyte infiltration was defined as the appearance of a single or a group of LCA positive cells within the epithelial compartment or at a direct physical contact with the basal cells.

As the disruptions were generally small and not readily appreciable, the physical status of the basal cell layers and leukocyte infiltration in a given acinus or duct were first photographed with 35 mm slide films by an investigator. Then, developed slide films were reviewed under a slide projector by at least two investigators. The interpretation of the physical status of each structure was based on the uniform conclusion of all reviewers. The disagreement was settled by either excluding the section or repeating the immunostaining on a new slide.

The rate and distribution of proliferating cells in ducts with and without focal basal cell layer disruptions were similarly evaluated as above with focusing on the clusters of multiple proliferating cells (at least four Ki-67 positive tumor cells) located within a given site.

To assess the possibility that a focal basal cell layer disruption and leukocyte infiltration are triggered by a localized basal cell death or degeneration, sections from each case were double immunostained for CK 34βE12 and

Table 1
Frequencies of leukocyte infiltration in acini and ducts with and without focal basal cell layer disruptions

Cell type	No. examined	No. of disruptions	No. of infiltration	*P*-value
Normal	85	3 (3.5%)	2 (66.6%)	
Normal in tumor	500	43 (8.6%)	38 (88.4%)	
Hyperplasia in tumor	800	82 (10.3%)	76 (92.7%)	
PIN in tumor	747	76 (10.2%)	69 (90.7%)	
Normal, hyperplasi and PIN[a]	201	0	67 (33.3%)	<0.01

[a] A total of 201 morphologically comparable normal appearing ($n = 43$), hyperplastic (HP, $n = 82$), and neoplastic (prostatic intraepithelial neoplasia, PIN, $n = 76$) acini and ducts without focal basal cell layer disruptions were used for comparison.

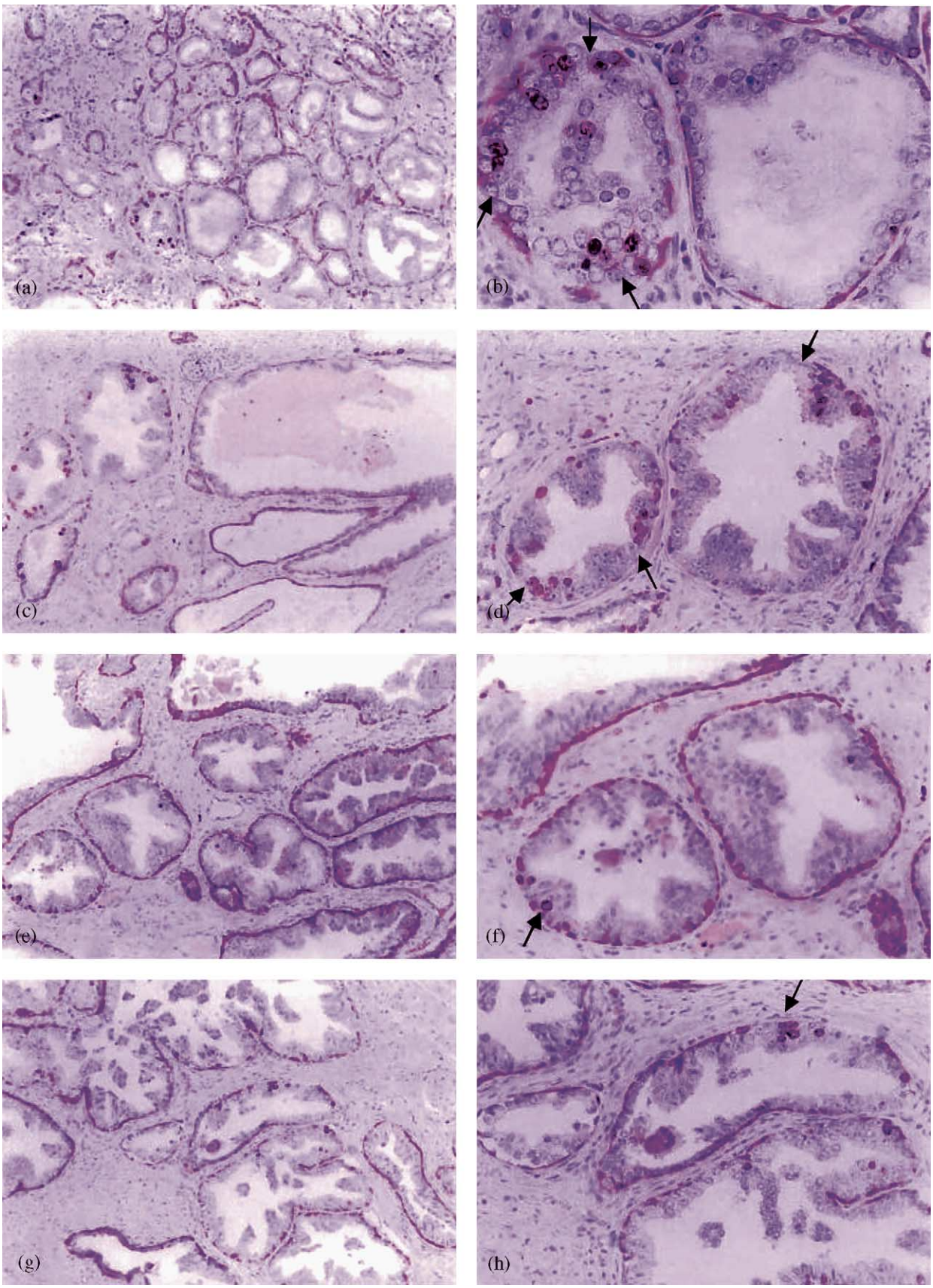

Fig. 2. Cell proliferation status in ducts and acini with and without focal basal cell layer disruptions. Sections were double immunostained for CK 34βE12 (red) and Ki-67 (brown). Arrows identify focal basal cell layer disruptions and associated proliferating cells: (a, c, e and g) 100×; (b, d, f and h) are a higher magnification of (a, c, e and g), 200×, respectively.

proliferating cell nuclear antigen (PCNA), whose expression in prostate appeared to be preferentially present in basal cells, based on our own experience. The proliferation status of basal cells in ducts and acini with and without focal disruptions, and near and away from the disruptions, were semi-quantitatively compared.

3. Results

Distinct focal basal cell layer disruptions and leukocyte infiltration were detected in both normal and tumor tissues. The size of the disruptions varied substantially, from a few cells (Fig. 1a and b) to over 80% of the entire basal cell layer (Fig. 1e and f). The rate and size of basal cell layer disruptions appeared to be independent of the size, length, and architecture of the ducts and acini, and also of the histologic type and grade of the lesions, but appeared to correlate with the extent and location of leukocyte infiltration.

Leukocyte infiltration occurred in four main forms: (1) a single or few leukocytes inter-positioning at or near the center of a single small disruption (Fig. 1a and b); (2) leukocytes lined along a basal cell layer with a large (Fig. 1c–f) or multiple disruptions; (3) sheet-, or belt-like leukocyte aggregates partially surrounding a duct or acinus (Fig. 1g and h); and (4) leukocytes scattered among epithelial cells of a duct or acinus without distinct basal cell layer disruption (not shown). A majority of the infiltrates are cytotoxic lymphocytes and natural killer (NK) cells, based on our most recent studies [20–22].

Of a total of 85 acini and ducts examined in five normal controls, 3 (3.5%) contained focal basal cell layer disruptions in one case. Two of the three focal disruptions were associated with leukocyte infiltration. Of 50 cases with co-existing normal ($n = 500$), hyperplastic ($n = 800$), and PIN ($n = 747$) ducts and acini examined, the frequencies of focal basal cell layer disruptions were 8.6, 10.3, and 10.2%, respectively, and the frequencies of leukocyte infiltration were 88.4, 92.7, and 90.7%, respectively (Table 1). Overall, of a total of 2047 acini and ducts examined in the tumor cases, 201 focal disruptions were identified. Of the 201 disruptions, 183 (91%) showed leukocyte infiltration, compared to 67 (33.3%) in 201 morphologically comparable (the same histological type and grade and similar architecture and size) acini and ducts without basal cell layer disruptions (Table 1).

A vast majority of the proliferating (Ki-67 positive) cells were located at or near the disruptions (Fig. 2). In addition, 22 clusters of multiple proliferating duct and acinar cells were seen directly overlying or near focally disrupted basal cell layers, but only four such clusters were seen in 201 morphologically comparable acini and ducts without basal cell layer disruptions (Table 2).

Basal cell layers adjacent to or surrounded by leukocytes and lymphocytes were often attenuated or fragmented, with substantial reduction of CK 34βE12 immunostaining, compared to those away from leukocytes (Fig. 3). Tumor cells immediately overlying focal basal cell layer disruptions were often morphologically distinct from their adjacent counterparts within the same duct or acinus in cell size, density, nuclear shape, and polarity (Fig. 3a, b, and e). Some of them appeared to invade the stroma (Fig. 3 b and e).

Table 2
Frequencies of clusters of multiple proliferating cells in acini and ducts with and without focal basal cell layer disruptions

Acinar and duct type	No. examined	No. of cluster (%)	*P*-value
With disruptions	201	22 (10.9%)	
Without disruptions	201	4 (2.0%)	<0.01

The frequency of PCNA expression in basal cells among cases and among different foci of the same case varied substantially. Of 50 ducts and acini with focally disrupted basal cell layers examined, 38 (76%) showed markedly reduced PCNA expression in the remaining basal cells, compared to 50 morphologically similar counterparts with intact basal cell layers. A vast majority of the basal cells immediately adjacent to focal disruptions had a markedly reduced PCNA expression, compared to their adjacent counterparts within the same duct, but away from disruptions (Fig. 4).

4. Discussion

The frequency and pattern of focal basal cell disruptions and leukocyte infiltration that were detected in prostate tumors in our current study are very similar to those of our previous studies in human breast tumors [13–16]. It appeared that focal basal or ME cell layer disruptions and leukocyte infiltration seem to be correlated events, which substantially impact the behavior and functions of overlying tumor cells, representing a potential trigger factor for tumor invasion.

A focal basal or ME cell layer disruption is likely caused by localized basal or ME cell death or degenerations and leukocyte infiltration, for several reasons. Leukocyte infiltration into tumor tissues is a common event, and that the number of infiltrated leukocytes appears to be linearly increased with tumor progression [23–25]. Furthermore, leukocytes contain a number of digestive enzymes that could specifically or non-specifically degrade both altered host cells and the basement membrane [26,27].

A localized basal cell death or degenerations and leukocyte infiltration could trigger tumor invasion through the following mechanisms:

1. Basal cells belong to a self-renewal population, and normally undergo proliferation and differentiation to replace aged or injured basal cells [28,29].
2. An external or internal insult, such as a localized trauma, chronic inflammation, or the exposure to lambda-

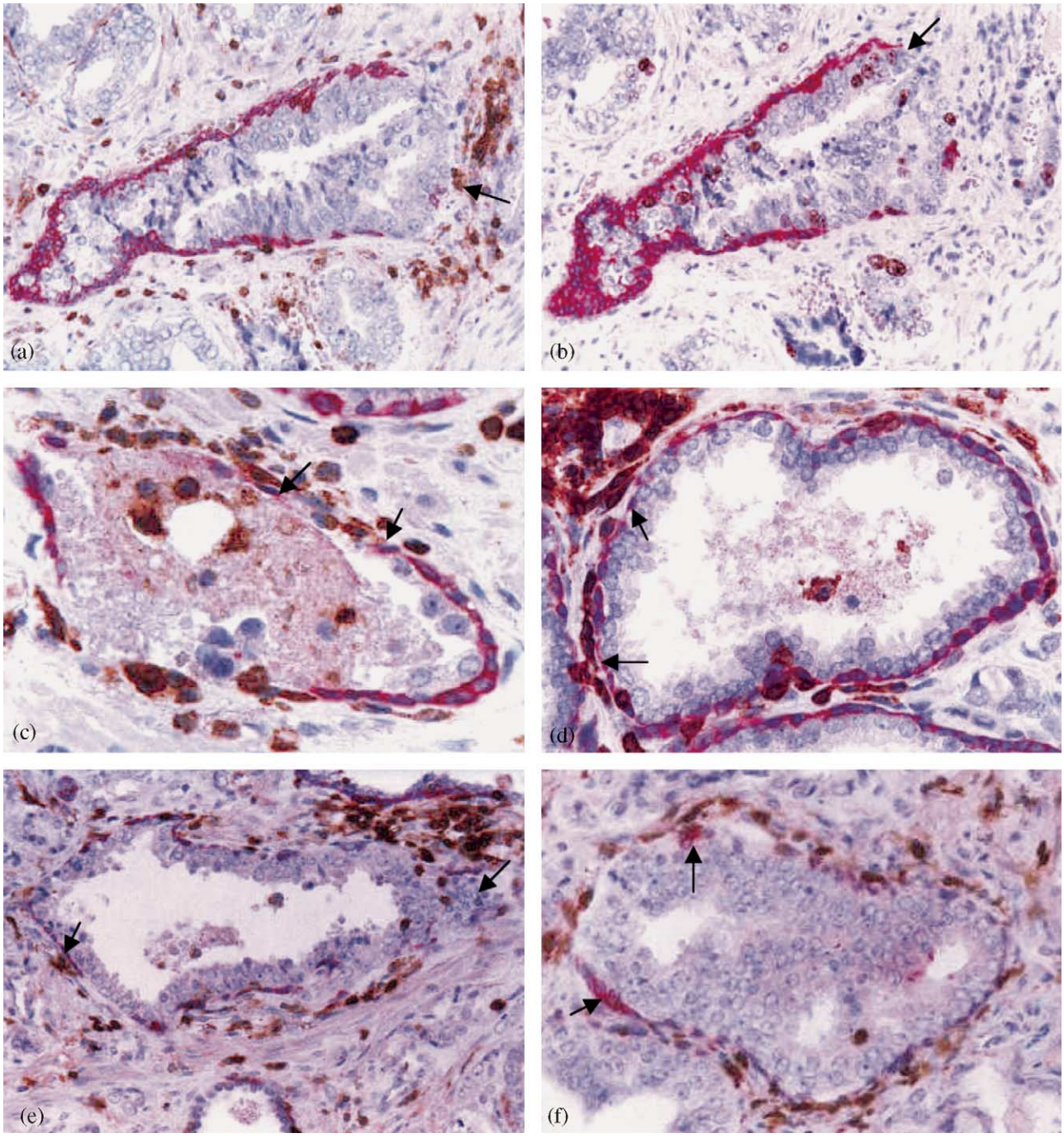

Fig. 3. Morphological and immunohistochemical alterations in cells adjacent to leukocytes. Sections were double immunostained for CK 34βE12 (red) and LCA or Ki-67 (brown). Arrows identify leukocytes or morphologically and immunohistochemically altered basal or tumor cells: (a) CK 34βE12 plus LCA; (b) a sequential section of (a), 34βE12 plus Ki-67; (c–f) 34βE12 plus LCA 300×.

carrageenan [30], directly disrupts the structural integrity of the basal cell layer or impairs the normal replacement process, resulting in a single or in a cluster of degenerated basal cells.

3. The degraded products of degenerated basal cells attract leukocytes, which migrate to the injured site and interact with altered basal cells.
4. Leukocytes release their digestive enzymes, leading to the physical destruction of altered basal cells and the adjacent basement membrane, as well as the formation of a foal disruption or gap that allows the direct contact between tumor and stromal cells.
5. The focal disruption in the basal cell layer and basement membrane results in an increased permeability of metabolism- and growth-related molecules to overlying and adjacent cells.
6. The altered micro-environment leads to variable consequences in overlying tumor and adjacent basal cells,

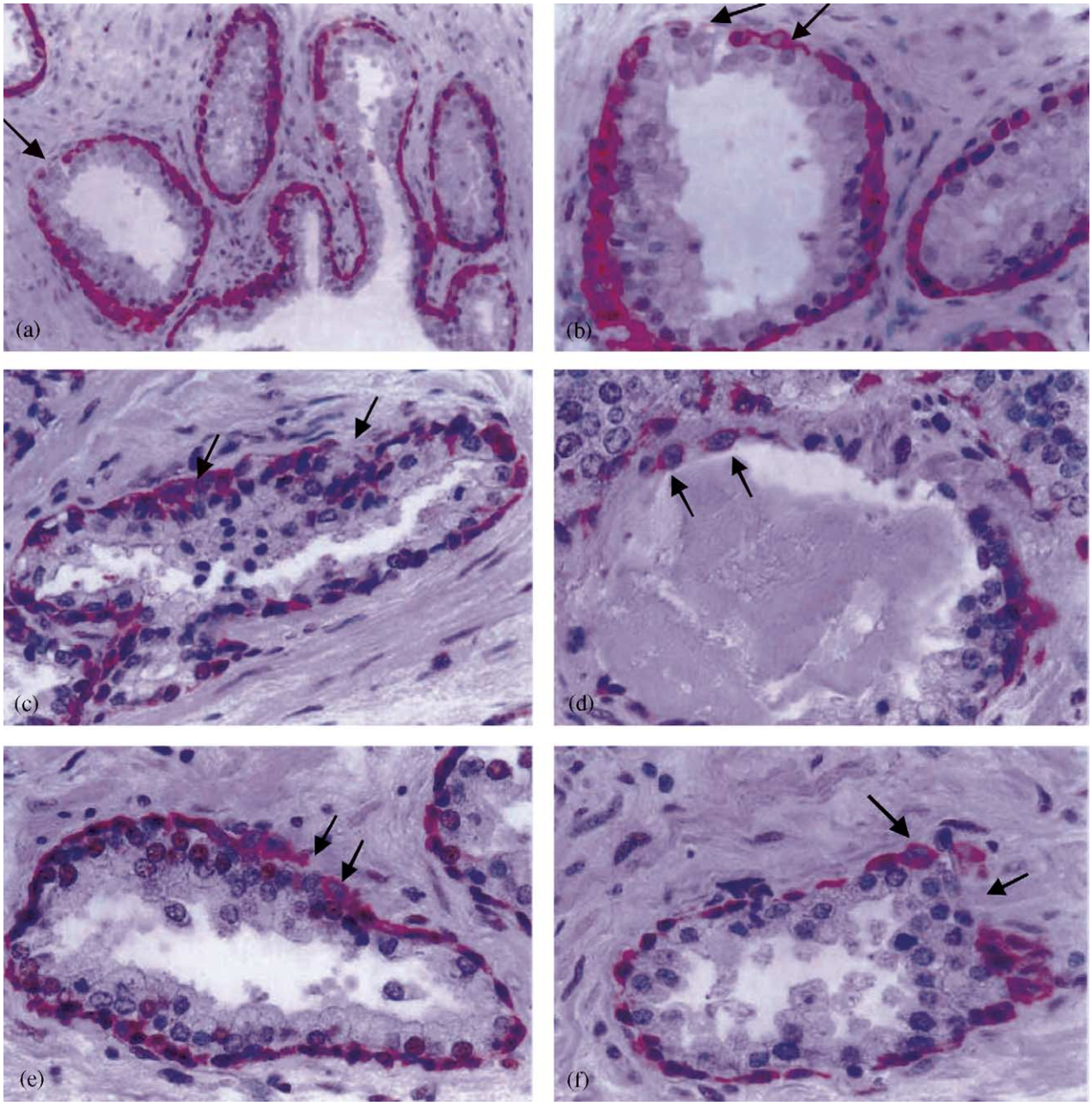

Fig. 4. The expression of PCNA in basal cells near and away from focal disruptions. Sections were double immunostained for CK 34βE12 (red) and PCNA (black). Arrows identify cells without PCNA expression. Note that most cells near the disruption are PCNA negative, while most cells away the disruption are PCNA positive: (a) 150×; (b) is a higher magnification of (a), 300×; (c–f), 300×.

depending on the nature of these cells. If the overlying tumor cells are fully differentiated, no substantial alteration might be detectable. In contrast, if they are uncommitted stem or progenitor cells, an extensive cell proliferation can be expected, which leads to the formation of a biologically more aggressive cell cluster that invades the stroma.

7. The cell cluster undergoes cytodifferentiation and release stage-restricted and invasion-associated molecules, triggering angiogenesis, tissue remodeling, and increasing production of growth factors in the stroma, providing a favorable environment for tumor cell growth [31–34].
8. The interactions between tumor and stromal cells lead to further degradation of the basal cell layer and basement membrane, and a further expansion and stromal invasion of the newly formed cell cluster [31–34].

Knowledge is lacking as to whether or to what extent our hypothesis reflects the intrinsic mechanism of basal cell disruptions and tumor invasion, for the following reasons: (1) our data are extracted from a small sample size, which

may not reflect the real status in a larger number of cases; (2) the underlying mechanism for each of hypothesized steps have not been elucidated; and (3) the specific subtype of leukocytes associated with focal basal cell layer disruptions has not been determined. However, given the facts that the disruption of the basal cell layers is a prerequisite for tumor invasion and that results from proteinase inhibitor-based human clinical trials disfavor the traditional proteolytic enzyme theory [11,12], our hypothesis might open a new window to explore the intrinsic mechanism of tumor invasion. Currently, several studies on a larger number of cases are in progress to explore the issues that were not addressed in this study. Our recent studies have revealed that basal cells near a focal disruption have a significantly lower rate of p63 expression than their counterparts away from the disruption, and that cytotoxic lymphocytes and NK cells are preferentially associated with focal basal cell layer disruptions [20–22].

Acknowledgments

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Inhibition of enzyme activity of and cell-mediated substrate cleavage by membrane type 1 matrix metalloproteinase by newly developed mercaptosulphide inhibitors

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MT1-MMP (membrane type 1 matrix metalloproteinase, or MMP-14) is a key enzyme in molecular carcinogenesis, tumour-cell growth, invasion and angiogenesis. Novel and potent MMP inhibitors with a mercaptosulphide zinc-binding functionality have been designed and synthesized, and tested against human MT1-MMP and other MMPs. Binding to the MT1-MMP active site was verified by the competitive-inhibition mechanism and stereochemical requirements. MT1-MMP preferred deep P1′ substituents, such as homophenylalanine instead of phenylalanine. Novel inhibitors with a non-prime phthalimido substituent had K_i values in the low-nanomolar range; the most potent of these inhibitors was tested and found to be stable against air-oxidation in calf serum for at least 2 days. To illustrate the molecular interactions of the inhibitor–enzyme complex, theoretical docking of the inhibitors into the active site of MT1-MMP and molecular minimization of the complex were performed. In addition to maintaining the substrate-specificity pocket (S1′ site) van der Waals interactions, the P1′ position side chain may be critical for the peptide-backbone hydrogen-bonding network. To test the inhibition of cell-mediated substrate cleavage, two human cancer-cell culture models were used. Two of the most potent inhibitors tested reached the target enzyme and effectively inhibited activation of proMMP-2 by endogenous MT1-MMP produced by HT1080 human fibrosarcoma cells, and blocked fibronectin degradation by prostate cancer LNCaP cells stably transfected with *MT1-MMP*. These results provide a model for mercaptosulphide inhibitor binding to MT1-MMP that may aid in the design of more potent and selective inhibitors for MT1-MMP.

Key words: cell-mediated substrate cleavage, enzyme inhibition kinetics, inhibitor–enzyme active-site interaction, matrix metalloproteinase (MMP), molecular modelling, novel synthetic inhibitor.

INTRODUCTION

MMPs (matrix metalloproteinases) are a family of zinc endopeptidases that play prominent roles during normal and pathological ECM (extracellular matrix) remodelling events, including cancer progression [1]. MT-MMPs (membrane type-MMPs) are a unique subset tethered to the cell membrane by a transmembrane domain or GPI (glycosylphosphatidylinositol) anchor [2]. The first identified MT-MMP, MT1-MMP, has been shown to play a central role in tumour-cell invasion and migration [3–6]. It hydrolyses constituents of the ECM directly [7–9] and activates proMMP-2 (progelatinase A) efficiently; activated MMP-2 cleaves the basement membrane type IV collagen [10–13]. Because MT1-MMP is highly localized at the leading edge of invading cancer cells and is a powerful proteolytic enzyme, it may be a good target for inhibition [14].

Synthetic MMPIs (MMP inhibitors) have been in development for more than a decade. Potent MMPIs have three general requirements: a functional group capable of co-ordinating to the enzyme active site Zn(II), at least one functional group participating in hydrogen bonding with the enzyme, and one or more side chains participating in favourable van der Waals interactions, particularly at the substrate specificity pocket (S1′ site) [15]. The most potent inhibitors reported have used a hydroxamic acid zinc-chelating group. Although this functionality affords good MMPI potency, many reports suggest the need for other functional groups owing to problems with oral bioavailability and toxicity [16–18]. Other MMPI functional groups include thiol, carboxylate, phosphinate and sulphodi-imine. Mercaptosulphide inhibitors that have been developed and characterized for several MMPs exhibit IC_{50} and K_i values comparable with those of well-known hydroxamic acids [19–23]. Mercaptosulphide peptidomimetic inhibitors are distinct from the hydroxamate peptidomimetics; they have different zinc-co-ordinating functionality, and the favoured stereochemistry at the P1′ site is an unnatural D-amino-acid. In the present study, the inhibition characteristics of MT1-MMP with novel mercaptosulphide inhibitors were determined, and a model of the mercaptosulphide inhibitor complexed to MT1-MMP is proposed to provide a rational approach towards inhibitor design. Furthermore, the efficacy of potent new MMPIs was tested in live cells, using two different models of substrate cleavage mediated by human cancer cells expressing endogenous and transfected functional MT1-MMP respectively.

EXPERIMENTAL

Materials

The fluorescence-quenched peptide substrate, Mca [(7-methoxycoumarin-4-yl)acetyl]-Pro-Leu-Gly-Leu-Dpa [*N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl]-Ala-Arg-NH_2, was purchased

Abbreviations used: cdMT1-MMP, catalytic domain of membrane type 1 matrix metalloproteinase; ConA, concanavalin A; Dpa, *N*-3-(2,4-dinitrophenyl)-L-2,3-diaminopropionyl; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); ECM, extracellular matrix; FN, fibronectin; GFP, green fluorescent protein; GPI, glycosylphosphatidylinositol; Mca, (7-methoxycoumarin-4-yl)acetyl-; MMP, matrix metalloproteinase; MMPI, MMP inhibitor; MT1-MMP, membrane type 1 MMP; MT1–GFP, MT1-MMP complexed to GFP; proMMP-2, progelatinase A; TCEP, tris-(2-carboxyethyl)phosphine; TIMP-2, tissue inhibitor of metalloproteinases-2.

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from Bachem. The mercaptosulphide inhibitors were synthesized and characterized as described previously [19,21,23,24]. The recombinant cdMT1-MMP (catalytic domain of MT1-MMP) was provided by Professor Harald Tschesche (Bielefeld University, Eastern Westphalia, Germany) [22,25]. MMP-1, -2, -3, -7 and -9 were described previously [21]. FITC was purchased from Pierce Biotechnology, and human FN (fibronectin) was from Invitrogen. All standard chemicals were purchased from Fisher Scientific with the exception of Ellman's reagent/DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)] and ConA (concanavalin A), which were from Sigma, and TCEP [tris-(2-carboxyethyl)-phosphine], which was from Calbiochem. Human fibrosarcoma cell line HT1080 and human prostate cancer cell line LNCaP were purchased from ATCC (Manassas, VA, U.S.A.) and were maintained in Dulbecco's modified Eagle's medium (Invitrogen).

Inhibitor-concentration determination

The reduced mercaptosulphide inhibitor concentration was determined with DTNB [20,26]. Absorbance was measured with a Shimadzu UV-260 UV–visible spectrophotometer with a 2 nm slit-width. Alternatively, for inhibitors with a phthalimido substituent, the total concentration of inhibitor was determined by absorbance at 219 nm. The molar absorption coefficient, ε_{219}, was determined for a standard, *N*-phthaloyl-L-phenylalanine, by measuring absorbance for concentrations up to 40 μM of four separate stock solutions. The ε_{219} was determined to be $(3.30 \pm 0.08) \times 10^4$ $M^{-1} \cdot cm^{-1}$.

Enzyme kinetics

Kinetic assays were performed with the peptide substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 (1 μM) as described previously [27,28]. The assays were performed in 50 mM Hepes buffer, pH 7.5, with 10 mM $CaCl_2$, 0.2 M NaCl and 0.05 % (w/v) Brij-35 (polyoxyethylene dodecyl ether) at 25 °C. Active cdMT1-MMP concentrations were determined by titration with standardized preparations of recombinant TIMP-2 (tissue inhibitor of metalloproteinases-2) [22]. For the inhibition assays, the inhibitors were incubated with enzyme for 15–30 min before adding substrate, to ensure equilibrium conditions. For those inhibitors with a phthalimido substituent, 1 mM TCEP (a reducing agent) was included to ensure complete reduction of the inhibitor to the free thiol. No effect on enzyme activity was noted for concentrations of TCEP up to 1 mM. The inhibitor dissociation constant, K_i, was determined as described previously [29] using the Morrison equation [30]:

$$\frac{v_i}{v_o} = \frac{[E]_o - [I]_o - K_i\,(app) + \sqrt{\{[I]_o + K_i\,(app) - [E]_o\}^2 + 4[E]_o K_i\,(app)}}{2[E]_o} \quad (1)$$

where v_i and v_o are the initial rates with and without inhibitor respectively, $[E]_o$ and $[I]_o$ are the initial enzyme and inhibitor concentrations respectively, and K_i (app) is the apparent K_i, which is equal to the true K_i under the conditions of substrate concentration ($[S] \ll K_m$ (the Michaelis constant) for competitive inhibition according to eqn (2):

$$K_i\,(app) = K_i\left(1 + \frac{[S]}{K_m}\right) \quad (2)$$

Inhibitor stability

The stability of the thiol was determined using the inhibition assay to quantify the concentration of active inhibitor. Selected inhibitors were incubated in calf serum at 37 °C. Aliquots were removed periodically, diluted with the assay buffer (described above) to 25 % serum, and the inhibition was tested against cdMT1-MMP. Control assays containing the same amounts of serum with no inhibitor were performed to obtain relative rates.

Modelling studies

The co-ordinates from the X-ray crystal structure of the cdMT1-MMP–TIMP-2 complex were downloaded from the Brookhaven Protein Data Bank (accession number 1BUV) [31]. MacroModel version 7.0 was used to remove the TIMP-2 molecule, and the inhibitors were theoretically docked into the active site of cdMT1-MMP by the following procedure: the MMP-1–hydroxamic acid inhibitor complex (PDB accession number 1HFC) [32] was modified at the inhibitor zinc-co-ordinating group to a mercaptosulphide functionality; the modified structure was energy-minimized according to procedures described below; the imidazolyl nitrogen atoms co-ordinated to zinc of the minimized structure were superimposed with those of cdMT1-MMP; and MMP-1 was deleted to leave the inhibitor bound in the cdMT1-MMP active site. Both sulphurs of the mercaptosulphide functionality were co-ordinated to zinc with zero-order bonds. The rationale for this zinc chelation was based primarily on earlier studies of the inhibition of MMP-1, -2, -3, -7, -8 and -9 with a mercaptosulphide inhibitor modified with an oxygen in place of the sulphide, which significantly reduced inhibitor potency. The mercaptosulphide inhibitor had IC_{50} values of 52, 1.4, 250, 33, 3.6 and 1.2 nM respectively, and the analogous inhibitor with oxygen had IC_{50} values of 1700, 330, 29000, 4800, 910 and 260 nM respectively (M. A. Schwartz, Y. Jin, D. R. Hurst and Q.-X. Sang, unpublished work).

Energy minimizations of the inhibitor and all enzyme residues within 7 Å (1 Å = 0.1 nm) were performed with MacroModel version 7.0. Briefly, the AMBER force field [33,34], modified to include parameters for Zn and Ca ([35–37]; M. A. Schwartz, Y. Jin, D. R. Hurst and Q.-X. Sang, unpublished work), was used with the PRCG (Polak–Ribiere conjugate gradient) method with a 0.05 gradient threshold in a GB/SA (generalized Born solvent/accessible surface area) continuum water solvent. A global minimization of the cdMT1-MMP–inhibitor complex was carried out using the MCMM (Monte Carlo multiple minimum) method (1000 steps), in which the inhibitor torsion angles at all relevant bonds were randomly varied and then minimized.

Gelatin substrate zymography and densitometry analyses of the bands

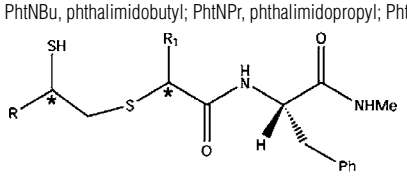
The procedures were reported in our previous papers [38,39]. In brief, HT1080 cells were pre-treated with different doses of the novel MMPIs in serum-free medium at 37 °C for 1 h, followed by stimulation with ConA (20 μg/ml) at 37 °C for 18 h. The spent conditioned media were collected and examined by gelatin zymography. The effect of the novel MMPI on proMMP-2 activation by MT1-MMP was documented by densitometry analysis for 62 kDa fully active MMP-2 using AlphaImager™ 2200 with AlphaEaseFC software (AlphaInnotech).

FITC–FN degradation assay

To observe cell-mediated protein-substrate degradation and cell migration, FN was conjugated to FITC in borate-buffered saline (pH 10) for 30 min at room temperature (approx. 23 °C). After extensive dialysis against PBS, FITC-labelled FN was coated on to glass coverslips by incubation with glutaraldehyde-cross-linked gelatin for 1 h at room temperature [39]. Human prostate cancer

Table 1 Inhibition constants for inhibition of MT1-MMP and other MMPs by mercaptosulphide inhibitors

Stereochemistry (*) for the C_α to SH is always listed first. Data were reported in [a] [22] and [b] [21]. iBu, isobutyl; PhMe, phenylmethyl; PhEt, phenethyl; PhtN, phthalimido; PhtNMe, phthalimidomethyl; PhtNBu, phthalimidobutyl; PhtNPr, phthalimidopropyl; PhtNPe, phthalimidopentyl; PhtNEt, phthalimidoethyl; PhtNEtNH, phthalimidoethylimino; PMP, paramethoxyphenyl.



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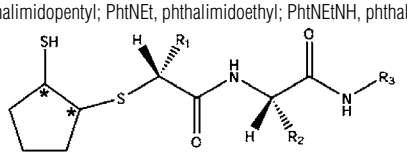

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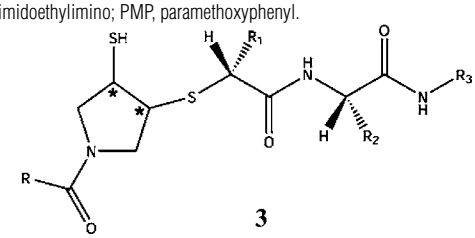

3

Compound	Identity	*	R	R_1	R_2	R_3	K_i (nM) MT1-MMP	MMP-1	MMP-2	MMP-3	MMP-7	MMP-9
1a	MAG-42	*R*	H	iBu			27	52	0.28	250	56	0.43
1b	MAG-58	*S*	H	iBu			160	400	34	3600	50	17
1c	MAG-148	*R*	H	PhMe			10000	12000	1400	83000	7300	1500
1d	MAG-133	*S*,*R*	Me	iBu			18[a]	13	0.35	46	15	0.21
1e	MAG-128	*R*,*R*	Me	iBu			70[a]	200	110	400	45	18
1f	MAG-292	*S*,*R*	PhtNEt	iBu			4.5	0.95	0.77	22	15	0.09
1g	MAG-254	*S*,*R*	PhtNBu	iBu			5.5	20	0.2	10	13	0.11
2a	MAG-182	*S*,*R*		iBu	PhMe	Me	24[a,b]	49[b]	1.1[b]	470[b]	40[b]	0.57[b]
2b	MAG-181	*R*,*S*		iBu	PhMe	Me	260[a,b]	680[b]	85[b]	2500[b]	710[b]	44[b]
2c	YHJ-73	*S*,*R*		PhEt	iBu	PMP	16[b]	> 12000[b]	20[b]	100[b]	1000[b]	8.6[b]
2d	YHJ-72	*R*,*S*		PhEt	iBu	PMP	380[b]	> 12000[b]	930[b]	150[b]	5500[b]	180[b]
3a	YHJ-294-2	*S*,*R*	NH_2	iBu	PhMe	Me	13[a,b]	100[b]	6.1[b]	360[b]	26[b]	1.2[b]
3b	YHJ-294-1	*R*,*S*	NH_2	iBu	PhMe	Me	3000[a,b]	5200[b]	430[b]	> 40000[b]	3500[b]	550[b]
3c	YHJ-265	1:1	Me	iBu	PhMe	Me	6.2	99	14	990	91	5.7
3d	YHJ-96	1:1	PhtNMe	iBu	PhMe	Me	70	110	17	300	50	4.9
3e	YHJ-97	1:1	PhtNPr	iBu	PhMe	Me	6	75	8.5	31	12	3.9
3f	YHJ-44	1:1	PhtNPe	iBu	PhMe	Me	7	52	1.7	1.9	11	0.98
3g	YHJ-223	1:1	PhtNEtNH	PhEt	PhMe	Me	3.7	190	1.8	13	250	0.35
3h	YHJ-224	1:1	PhtNEtNH	PhEt	iBu	PMP	3.1	> 3000	31	1.5	76	4.6
3i	YHJ-132	*S*,*R*	PhtNEtNH	iBu	PhMe	Me	1.2	8.8	0.7	6	6.5	1.1
3j	YHJ-133	*R*,*S*	PhtNEtNH	iBu	PhMe	Me	430	1900	90	1100	670	122

cell line LNCaP cells stably transfected with MT1–GFP (MT1-MMP–green fluorescent protein) cDNA construct were generated as described previously [39]. LNCaP cells expressing the MT1–GFP chimaera were plated onto FITC–FN coverslips and incubated with vehicle control (DMSO), TIMP-2, YHJ-132 or YHJ-294-2 at different concentrations in serum-free medium at 37 °C for 18 h. The cells were then fixed with 4 % (w/v) paraformaldehyde in PBS and examined under a fluorescent microscope (Olympus, IX-70). The substrate degradation was demonstrated by loss of fluorescence of FITC-labelled substrate and cell migration was determined by observing the tracks of digested substrate on the same slide. MT1–GFP-expressing cells digested FN and migrated over digested substrate. The dark lines on the green FITC–FN background indicate substrate degradation and cell migration. The cells are also green because of the GFP expression together with MT1-MMP.

RESULTS

Mechanism of interaction

The inhibitors (Table 1) were designed with a zinc-co-ordinating functionality and would be predicted to bind in the active site groove of MMPs based on structures of similar hydroxamate inhibitors complexed with MMP-1. However, detailed inhibition mechanisms have not been determined experimentally, and alternative modes of inhibition must be considered. To verify a competitive mechanism of inhibition, a prototypical inhibitor, MAG-182, was assayed with cdMT1-MMP at several substrate concentrations. A linear form of the Morrison equation derived by Henderson [40] (eqn 3) was applied:

$$\frac{[I]_o}{\left(1-\frac{v_i}{v_o}\right)} = [E]_o + K_i\,(\text{app})\left(\frac{v_o}{v_i}\right) \quad (3)$$

where $[I]_o$ and $[E]_o$ are the total inhibitor and enzyme concentrations, and v_i and v_o are the rates with and without inhibitor respectively. The Morrison plot and Henderson plot are shown in Figure 1. The linear increase in K_i (app) with increasing substrate concentration is indicative of competitive inhibition, as shown by eqn (2). Additionally, a well-known ACE (angiotensin-converting enzyme) inhibitor, captopril {*N*-[(*S*)-3-mercapto-2-methylpropionyl]-L-proline}, which is also a thiol compound, was used as a negative control and found to have a K_i value of 4 mM. Therefore the mercaptosulphide inhibitors are interacting exclusively at the enzyme active site.

The competitive mechanism of inhibition does not distinguish between primed and unprimed binding. Members of the MMP family are known to have either a deep or a shallow S1′ pocket that is easily probed by modifying the P1′ substituent. Shallow-pocket MMPs, MMP-1 and -7, have K_i values that are an order of magnitude higher compared with deep-pocket MMPs, such as MT1-MMP, for inhibitors with an extended-chain P1′ substituent. The inhibition by MAG-182 and YHJ-73 is compared with MMP-1 and -7 and MT1-MMP in Table 1. Respective K_i values

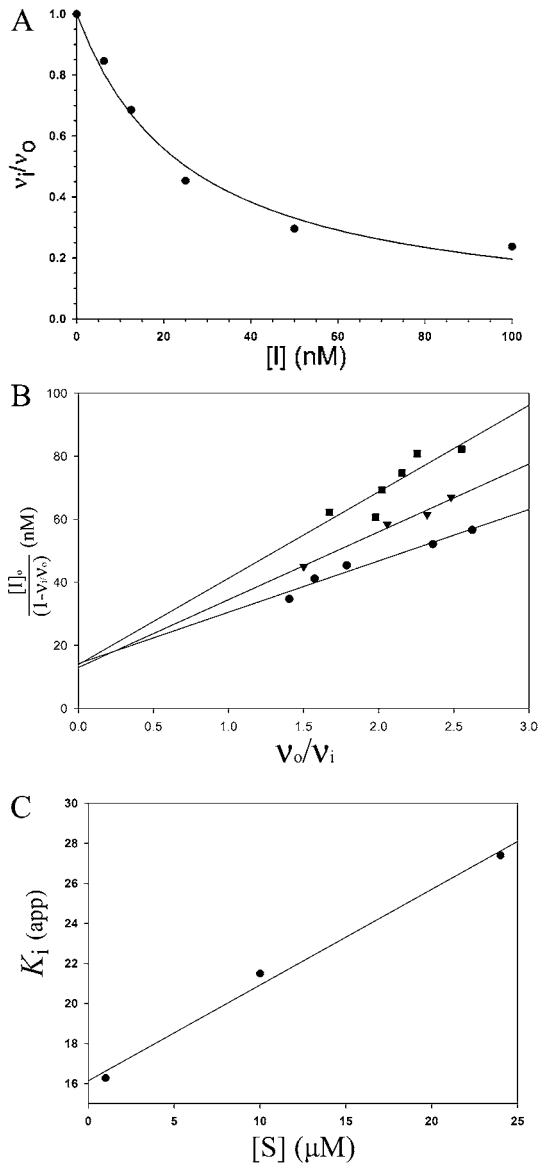

Figure 1 Competitive inhibition mechanism

(**A**) A typical inhibition curve fitted with the Morrison equation (eqn 1) is shown for MAG-182. (**B**) The inhibition assay was also performed with substrate concentrations of 1, 10 and 24 μM and plotted according to the Henderson equation (eqn 3), described in the text. (**C**) A linear increase in the slope, K_i (app), is indicative of competitive inhibition, according to eqn (2).

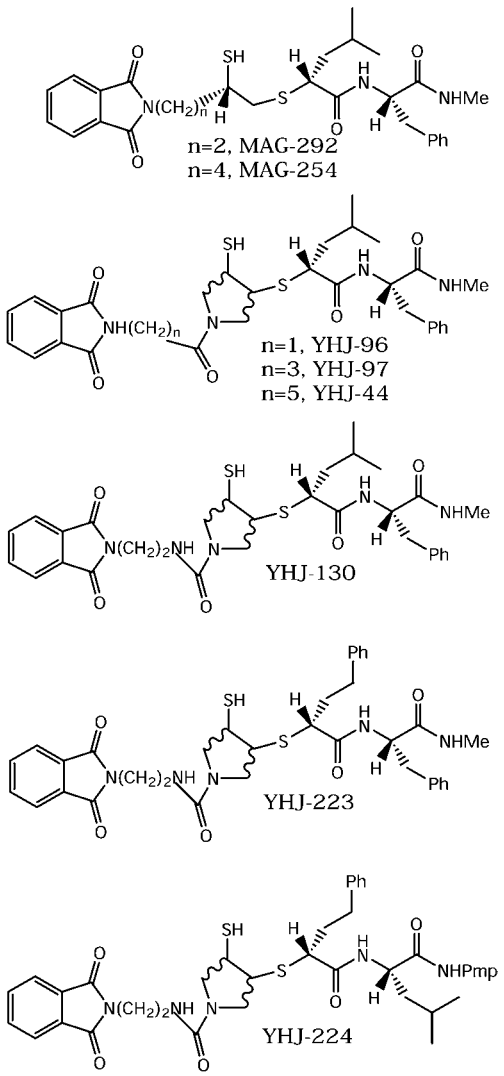

Figure 2 Novel mercaptosulphide inhibitors containing a phthalimido substituent

YHJ-130 is the 1:1 mixture of YHJ-132 and YHJ-133, as shown in Table 1.

for MMP-1 are 49 nM and 12 μM; for MMP-7, 40 nM and 1 μM; and for MT1-MMP, 24 nM and 16 nM [21]. The significant decrease in inhibition potency with a homophenylalanine side chain, compared with leucine, against MMP-1 and -7 demonstrates that the P1′ substituent is interacting at the S1′ pocket.

To ensure that the binding interactions are specific, the stereochemical dependence of inhibition was probed. Variation in the stereochemistry of the inhibitor near the zinc-co-ordinating functionality and at the P1′ side chain led to variation in inhibition. The C_α to SH favours the absolute *S* configuration (compare MAG-133 with MAG-128), and the derivatives containing a cyclopentyl or pyrrolidinyl ring significantly favour the *S*,*R* configuration (C_α,C_β to SH respectively; compare MAG-182 with MAG-181, YHJ-73 with YHJ-72, and YHJ-294-2 with YHJ-294-1). The stereochemical requirements at the P1′ site favour the absolute *R* configuration corresponding to the unnatural D-amino-acid derivative (compare MAG-42 with MAG-58). These results confirm that stereochemistry is important for inhibitor potency and provide additional support for the inhibitor interacting specifically and exclusively at the active site of the enzyme.

Probing the non-prime site

From the crystal structure of cdMT1-MMP, the non-prime site does not have well-defined pockets comparable with S1′. However, it was found that a phthalimido substituent (Figure 2) added to the C_α to SH of MAG-42 (MAG-292) increased the potency by a factor of five (Table 1). Compared with a methyl group (MAG-133), the potency is increased by a factor of four. To probe the possibility of a specific interaction with cdMT1-MMP, the tether length attaching the phthalimido substituent was increased by two carbons (MAG-254). No change was noted in the K_i value, suggesting that the increase in potency is because of non-specific hydrophobic interactions.

A new series of mercaptosulphide inhibitors that incorporate a pyrrolidine ring at the zinc-co-ordinating functionality was designed to increase water-solubility and possibly provide

structural features that would allow for greater selectivity of inhibition (Table 1, compound 3) [21,23]. These derivatives displayed identical stereochemical requirements for potency with the previously designed mercaptosulphides (compounds 1 and 2). A phthalimido substituent was also added, with varying tether lengths, to the N-acetyl of these compounds, with no change in potency (YHJ-265, -97 and -44). However, a 10-fold increase in K_i was noted for the *N*-phthalimidomethylacetyl (YHJ-96). This suggests that YHJ-96 is sterically hindered by the short attachment of the phthalimido substituent, or possibly that π–π interactions between the phthalimido and an aromatic residue of the enzyme affect inhibitor binding. Incorporating a urea group (YHJ-132, -133, -223 and -224) did not affect the K_i, although the water-solubility would be significantly increased.

The mercaptosulphide inhibitors may be readily air-oxidized in buffer and typically lose their effectiveness as MMPIs over time. Stability experiments with the non-cyclic mercaptosulphide, MAG-42, in buffer showed complete loss of inhibitor activity within 45 min at 25 °C. Incorporation of the cyclopentyl ring at the mercaptosulphide functionality, MAG-182, increased inhibitor stability by approx. 6 h. Pyrrolidinyl derivatives of these inhibitors have improved water-solubility and may be candidates for *in vitro* cell and *in vivo* animal studies (M. A. Schwartz, Y. Jin, D. R. Hurst and Q.-X. Sang, unpublished work). Moreover, the phthalimidoethylurea derivative, YHJ-132, which is the most potent of these inhibitors towards cdMT1-MMP, was stable for at least 2 days in calf serum at 37 °C (M. A. Schwartz, Y. Jin, D. R. Hurst and Q.-X. Sang, unpublished work). The simple urea derivative, YHJ-294-2, was stable for approx. 4–6 h. MAG-42 and MAG-182 were tested in calf serum and were found to have stability similar to that found in the buffer experiments.

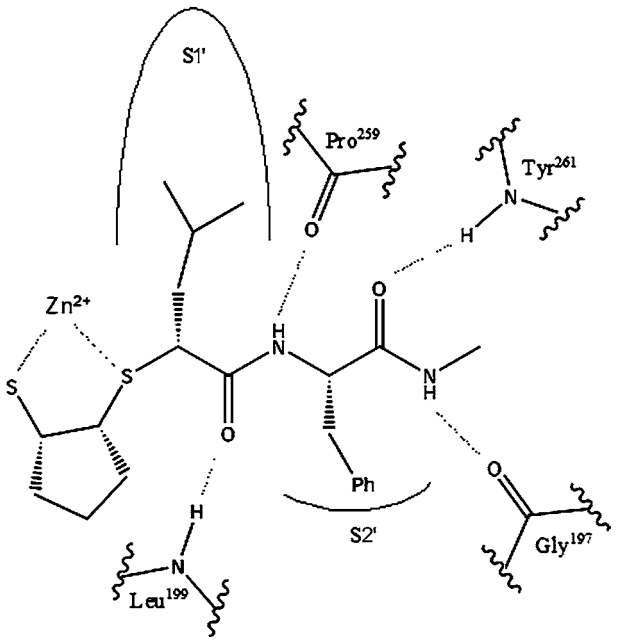

Figure 3 A model for inhibitor binding

MAG-182 is shown with key enzyme residues that are within hydrogen-bonding distance. The side chains of the inhibitor maintain important van der Waals interactions with the S1′ and S2′ pockets.

Proposed model

These mercaptosulphide inhibitors appear to be structurally similar to some well-characterized hydroxamate peptidomimetic MMPIs and might be predicted to interact correspondingly; however, the mercaptosulphides are distinct from the hydroxamate peptidomimetics with regard to the zinc-co-ordinating functionality and the favoured unnatural D-amino-acid stereochemistry at the P1′ site. Molecular modelling was employed for structural analysis of the binding of the mercaptosulphide inhibitors into the cdMT1-MMP active site. The enzyme–inhibitor complexes were globally minimized so that interactions important for inhibitor potency could be identified. Rational explanations for the disparity between K_i values of structurally similar inhibitors were sought and identified.

The three general requirements for MMPI potency are achieved with mercaptosulphide inhibitors: a zinc-binding functionality, multiple hydrogen bonds and favourable van der Waals interactions with the S1′ pocket. The cdMT1-MMP complexed with the inhibitor MAG-182 is illustrated in Figure 3. The inhibitor interacts with the primed side of the enzyme active site, with the P1′ leucine side chain oriented with one terminal methyl in line with the S1′ pocket. Four hydrogen bonds between the peptide backbone of the mercaptosulphide inhibitors and the enzyme active site were identified. The P1′ carbonyl hydrogen bonds with the NH of Leu^{199}, the P2′ NH with the carbonyl of Pro^{259}, the P2′ carbonyl with the NH of Tyr^{261} and the terminal NH with the carbonyl of Gly^{197}. Importantly, the Phe^{198} between the hydrogen-bonding residues Gly^{197} and Leu^{199} is co-ordinated to a structurally important calcium ion [31]. This severely limits the flexibility of these residues.

Substitution at the P1′ site demonstrated the largest difference in K_i values. Even though a phenylalanine side chain would be predicted to show potency similar to that of a leucine side chain based on previous substrate studies [41], comparison of the inhibition by two such inhibitors (MAG-42 and -148) actually showed more than a 350-fold difference in K_i. These two inhibitors complexed with cdMT1-MMP were superimposed (Figure 4A). The inhibitors overlay quite well, with the exception of the P1′ carbonyl, which is a key hydrogen-bonding group with the NH of Leu^{199}. The N to O distance is 3.2 and 3.6 Å for MAG-42 and -148 respectively. The disruption of this hydrogen bond is owing to the restricted orientation of the phenylalanine side chain by the well-defined S1′ pocket, along with the restricted flexibility of Leu^{199} due to co-ordination of the adjacent residue, Phe^{198}, to a structural calcium ion. The respective inhibitor with a homophenylalanine side chain superimposed with MAG-42 maintains all of the necessary hydrogen-bonding interactions (Figure 4B). The C_1 phenyl ring atom is located in the same position as the terminal methyl carbon that is pointing in the direction of the S1′ pocket. Therefore there is no distortion of the inhibitor backbone, and all necessary interactions are maintained. The distortion of the backbone in MAG-148 is a result of the steric restriction of the phenyl ring in the S1′ pocket. Disruption of one hydrogen bond, with all other interactions being the same, could account for a free energy change of the order of 8–21 kJ/mol. The free energy is related to K_i by eqn (4):

$$\Delta G^0 = -RT \ln K_i \quad (4)$$

and a 10-fold difference in K_i corresponds to a free energy change of 5.69 kJ/mol, 100-fold corresponds to 11.38 kJ/mol, and 1000-fold corresponds to 17.07 kJ/mol. A reasonable explanation for the 350-fold difference in K_i between MAG-42 and -148 is disruption of a single hydrogen bond. This is the first report to provide a rationale for the large disparity between these side chains in terms of either substrate specificity or inhibition studies.

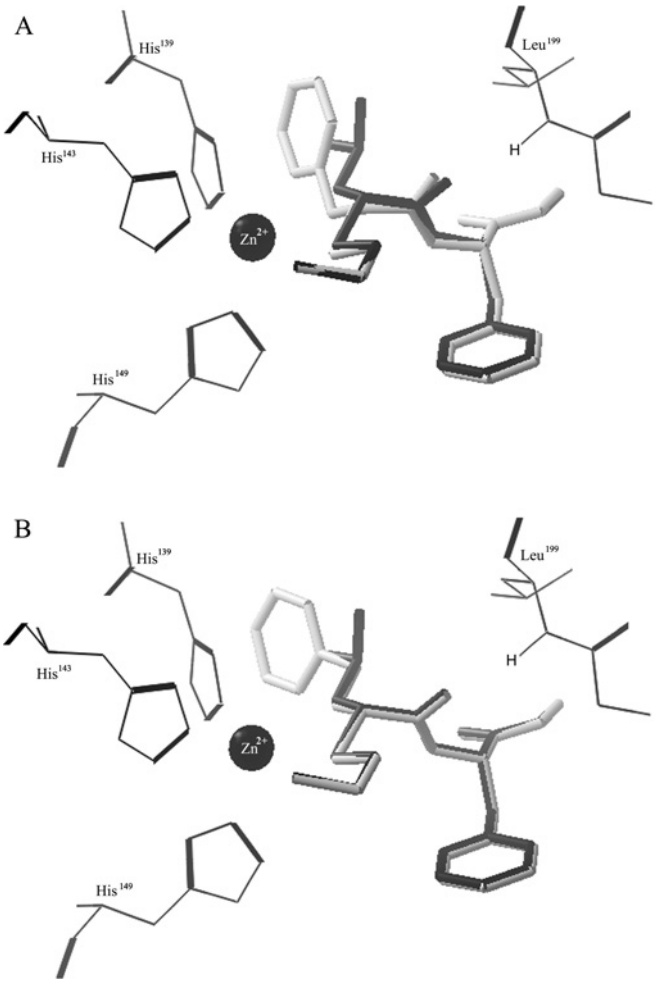

Figure 4 Modification of the P1′ substituent

The superimposed complexes of cdMT1-MMP with inhibitors modified at the P1′ site reveal reasons for the significant K_i differences. The inhibitors are shown as black or grey sticks, the enzyme residues as wire, and the catalytic zinc is black CPK (Corey–Pauling–Koltun). (**A**) MAG-42 (black) and -148 (grey) are superimposed. A key hydrogen bond with Leu^{199} and MAG-42 has an N to O distance of 3.2 Å. This hydrogen bond is not achieved by MAG-148, with an N to O distance of 3.6 Å. (**B**) MAG-42 (black) and an inhibitor modified with a phenethyl P1′ substituent (grey) are superimposed. With this P1′ substituent, all interactions important for inhibitor potency are conserved.

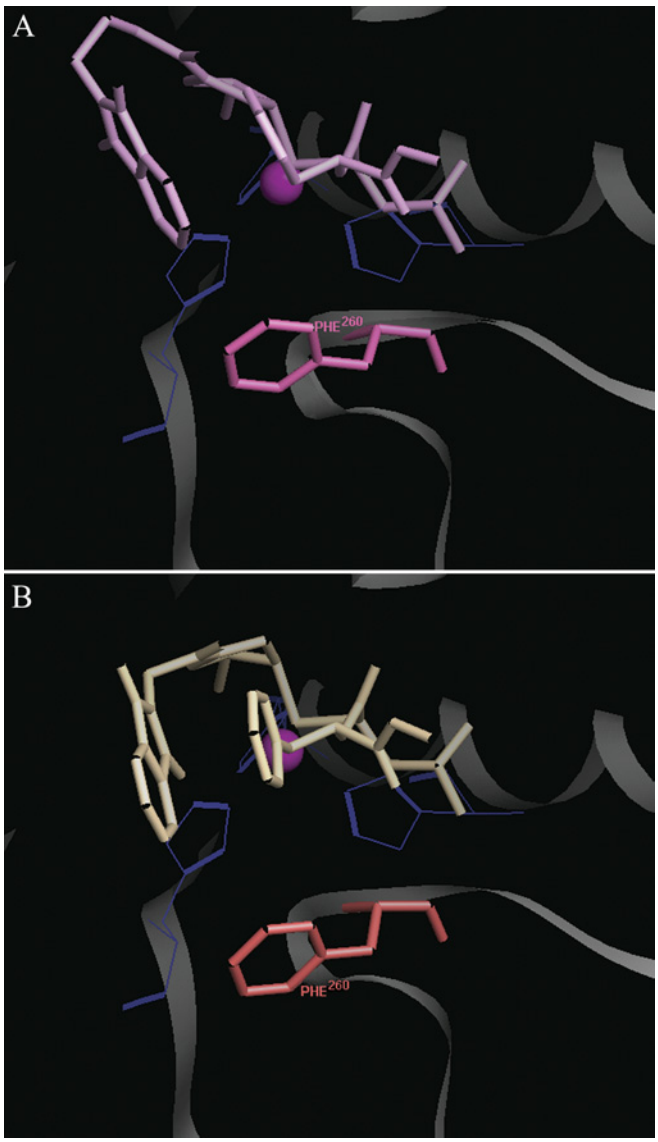

Figure 5 Edge-to-face aromatic–aromatic interaction

(**A**) The model of inhibitor YHJ-97 (purple stick) is shown complexed with cdMT1-MMP. The phenyl ring centroid-to-centroid distance between the phthalimido and Phe^{260} is 5.5 Å. Zinc is shown as magenta CPK (Corey–Pauling–Koltun) with the three co-ordinating histidines as blue wire. (**B**) The model of inhibitor YHJ-96 (beige stick) is shown complexed with cdMT1-MMP. The phthalimido substituent does not interact with any residues of cdMT1-MMP.

The 10-fold difference in K_i for YHJ-96 and -97 was also very intriguing. These inhibitors differ only in the tether length for the attachment of the phthalimido substituent at the non-prime site. Modelling of YHJ-97 showed an edge-to-face aromatic–aromatic interaction between the phthalimido ring and Phe^{260} that was not identified for YHJ-96 (Figure 5). The YHJ-97 phthalimido ring is oriented towards the centre of the phenyl ring of Phe^{260}, with a distance of 5.5 Å between the two phenyl ring centroids. The preferential distance between phenyl ring centroids is 4.5–7 Å for this type of interaction and may contribute up to 8.4 kJ/mol of free energy [42], which correlates well with the 10-fold difference in K_i between these inhibitors.

Inhibition by MMPIs of MT1-MMP-mediated substrate cleavage in cells

Two potent broad-spectrum and relatively stable novel MMPIs, YHJ-132 and YHJ-294-2, have been tested in two different human cancer-cell culture systems to examine their inhibition efficacy in live cells. The first model is the human fibrosarcoma HT1080 cell culture system. HT1080 cells produce endogenous active MT1-MMP upon treatment with 20 μg/ml ConA. The endogenous MT1-MMP can cleave and activate endogenous proMMP-2 to form the 64 kDa intermediate. This activation was blocked by these two inhibitors in a dose-dependent manner, as shown by the accumulation of proMMP-2 zymogen (Figure 6). The dynamic concentration changes of the 64 kDa intermediate were contributed by both MT1-MMP, which converted 72 kDa zymogen into the 64 kDa intermediate, and self-catalysed intermolecular cleavage of the 64 kDa enzyme to the 62 kDa active MMP-2. At an inhibitor concentration of 200 nM, the generation of the 64 kDa intermediate was partially blocked, and that of the 62 kDa active MMP-2 was almost completely blocked by these two inhibitors. Normally, the 64 kDa intermediate is

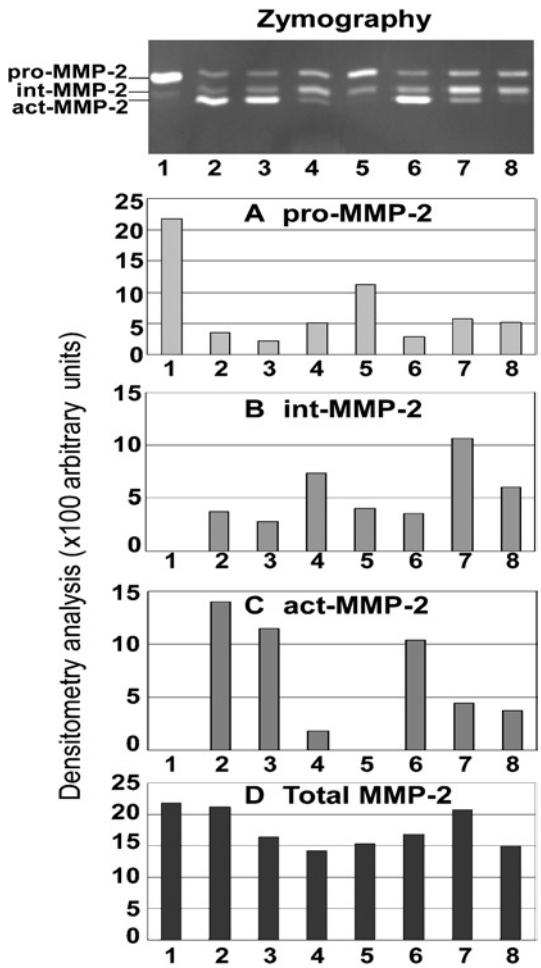

Figure 6 Dose-dependent inhibition of proMMP-2 activation by two novel MMPIs in a cell culture system as shown by a representative gelatin zymogram and densitometry analyses using Alphalmager™ 2200

The conditioned media of HT1080 cells treated with or without different doses of the novel MMPIs plus 20 μg/ml ConA were examined by gelatin zymography. The molecular masses of latent, intermediate and fully activated MMP-2 were demonstrated as 72 kDa pro-MMP-2, 64 kDa int-MMP-2, and 62 kDa act-MMP-2 respectively. Lane 1, HT1080 control; lane 2, HT1080 with ConA; lanes 3–5, HT1080 with ConA and 10, 100 or 200 nM YHJ-132 respectively; lanes 6–8, HT1080 with ConA and 10, 100 or 200 nM YHJ-294-2 respectively.

quickly converted into the 62 kDa active form; at 100 nM of each of these two inhibitors, this conversion was effectively blocked, but the inhibition of the conversion of the 72 kDa zymogen into the 64 kDa species was incomplete (Figure 6). From these results, it appears that the 64 kDa form is 'stabilized' by these inhibitors, indicating that these two inhibitors are less effective on MT1-MMP than on MMP-2, which is consistent with the enzyme-inhibition parameters, demonstrating that these two inhibitors are slightly less potent against MT1-MMP than MMP-2 (Table 1). The K_i values for YHJ-132, YHJ-294-2 and some other mercaptosulphide inhibitors against MMP-1, 2, -3, -7, -9 and MT1-MMP are shown in Table 1.

The second model utilizes the human prostate cancer cell line LNCaP cells expressing stably transfected MT1–GFP chimaera. These cells were plated on to FITC–FN glass coverslips. YHJ-132 and YHJ-294-2 partially or completely inhibited MT1-MMP- and perhaps other MMP-induced substrate degradation at 5 μM and 10 μM, but were much less effective in blocking cell migration (Figure 7). TIMP-2, at 1 μM, completely inhibited cell-mediated substrate cleavage and partially impeded cell migration. These results are consistent with our previous report that MMPIs did not effectively stop cell migration, owing to MMP-independent migration mechanisms such as phagocytosis-mediated cell movement and migration [39]. These data demonstrate that these novel synthetic inhibitors are able to reach MT1-MMP and other MMPs produced by live cells and block their substrate digestion.

DISCUSSION

In the present study, the inhibition profile, mechanisms and requirements for potency of mercaptosulphide inhibitors against the target enzyme, cdMT1-MMP, have been investigated. Synthetic MMPIs have been developed and characterized extensively with collagenase 1 and 2 (MMP-1 and -8), gelatinase A and B (MMP-2 and -9), stromelysin 1 (MMP-3), and matrilysin (MMP-7), but few inhibition studies have included MT1-MMP. The most potent inhibitors developed by other researchers contain a hydroxamic acid zinc-chelating group, and these compounds have been the lead agents for clinical trials. The results of some clinical trials have been disappointing for many reasons; for example, the trial design may not have been optimal and the hydroxamate inhibitors may not be specific [43–47]. There is an urgent need for developing MMPIs with novel zinc-binding groups [48–50]. Mercaptosulphide inhibitors have been developed and characterized for many MMPs and exhibit IC_{50} and K_i values approaching those of well-characterized hydroxamates [19–23]. The detailed mechanisms of interaction of these inhibitors with a MMP have not been previously elucidated.

The mercaptosulphide inhibitors were demonstrated to bind reversibly and competitively to MMPs. In agreement with other MMP family members, there is strong stereoselectivity at the P1′ and zinc-binding groups. This observation correlates with structural studies that indicate that MMPs have a highly conserved active site [51]. Stereochemistry at the P1′ site favours the absolute *R* configuration corresponding to the unnatural D-amino-acid derivative. Around the mercaptosulphide zinc-co-ordinating functionality, the *S*,*R* stereochemistry is favoured (C_α,C_β to thiol). The non-prime site was probed with a phthalimido substituent. The inhibition potency of inhibitors containing this substituent was comparable with, and in some cases improved upon, that of similar inhibitors without the non-prime phthalimido. An exception was noted with YHJ-96, which was 10-fold less potent than YHJ-97. These inhibitors differ only in the tether length for the attachment of the phthalimido (methyl and propyl respectively). The S1′ pocket is the most well-defined pocket and is deep in MT1-MMP compared with other MMPs. This pocket may simply accommodate long hydrophobic groups at the inhibitor P1′ site and therefore a phenylalanine side-chain inhibitor should be approximately equal in potency when compared with leucine. However, MAG-148, which contains a P1′ phenylalanine side chain, was found to be less potent (more than 350-fold) than the corresponding inhibitor MAG-42 with a leucine side chain. This result could not be predicted from the descriptions posted in the literature regarding inhibitor binding. It was therefore necessary to evaluate the experimental results with molecular modelling to propose rational explanations for the requirements of inhibitor potency.

The overall inhibition by the mercaptosulphide compounds of cdMT1-MMP follows the general requirements of other MMP-family members, with the exception of YHJ-96. Although the non-prime site is not of particular importance for selectivity, the 10-fold difference in K_i between YHJ-96 and -97 is interesting. An aromatic–aromatic interaction was identified

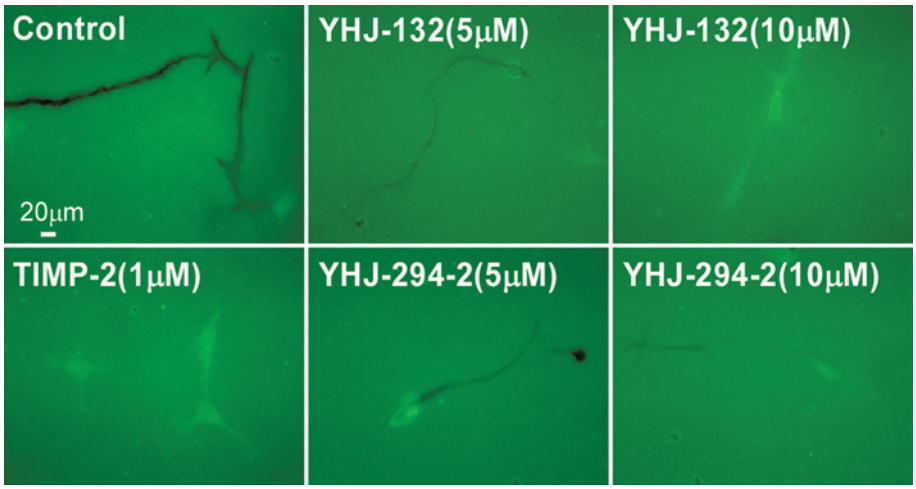

Figure 7 Inhibition of cell-mediated substrate degradation by two novel MMPIs

LNCaP cells stably transfected with the MT1–GFP chimaera were plated on to FITC–FN coverslips and incubated with vehicle control (DMSO), TIMP-2, YHJ-132 or YHJ-294-2 at different concentrations in serum-free medium at 37 °C for 18 h. The cells were then fixed and examined under fluorescence microscopy. The substrate degradation was demonstrated by loss of fluorescence of FITC-labelled substrate, and cell migration was determined by observing the tracks of digested substrate on the same slide. MT1–GFP-expressing cells digested FN and migrated over digested substrate. The dark lines on the green FITC–FN background indicate substrate degradation and cell migration. The cells are also green because of the GFP expression together with MT1-MMP. The positive control, 1 μM TIMP-2, completely blocked the FN degradation and partially inhibited cell migration. New MMPIs, YHJ-132 and YHJ-294-2, partially or completely inhibited MT1-MMP-induced substrate degradation at 5 μM and 10 μM, but were much less effective in stopping cell migration.

between YHJ-97 and Phe^{260} that was not seen with YHJ-96. This would explain the 10-fold difference in potency against cdMT1-MMP. MMP-3 was the only other MMP that showed this behaviour. It is not clear whether MMP-3 interacts with these inhibitors in a similar fashion; however, Cohen and colleagues recently reported π–π stacking interactions at the non-prime site for the inhibitor futoenone complexed with MMP-3 [52]. This inhibitor is selective for MMP-3 over other MMPs owing to a unique tyrosine residue. The cdMT1-MMP Phe^{260} appears to be located at the S2′ site. Therefore the phthalimido substituent in YHJ-97 may be interacting at the S2′ site and not in the non-prime site as would have been predicted. Although YHJ-97 is not selective for cdMT1-MMP compared with other MMPs, the 10-fold preference of cdMT1-MMP for YHJ-97 over YHJ-96 could be explained rationally with modelling. Extension of the tether length to five carbons, as in YHJ-44, did not affect inhibitor potency compared with YHJ-97.

The S1′ pocket has been the most-characterized site in MMPs, especially with regards to inhibition studies. Although MT1-MMP is known to have a deep S1′ pocket, the phenylalanine side-chain inhibitor, MAG-148, was more than 350-fold less potent compared with the leucine side-chain inhibitor, MAG-42. Four hydrogen bonds between the peptide backbone of mercaptosulphide inhibitors and the enzyme active site appear to be necessary for inhibition potency. Disruption of one of these bonds, with all other interactions being the same as predicated, as in the case of MAG-148, could account for a free energy change of the order of 8–21 kJ/mol. The cdMT1-MMP active site is rigid at the Leu^{199} hydrogen-bonding site owing to the structural calcium metal that is co-ordinated to the adjacent residue, Phe^{198}. This calcium-binding site is highly conserved among the MMPs and may be the reason why MAG-148 is not favoured in any of the deep-pocket MMPs tested. The present paper is the first report to provide a rationale for the large disparity between these side chains in terms of either substrate specificity or inhibition potency.

A new series of inhibitors incorporating a pyrrolidinyl ring at the zinc-binding functionality (Table 1, compound 3) has been developed recently [21,23]. These compounds have increased water-solubility, while maintaining inhibitor potency. The stereochemical requirements of these inhibitors are the same as for the previously designed inhibitors (Table 1, compounds 1 and 2) and demonstrate binding in the same orientation. The increased water-solubility may correspond to an improved oral bioavailability that must be considered for future drug design. Therefore these compounds may be ideal candidates for future *in vivo* cancer-cell invasion and metastasis studies.

The prominent roles of MMPs in diseases have led to aggressive targeting of these enzymes by pharmaceutical companies. Despite all the advancements in MMPI research, and particularly with hydroxamic acids, clinical trials have been disappointing. Recent papers suggest the need for specific targeting of MT1-MMP in cancer invasion [5,6]. To increase our understanding of MMPIs, and particularly mercaptosulphide inhibitors, we have characterized them with a potential target, MT1-MMP, in enzyme-inhibition kinetic and mechanistic studies. Moreover, we have demonstrated that these inhibitors have reached their target enzyme on the cell surface and blocked the substrate cleavage by MT1-MMP in two different human cancer-cell culture systems, with functional endogenous and exogenous MT1-MMP expression respectively. Owing to the exceptionally significant roles that MT1-MMP plays, not only in cancer-cell invasion and growth [1–8], but also in conferring tumorigenicity on non-malignant epithelial cells [53,54], it is essential to make efforts to develop novel potent and selective inhibitors to target MT1-MMP. Our studies might identify enzyme–inhibitor interactions that assist the rational design of new generations of compounds with increased potency, selectivity, stability and water-solubility.

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A subset of in situ breast tumor cell clusters lacks expression of proliferation and progression related markers but shows signs of stromal and vascular invasion☆

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Abstract

Background: Our previous studies in pre-invasive mammary tumors revealed that estrogen receptor negative cell clusters (ER NCC) overlying focally disrupted myoepithelial (ME) cell layers showed a significantly higher rate of genetic abnormalities and cell proliferation than adjacent cells without ME cell layer disruptions. A subset of these ER NCC, however, completely lacked expression of Ki-67, a most commonly used marker for cell proliferation. The purpose of this study was to further elucidate the immunohistochemical and morphological profiles of these ER NCC. *Methods:* Fifteen cases with such ER NCC were selected from our previous studies and assessed with a panel of commonly used biomarkers for cell proliferation, tumor progression, and normal stem cells. *Results:* Immunohistochemically, in addition to Ki-67 and ER, these ER NCC completely lacked expression of all other proliferation and progression related markers that were distinctly expressed in adjacent cells within the same duct but overlying the non-disrupted ME cell layer. These ER NCC also lacked expression of all normal stem cell-related markers tested. These cell clusters, however, showed a higher and atypical expression of c-erb-B2, compared to their adjacent counterparts. Morphologically, these ER NCC were generally arranged as triangle shaped structures penetrating into the stroma, similar to micro-invasive lesions. About 15% of these ER NCC appeared to directly spread into blood vessel-like structures. These ER NCC and their possible derivatives within the stroma and blood vessels-like structures shared the same morphologic and immunohistochemical features. No comparable ER positive cell clusters were identified in any of the cases. *Conclusions:* These findings suggest that these ER NCC and their possible derivatives are likely regulated by yet to be defined molecules and mechanisms, and they are unlikely to respond to currently available anti-mitotic agents.

Keywords: Estrogen receptor; Myoepithelial cells; Anti-mitotic agents; Stromal and vascular invasion; Stem cells; Breast tumor cells; Ductal carcinoma in situ; Morphologic features; Progression-related markers; Tumor invasion and metastasis; Vascular invasion; Immunostataining; Matrix metalloproteinase-26; c-erb-B2 protein; Cytokeratin; Basement membrane disruption; Genetic alterations

☆ The opinions and assertions contained herein represent the personal views of the authors and are not to be construed as official or as representing the views of the Department of the Army or the Department of Defense.

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1. Introduction

The epithelium of the normal human breast and in situ breast tumors is physically separated from the stroma by both a myoepithelial (ME) cell layer and the basement membrane [1–3]. ME cells are joined by intercellular junctions and adhesion molecules, forming a continuous layer that encircles the entire duct system, and a discontinuous layer or a basket-like structure that covers a vast majority of lobules and terminal duct-lobular units [1–3]. The basement membrane is composed of a group of fibrous proteins embedded in a hydrated polysaccharide gel, forming a continuous lining surrounding and attaching to ME cells via hemidesmosomes and focal adhesion complexes [1–3]. This architectural feature confers ME cells and the basement membrane two essential functions. First, as the epithelium is normally devoid of lymphatic and blood vessels and totally depends on the stroma for its metabolism and survival needed materials, the ME cell and basement membrane function as gatekeepers, directly regulating the communication between these two cellular compartments. Second, due to the physical interposition of the ME cell layer and basement membrane between the stroma and epithelium, ductal tumor cells must first penetrate the ME cell layer and then followed by the basement membrane, in order to reach the stroma for invasion or metastasis.

A generally accepted hypothesis for the direct cause of basement membrane disruptions and tumor invasion has been attributed primarily, if not solely, to over-production of proteolytic enzymes by tumor or stromal cells [4]. This hypothesis alone, however, may not reflect the intrinsic mechanism of tumor invasion, as results from recent worldwide clinical trials with a wide variety of corresponding enzyme inhibitors have been disappointing [5,6].

While attempting to identify early signs of ME layer disruptions and precursors of invasive lesions, we have carried out a number of studies, focusing on the correlation between the structural integrity in ME layers and the immunohistochemical and genetic profiles in adjacent epithelial cells.

In double immunostained sections from 220 patients with estrogen receptor (ER) positive, non-invasive breast tumors, we detected a total of 405 focal ME cell layer disruptions, defined as the absence of ME cells resulting in a gap equal to or greater than the combined size of three ME or epithelial cells [7]. Of these disruptions, 350 (86.4%) were overlaid by cell clusters with no or substantially reduced ER expression, in contrast to adjacent cells within the same duct, which expressed a high level of ER and overlaid a non-disrupted ME cell layer [7]. Compared to their adjacent counterparts within the same duct, most ER negative cell clusters (ER NCC) overlying focally disrupted ME cell layers displayed several unique features, including a substantially higher frequency of loss of heterozygosity at multiple chromosomal loci, a significantly higher expression of tumor progression and invasion related genes, and a significantly higher index of Ki-67 positive cells [7–10], seemingly representing a biologically more aggressive cell clone or the precursor of invasive lesions.

About 10% of these ER NCC in some cases, however, were completely devoid of Ki-67 immunoreactive cells, in a sharp contrast to both the adjacent ER positive cells within the same duct and other ER NCC in different ducts, which showed a markedly elevated number of Ki-67 positive cells [11,12]. These unusual ER NCC, however, exhibited distinct signs of growth, and stromal or vascular invasion [13–15].

As it has been well documented or suggested that ER negative tumors have a substantially worse prognosis [16–18], and deregulated cell proliferation is a direct cause of breast malignancies [19,20], this study attempted to further elucidate the immunohistochemical and morphological features of these ER NCC. The primary goal of this study was to determine whether these unusual ER NCC would have a unique immunostaining pattern for currently available proliferation, tumor progression, and other related markers that have been linked to breast tumor progression and invasion.

2. Materials and methods

Formalin-fixed, paraffin-embedded human breast tissues with ductal carcinoma in situ ($n = 15$) containing focally disrupted ME cell layers and ER NCC with no Ki-67 immunoreactive cells were selected from over 400 pre-invasive lesions from our previous studies [7–10]. Consecutive sections at 4–5 μm thickness were placed on positively charged microscopic slides, and stained with H&E for morphologic classification based on our published criteria [21]. Immunohistochemical staining was carried out, using our published protocols [22,23]. Briefly, sections were incubated at 70–80 °C for 1 h, deparaffinized with xylene, and washed with ethanol and water. Deparaffinized sections were incubated in 1× antigen retrieval solution (Biocare Medical, Foster City, CA) overnight at 60–70 °C, washed in water and phosphate-buffered saline (PBS), treated with 3% hydrogen peroxide and normal serum, and incubated with a primary antibody. After incubation with the primary antibody, sections were washed with PBS, and then sequentially incubated with the corresponding secondary antibody, avidin–biotin–peroxidase solution, and substrate diaminobenzidine (Vector, Burlingame, CA). For elucidation of a new antigen, immunostained sections were thoroughly washed with PBS, and then incubated with a new antibody. The antigen and antibody complex was detected with a corresponding secondary antibody, avidin–biotin–alkaline phosphatase detection kit, and Zymed AP-red substrate kit (Zymed Laboratories Inc., South San Francisco, CA).

To identify focal ME cell layer disruptions and to determine the size of associated ER NCC, sections 1, 11, and 21 from each case were double immunostained for ER, Ki-67, and smooth muscle actin (Vector, Burlingame, CA). ER NCC were

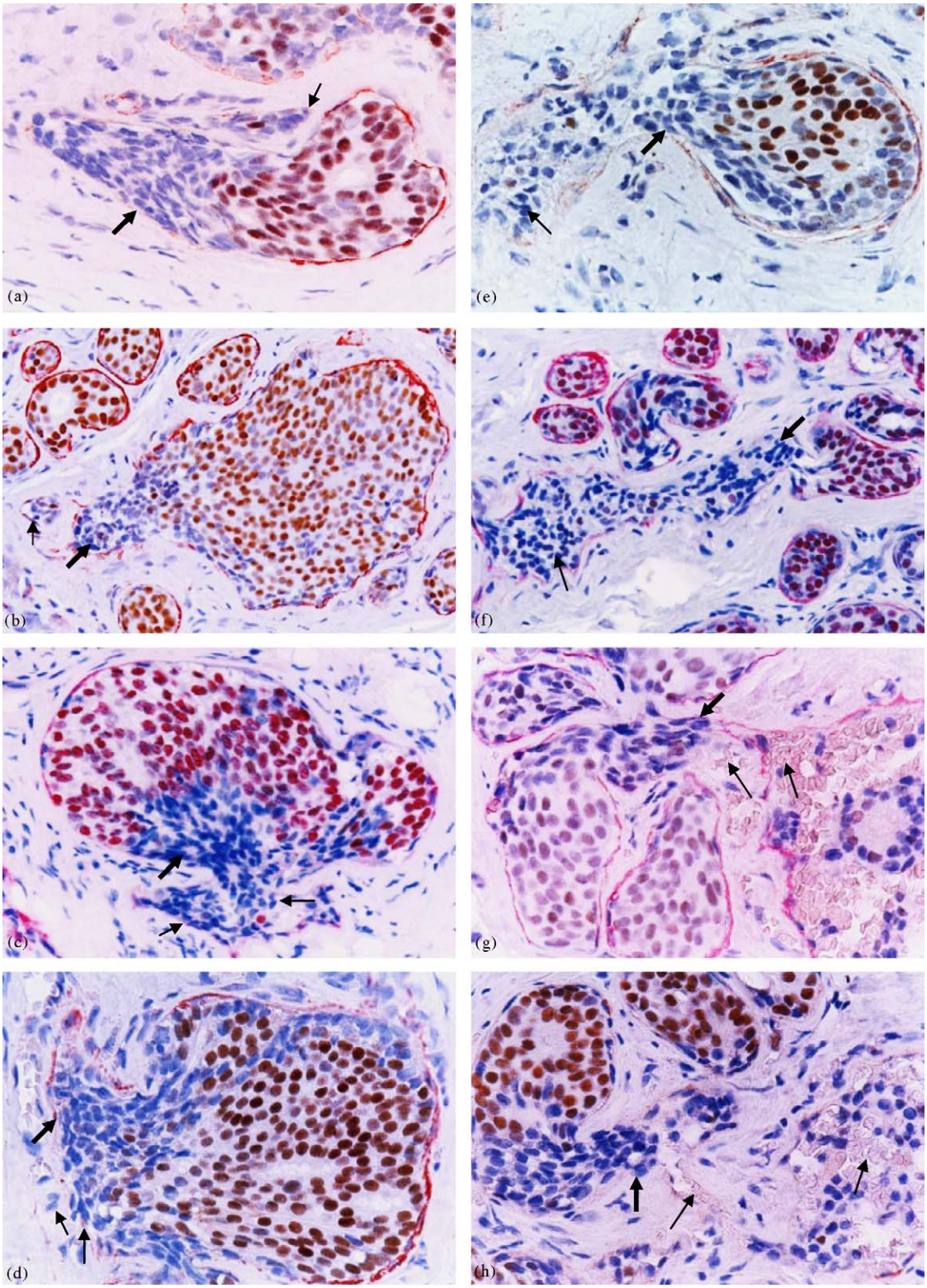

Fig. 1. Potential signs of stromal and vascular invasion of ER negative cell clusters (ER NCC). Sections were double immunostained for smooth muscle actin (SMA, red) and ER and Ki-67 (brown). Larger arrows identify ER NCC, and smaller arrows identify micro-invasive lesions and blood vessel-like structures or red blood cells. Note that cells within the vessel-like structures exhibited a non-cohesive or ''floating'' appearance (400×).

defined as large if they were appreciable in more than 10, or as small if they were present in fewer than 10 sections.

To assess the proliferation status, additional proliferation-related markers, including cyclin A, cyclin D1, p27, and proliferating cell nuclear antigen (PCNA) [24–26] were used.

To assess the progression status, the expression of matrix metalloproteinase-26 (MMP-26, which is seen in near all DCIS), beta protein 1 (BP1, a homeotic protein detectable in over 80% of invasive breast tumors), and c-erb-B2 (an oncoprotein associated with aggressive behavior) [27–29], were assessed.

To assess whether these ER NCC belong to progenitor of cells, the expression of three normal stem cell related molecules, cytokeratin 5, breast cancer resistant protein (BCRP), and CD 44 (30–32) was evaluated.

To identify the histological origin of these ER NCC, sections were immunostained with stromal and epithelial phenotypic markers, vimentin, smooth muscle actin (SMA), cytokeratin (CK) AE1/AE3, and epithelial specific antigen (ESA) (Vector, Burlingame, CA).

3. Results

Distinct focal ME cell layer disruptions and associated ER NCC with no Ki-67 immunoreactive cells were present in all cases. The number and size of these clusters varied substantially among cases. Morphologically, cells within or among ER NCC were identical or similar, but a vast majority of them were noticeably smaller in size

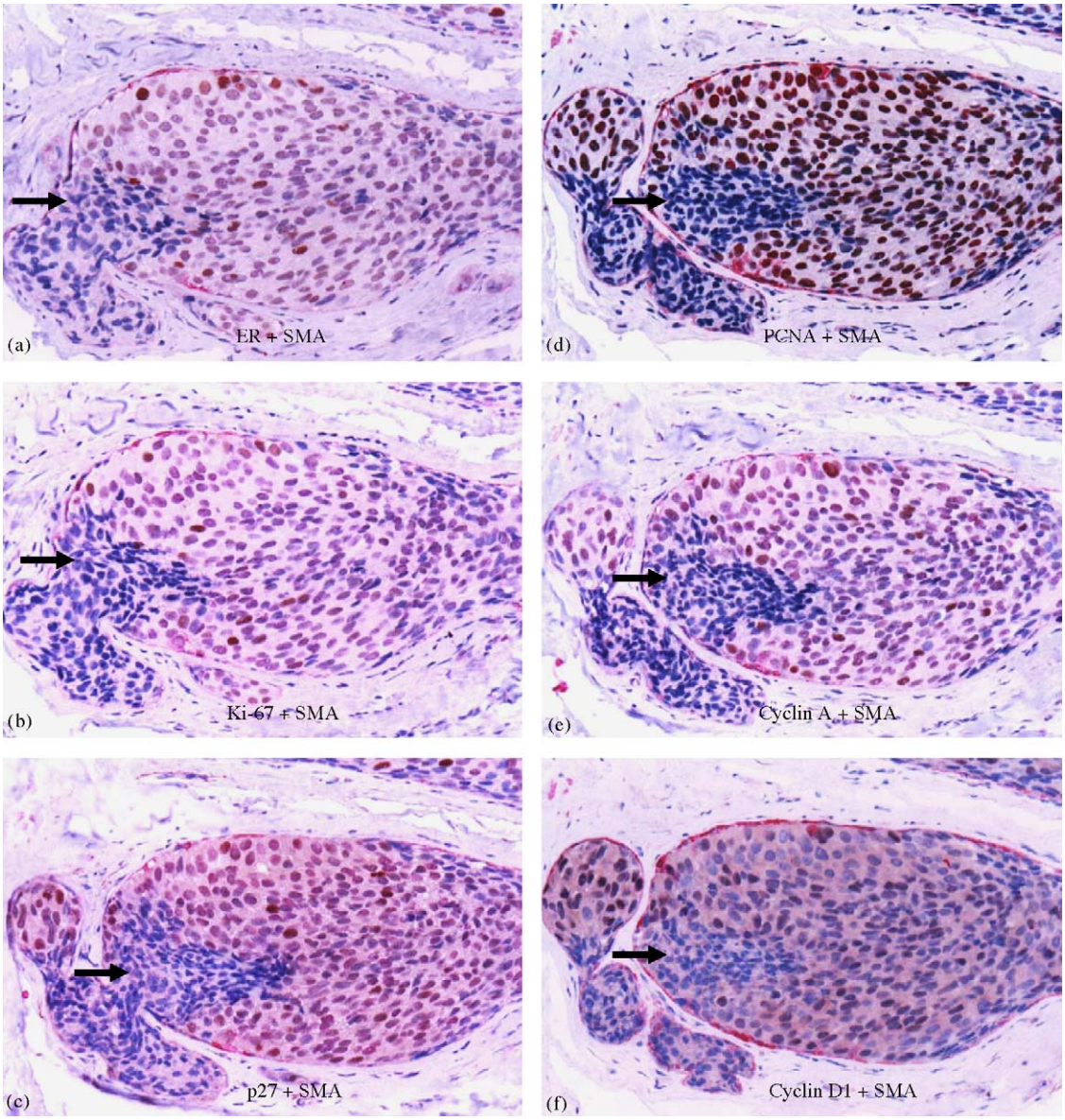

Fig. 2. The lack of expression of proliferation related markers in ER NCC. Consecutive tissue sections were double immunostained for SMA (red) and different proliferation related markers (brown). Arrows identify focal ME cell layer disruptions and ER NCC (200×).

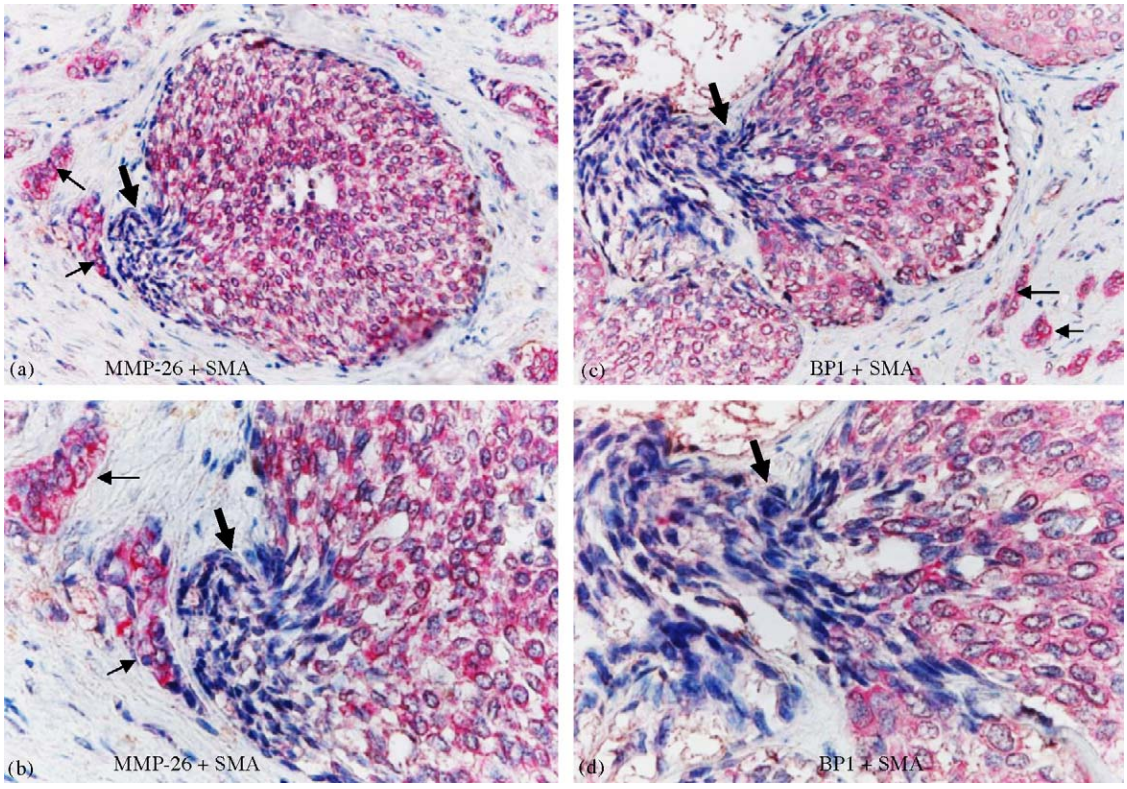

Fig. 3. The lack of the expression of matrix metalloproteinase-26 (MMP-26) and beta protein 1 (BP1) in ER NCC. Sections were double immunostained for SMA (brown) plus MMP-26 or BP1 (red). Larger arrows identify focal ME cell layer disruptions and ER NCC; smaller arrows identify micro-invasive lesions. (a) and (c) 200×; (c) and (d) are a higher magnification of (a) and (c), respectively, 400×.

with dark, elongated, or irregular nuclei and were often densely packed, compared to adjacent cells without ME cell layer disruptions (Fig. 1). These ER NCC were generally arranged as triangle-shaped structures penetrating into the stroma, comparable to microinvasive lesions (Fig. 1). Cells in about 15% of these ER NCC exhibited a non-cohesive or ''floating'' appearance and seemed to directly spread into blood vessel-like structures (Fig. 1). These vessel-like structures often contained distinct red blood cells (Fig. 1). These ER NCC and adjacent micro-invasive lesions and cells within vessel-like structures were immunohistochemically and morphologically similar (Fig. 1).

Immunohistochemically, in addition to ER and Ki-67, these ER NCC also completely lacked expression of other proliferation related markers, including p27, PCNA, cyclin A, and cyclin D1 that were distinctly present in their adjacent counterparts within the same duct (Fig. 2). These ER NCC were also devoid of tumor progression related markers MMP-26 and BP1, in contrast to adjacent cells within the same duct and adjacent invasive lesions, which were strongly immunoreactive to these markers (Fig. 3). In five selected cases, these ER NCC were also devoid of expression of stem cell related markers, CD44, BCRP, and cytokeratin 5, but they were weakly positive to epithelium specific antigen and cytokeratins AE1/AE3 (Fig. 4), suggestive of the epithelial origin.

On the other hand, a subset of these ER NCC in nine cases were very strongly immunoreactive to c-erb-B2, in a sharp contrast to their adjacent counterparts within the same duct, which were largely negative or weakly positive to this marker (Fig. 5). The c-erb-B2 protein, however, was predominantly in the cytoplasm, rather than the typical membrane localization (Fig. 5).

These ER NCC were totally negative for the stromal specific markers vimentin and SMA in all cases (data not shown). No morphologically or immunohistochemically comparable ER positive cell clusters were found in any of the cases.

4. Discussion

Our previous studies revealed that a subset of cell clusters overlying focally disrupted ME cell layers completely lacked the expression of ER and Ki-67 [11,12]. Our current study further revealed that these clusters possess additional unique features: [1] signs of stromal and vascular invasion; [2] the lack of other proliferation related markers; [3] the lack of tumor progression related markers; [4] the lack of normal stem cell related markers; [5] an elevated and atypical expression of c-erb-B2, an onco-protein. To the best of our knowledge, such or similar cell clusters and growth pattern have not been previously reported.

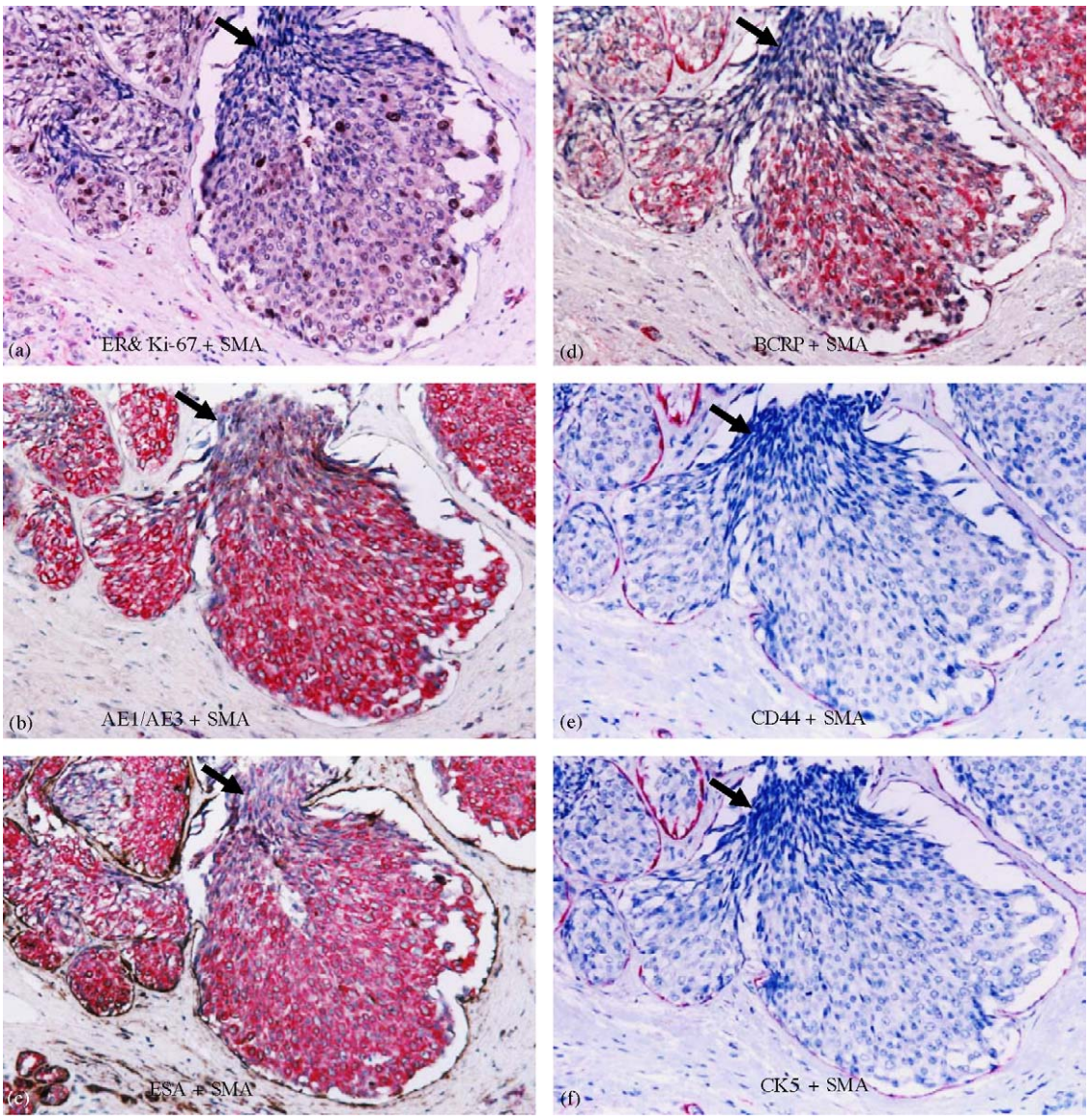

Fig. 4. The expression status of normal stem and epithelium phenotypic markers in ER NCC. Consecutive sections were double immunostained with SMA and normal stem cell related and epithelium phenotypic markers. Arrows identify ER NCC (250×). AE1/AE3: cytokeratins AE1/AE3; ESA: epithelial specific antigen; BCRP: breast cancer resistant protein; CK5: cytokeratin 5.

These findings are hard to reconcile with those of our previous studies, which have suggested that focal ME cell layer disruptions could potentially trigger cell proliferation and tumor progression through two different mechanisms. First, as the ME cells manufacture a number of tumor suppressors, including maspin, p63, and Wilms' tumor 1 [33–35], a focal ME cell layer disruption or pathologic alteration could potentially result in a localized loss of these growth inhibitors, consequently leading to the loss of paracrine inhibitory functions on adjacent tumor cells. Second, because both metabolism and growth related materials needed by epithelial cells must first pass through the basement membrane and the ME cell layer, a focal ME layer disruption is likely to result in an increased permeability for these materials, which could confer growth advantages on cells near focal ME disruptions. Our recent studies have shown that a vast majority of proliferating cells are located at or near focal ME cell layer disruptions, and that ER negative cell clusters overlying focal ME disruptions have a significantly higher expression of tumor progression and invasion related genes, compared to ER positive cells within the same duct [7–10].

All the molecules that are not detectable in these ER NCC have been found to play significant roles in tumor progression and invasion. It has been well documented that breast tumor progression is paralleled by a progressive hormonal independence, and that ER negative tumors have a significantly worse prognosis than ER positive tumors [16–18]. Deregulated expression of proliferation related molecules, including Ki-67, PCNA, cyclin A, cyclin D1, and p27 is linked to tumor aggressiveness, or is even considered as direct causes of malignancies [24–26]. The expression of MMP-26 and BP1

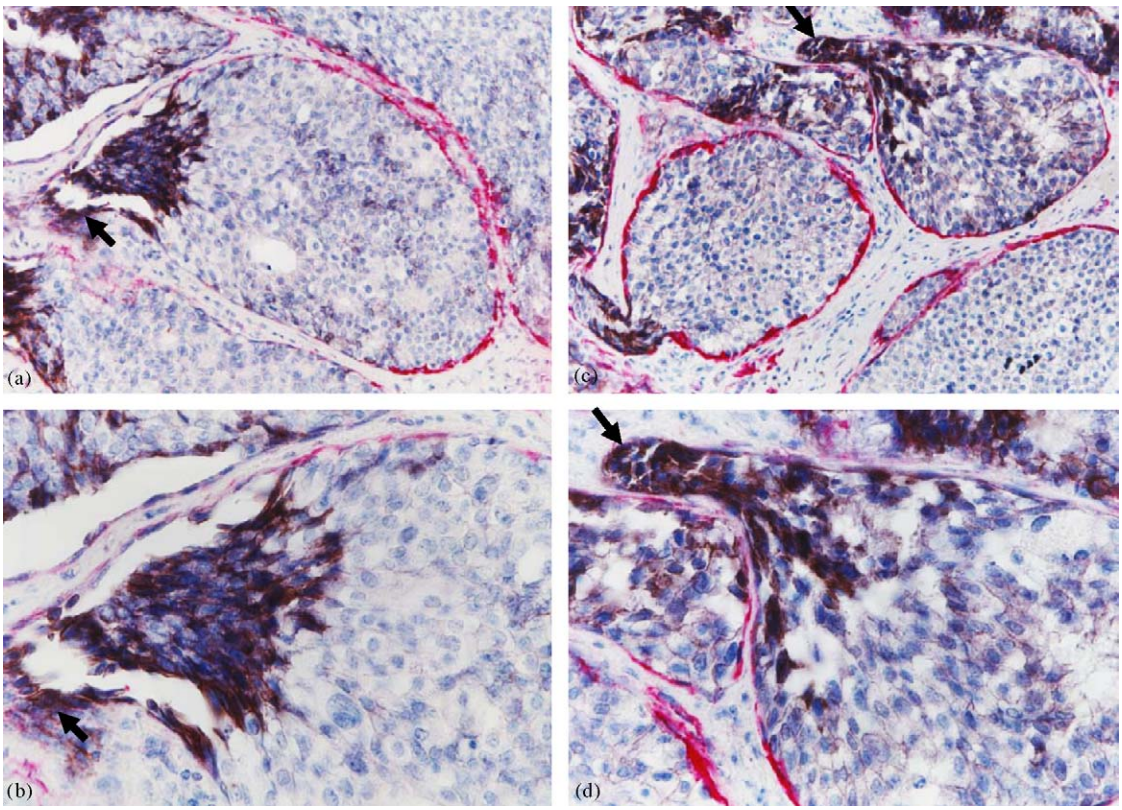

Fig. 5. Elevated and atypical expression of c-erb-B2 in ER NCC. Sections were double immunostained for c-erb-B2 (black) and SMA (red). Arrows identify ER NCC. (a) and (c) 200×; (b) and (d) are a higher magnification of (a) and (c), respectively, 400×.

is detectable in near all DCIS and in over 80% of invasive breast tumors, respectively [27–29], seemingly representing reliable markers for breast tumor progression and invasion. Cytokeratin 5, CD44, and BCRP have been suggested to be potential markers for breast stem cells, which are believed to be a basis of breast pathology [30–32].

The mechanism for the lack of cell proliferation, tumor progression and invasion, and normal stem cell related markers in these ER NCC is unknown. The most likely reason is that these ER NCC might represent mutated progenitor or stem cells that differ genetically and biochemically from both differentiated and normal stem cells, whereas the markers used in our current study are not designed to recognize mutated or newly formed biomolecules. Previous studies have shown that mutations of the E-cadherin gene, including in-frame deletions in exons 8 or 9, or a point mutation in exon 8, not only substantially impact the expression of the cellular adhesion molecules and cell morphology, but also markedly influence the beta-catenin localization and susceptibility to cytoskeletal alterations, as well as the accumulation of apoptosis-related proteins [36–38]. Previous studies have also demonstrated that mutational and/or functional alterations in certain genes, including CD44 and p53, could result in multiple isoforms of proteins, which have different structures and sub-cellular distributions and require different antibodies for their detections [39–41]. In addition, previous studies have suggested that a mutated stem cell is the common cellular origin of teratocarcinomas and cancers, and that a functional breast could be formed from a single cell [42,43]. Most recent studies have further suggested that stem cells and other aggressive tumor cells are capable of generating their own vascular structures [44–47].

Alternatively, these ER NCC are newly formed and are not mature enough to express "adult" molecules, or the expression level is too low to be detected with routine methods. Our previous studies with light and electron microscopic immunocytochemistry combined with autoradiography in adult submandibular glands (which are structurally similar to the breast) have demonstrated that newly formed cell clusters or secretory structures by both normal replacement and regeneration are not only morphologically different from their adjacent normal adult counterparts, but also completely lack the expression of several secretory proteins that are heavily expressed in their adjacent adult counterparts [48,49]. The morphology and protein level in these newly formed structures, however, increasingly resembled the adult counterparts with time, and became indistinguishable from normal adult structures after two months [48,49]. Similar changes have been reported in human breast tissues [50–52].

In either case, the consistent lack of these molecules that are distinctly present in their adjacent counterparts within the same duct appears to have several important implications. First, it suggests that cell clusters overlying focally disrupted

ME cell layers are heterogeneous, consisting of at least three phenotypically different cell types: ER positive, ER negative with Ki-67 expression, and ER negative without Ki-67 expression. As these cells have substantially different immunohistochemical and morphological profiles [7–10], their contributions to the development of invasive lesions are likely to be significantly different. Second, these ER NCC could potentially advance to invasive or metastatic lesions without detectable expression of tumor progression related markers, simulating idle benign lesions. Third, these ER NCC are likely to resist currently available anti-mitotic agents, representing potential ''seeds'' for recurrent and drug resistant tumors. Therefore, microdissection of these cell clusters and adjacent cells within the same duct for genetic and biochemical comparisons could potentially lead to identification of the specific molecules that trigger early events of ME layer and basement membrane disruptions, tumor invasion, and metastasis. The development of antibodies or chemical agents to target these cells might provide a more effective and less toxic means to block tumor invasion or metastasis at early stages. In addition, microdissection of these cell clusters from frozen tissues for tissue culture might lead to the establishment of useful cell lines that benefit stem cell research.

An elevated and atypical expression of c-erb-B2 is very likely to reflect genetic alterations and aggressive behavior in these cell clusters. Previous studies in preformed, growth-arrested mammary acini cultured in three-dimensional basement membrane gels revealed that the activation of c-erb-B2 led to the re-initiation of proliferation and alterations of acinar structures, including the disruptions of tight junctions and changes of the cellular polarity [53]. More recent studies have further shown that the phenotypic consequence of c-erb-B2 overexpression was the shortening of the G1 phase and early S phase entry of the cell cycle, which lead to hyperproliferation [54]. This effect is believed to be mediated through up-regulation of cdk6 and cyclins D1 and E, and an enhanced degradation and re-localization of p27 [54,55].

The intrinsic nature of these unusual ER negative cell clusters, however, cannot be conclusively determined at present for three main reasons: (1) no published findings are available for comparisons; (2) the sample size is suboptimal for a meaningful statistical analysis; (3) the clinical significance is unknown. Currently, a number of studies are in progress, to further characterize these ER NCC, using more both immunohistochemical and genetic approaches.

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Isoelectric Point-Based Prefractionation of Proteins from Crude Biological Samples Prior to Two-Dimensional Gel Electrophoresis

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Two-dimensional gel electrophoresis (2-DE) is used to compare the protein profiles of different crude biological samples. Narrow pH range Immobilized pH Gradient (IPG) strips were designed to increase the resolution of these separations. To take full advantage of IPG strips, the ideal sample should be composed primarily of proteins that have isoelectric point (p*I*) values within the pH range of the IPG strip. Prefractionation of cell lysates from a human prostate cancer cell line cultured in the presence or absence of epigallocatechin-3-gallate was achieved in fewer than 30 min using an anion-exchange resin and two expressly designed buffers. The procedure was carried out in a centrifuge tube and standard instrumentation was used. The cell lysates were prefractionated into two fractions: proteins with p*I* values above 7 and between 4 and 7, respectively. The fractions were then analyzed by 2-DE, selecting appropriate pH ranges for the IPG strips, and the gels were compared with those of unprefractionated cell lysates. Protein loading capacity was optimized and resolution and visualization of the less abundant and differentially expressed proteins were greatly improved.

Keywords: anion-exchange resin • batch chromatography • immobilized pH gradient • human prostate cancer cell lysates • green tea polyphenol • differentially expressed proteins • protein loading capacity • resolution and visualization • prefractionation • two-dimensional gel electrophoresis

1. Introduction

Two-dimensional gel electrophoresis (2-DE) is an analytical technique that simultaneously separates thousands of proteins and allows comparative protein profiling between different crude biological samples. Although labor intensive, this technique is still the predominant method for protein profiling. The existing 2-DE gel methods are incapable of detecting the majority of protein components in complex proteomes such as mammalian cells, tissues, and biological fluids.[1] To increase the resolution of 2-DE separation, long Immobilized pH gradient (IPG) strips with narrow pH ranges have been designed. After separating the proteins by 2-DE, the resultant spots can be cut, destained, digested by trypsin, and analyzed by Matrix-Assisted Laser Desorption Ionization-Time-of-Flight−Mass Spectrometry (MALDI-TOF−MS). Tools such as ExPASy, that scan the different protein databases available, allow for the identification of the protein based on its isoelectric point (p*I*), molecular mass (M_r), and the masses of the different peptides generated from the trypsin digestion of this protein.[2−7] The p*I* and M_r can be estimated from the location of the spot on the gel. The masses of the peptide fragments are measured by MALDI-TOF−MS.

One of the main limitations that might hinder the identification of a protein separated by 2-DE is its low abundance in the sample. This limitation is a result of the finite protein loading limits on commercially available Immobilized pH Gradient (IPG) strips, where the first dimension of the separation occurs. A protein placed in a medium with a pH gradient (i.e., IPG) and subjected to an electric field will move toward the electrode of opposite charge. As it migrates, the protein will arrive at the point in the pH gradient equal to its p*I*, and there, being net zero-charged (zwitterions, neutral), it will stop migrating.[8]

The maximum protein load on an 11 cm IPG strip is less than 300 μg, but when an IPG strip (pH 7 to 10) is used, many proteins contained in the loading capacity, particularly those with pIs lower than 7 and higher than 10, will not be separated. Proteins outside the boundaries of the pH gradient will be electrostatically pushed to either end of the IPG strip because they remain net positively or negatively charged at every point of the IPG polyacrylamide gel. Since the proteins with p*I* values between 7 and 10 are a minority in the sample, many will be dragged by the overwhelming majority of proteins migrating to the ends of the strip. At the end of the separation, the amount of proteins profiled represents a fraction well below the original mass that was loaded. To overcome this limitation, prefractionating a sample prior to 2-DE is a prerequisite.

Several prefractionation techniques prior to 2-DE have been used, including nonp*I* based techniques such as reversed-phase high-performance liquid chromatography (RP-HPLC).[9] These methods allowed for the increased protein loading, but lacked compatibility between the p*I* of the proteins fractionated with the pH range of the IPG strip used. Anion exchange column

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chromatography has been used to prefractionate the proteome prior to 2-DE. One of the restrictions of separating proteins using anion exchange column chromatography is the inability of using high concentrations of urea in the extraction reagent or in the buffers.[10] This high concentration of urea is required to solubilize the proteins that have a low aqueous solubility but when injected in a column chromatography will generate a high backpressure. This prefractionation is therefore limited to the soluble proteins. Other p*I*-based methods that use isoelectric membranes[11] were successful in prefractionating the sample into narrow p*I* fractions, but these methods take hours and require specialized instrumentation. A successful prefractionation that uses common instruments and that is performed in a relatively short time (fewer than 30 min) will be more advantageous, minimizing the hard labor and expense of 2-DE: two factors that are already considered disadvantages of this technique.

In this study, we prefractionate a human prostate cancer cell line, LNCaP cell lysates into two fractions: the first containing proteins with p*I*'s higher than 7, and the second containing proteins with p*I*'s between 4 and 7. These fractions were then applied to 11 cm IPG strips with a pH gradient range appropriate for the p*I*s of the proteins present in the samples. LNCaP cells were then subjected to treatment by epigallocatechin-3-gallate (EGCG), a green tea polyphenol known to induce a change in the protein expression and apoptosis/programmed cell death of LNCaP cells.[12,13] This prefractionation was carried out using Polybuffer Exchanger 94 (PBE 94) and two expressly designed buffers. The buffers used were originally used as anion exchange column chromatography buffers.[14]

2. Materials and Methods

Ready IPG Strips, pH 4−7 and 7−10, 11 cm long (cat. no. 163-2015); 100 × Biolyte 3/10 ampholyte (cat. no. 163-2094) and dithiothreitol (cat. no. 161-0611) were purchased from Bio-Rad. Tris (cat. no. BP152-5), acetic acid, glacial (cat. no. A38^{c}-212), were purchased from Fisher Scientific. Polybuffer Exchanger 94 (PBE 94 cat. no. 17071201) was purchased from Amersham Biosciences Corporation. Dulbecco's Modified Eagle's Media (cat. no. D-2902), bis-tris propane (cat. no. B-9410), pyridine (cat. nos. 27040−7), chloroacetic acid (cat. nos. 40, 292−3), lactic acid (cat. nos. 25, 247−6), tricine (cat. nos. T-0377), sodium dodecyl sulfate (cat. no. L-4509), urea (cat. no. U-6504), and bromophenol blue (cat. no. B-7920) were purchased from Sigma-Aldrich. Piperazine anhydrous, 99% (cat. nos. 13129−1000) was purchased from Acros. CHAPS (cat. no. 220201) was purchased from Calbiochem.

2.1. Cell Culture, Treatment, and Lysis. Human prostate cancer cell line that was from a prostate cancer patient with lymph node metastasis, LNCaP cells (CRL-1740) was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and was maintained in Dulbecco's Modified Eagle's Media (DMEM) (Sigma, St. Louis, MO) without phenol red and was supplemented with 3.7 mg/mL $NaHCO_3$, 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 100 U/mL of Penicillin, and 100 μg/mL of Streptomycin (Cambrex, Walkersville, MD). Cells were grown in a 100 × 20 mm tissue culture plate with 10 mL of media at 37 °C in a humidified atmosphere consisting of 5% CO_2. EGCG was purchased from Sigma (St. Louis, MO) and dissolved in double distilled water to the final concentration of 50 mM. Cells were treated with 50 μM of EGCG for 24 h when they were at exponentially growing phase. The control was treated with the same volume of water. The volume of added EGCG stock solution was 0.1% of the total culture media. Cultured cells were washed three times with phosphate buffered saline (PBS), then lysed with 1 mL of lysis buffer as described,[15] though modified to contain 5 M urea, 2 M thiourea, 1% octylglucopyranoside (OG), 0.25% w/v Biolyte 3−10 ampholyte, 12.5% water saturated isobutanol, 10% 2-propanol, 5% glycerol, and 50 mM dithiothreitol (DTT). Cells were removed with a cell scraper and transferred to a centrifuge tube. Cells were vortexed for 5 min followed by centrifugation at 17 000 × *g* for 20 min. The supernatant was recovered and the precipitate was discarded.

2.2. Titration of the PBE 94 Resin with Start and Elution Buffers. PBE 94 was originally designed as a packing resin for anion exchange columns utilized as a liquid separation method for fractionating proteins according to their p*I* values.[16−19] Chromatofocusing is an ion-exchange chromatography technique in which proteins are bound to an anion exchanger (PBE 94), then eluted by a continuous decrease of the buffer pH so that proteins elute in order of their isoelectric points.[17] Polybuffer Exchanger 94 was packed in a 250 × 5 mm column. The buffers used are described previously.[14] The column was first equilibrated with Start buffer A (12.5 mM bis-tris propane and 12.5 mM piperazine in water-adjusted to pH 8 using HCl) at a flow rate of 1 mL/min until the eluted solution had a stable pH of 8. The concentration of Elution buffer B (12.5 mM pyridine, 12.5 mM acetic acid, 12.5 mM lactic acid and 12.5 mM chloroacetic acid in water, pH 3.3) was then set from 0 to 100% over 60 min, held on 100% for 30 min, and a 0.1 M HCl solution was pumped at the end to reach extremely acidic pH. The column was quickly re-equilibrated with buffer A to avoid any damage secondary to the low pH. A pH meter was used to monitor the pH change of the eluting solution.

2.3. Prefractionation of the LNCaP Cell Lysates Using PBE 94. Instead of packing PBE 94 into a column, 1 mL of this resin was pipetted in a centrifuge tube, followed by adding 350 μL of a solution composed of 50% buffer A and 50% buffer B. The tube was then vortex mixed for 30 s, centrifuged at 5000 × *g* for 30 s, and the supernatant was discarded. This washing step was repeated twice. LNCaP cell lysate (330 μL) was then added to the resin along with 150 μL of a solution composed of 50% buffer A and 50% buffer B. The tube was vortex mixed for 30 s, centrifuged at 5000 × *g* for 30 s, and the supernatant was collected. The resin was washed three times with 350 μL of the same buffer (50% Buffer A, 50% Buffer B), with each iteration followed by vortex mixing for 30 s, centrifugation at 5000 × *g* for 30 s, and supernatant recovery. The four supernatant fractions were then combined, vacuum-dried, and reconstituted in a sample buffer, which is the same as the lysis buffer described above. Protein concentration was determined as described below prior to the 2D-gel analysis. The resin was then washed four times with 350 μL of buffer B, followed by vortex mixing for 30 s and centrifugation at 5000 × *g* for 30 s, and the supernatant was collected after each repeat.

2.4. Protein Assay. The protein concentration of the cell lysates and fractions collected was determined using bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, IL) using bovine serum albumin (BSA) as standards according to the manufacturer's instruction. The concentrations of the proteins in the cell lysates and in the fractions were determined using the standard curve generated by the absorbance at 562 nm of standards in the *Y* axis versus the known concentration of the standards in the *X* axis.

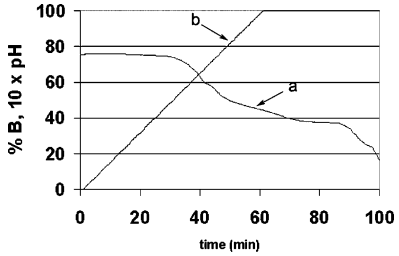

Figure 1. (a) pH gradient generated by the mixing of the start buffer (pH 8) and the elution buffer (pH 3.3) and (b) the concentration of the elution buffer, set from 0 to 100% in 60 min, maintained at 100% for 30 min, followed by a 0.1 M HCl solution at the end to reach extremely acidic pH.

2.5. Two-Dimensional Gel Electrophoresis. 2.5.1. Isoelectric Focusing. Prefractionated samples were treated using Centricon-3 (Millipore, Danvers, MA), according to the manufacturer's instructions, to exchange their buffers with the rehydration buffer, which is the same as the lysis buffer described above. The samples were rehydrated at 50 V for 16 h at 20 °C using 11 cm long Immobilized pH Gradient (IPG) strips, pH 4 to 7, 7 to10, or 3 to10. Proteins were focused at 250 V for 15 min, and then 8000 V was maintained for a total of 60 000 Vh per gel.

The strips were then equilibrated for 10 min in 2.5 mL of a solution constituted of 375 mM Tris-HCl pH 8.8, 6 M urea, 2% SDS, and 2% dithiothreitol. After this first equilibration, the strips were equilibrated for another 10 min in 2.5 mL of a second equilibration buffer constituted of 375 mM Tris-HCl pH 8.8, 6 M urea, 2% SDS, and 2% iodoacetamide.

2.5.2. SDS−Polyacrylamide Gel Separation. The equilibrated IPG strips were washed with cathode buffer (0.1 M Tricine, 0.1 M Tris-HCl pH 8.2, and 0.1% SDS) and placed onto a 10% Tris-HCl Criterion gel (Bio-Rad, Hercules, CA). The anode buffer consisted of 0.2 M Tris-HCl pH 8.9. Gels were electrophoresed at 50 V for 30 min, then at 100 mA/gel until the end of the separation. Gels were silver stained for visualization of the proteins.

2.5.3. Silver Staining of Proteins. All the gels were silver stained according to the following protocol: Gels were fixed in 50% methanol (v/v) and 12% acetic acid (v/v) for 2 h, then washed 3 times in 50% ethanol (v/v). The duration of each wash was 20 min. Gels were then incubated in a 0.02% sodium thiosulfate solution (w/v) for 1 min, followed by four 1-min washes in water. Gels were then placed in a solution composed of 0.2% silver nitrate (w/v) and 0.075% (v/v) formaldehyde for a period of 20 min, followed by three 1-min washes in water. Gels were then developed in a 6% sodium carbonate (w/v), 0.005% formaldehyde (v/v), and 0.004% sodium thiosulfate (w/v) solution until the protein bands were visualized. An 1% acetic acid solution was added to stop the staining reactions.

2.6. Imaging Analysis. The numbers of individual spots on the gels were determined using ImageMaster 2D Platinum (Amersham Biosciences Corporation, Uppsala, Sweden). The parameters used were: Smooth 5, Min. Area 20, Saliency 1.0000.

3. Results

Figure 1 shows the pH gradient generated by titration of the anion-exchange resin with buffers A and B. A gradual increase in the concentration of buffer B resulted in a gradual decrease in the pH of the eluted solution. The same resin (PBE 94) and buffers were used for the batch chromatography prefractionation. By adjusting the pH of the solution containing the resin and the whole cell lysate to a value slightly below 7 using buffers A and B, all proteins with a p*I* higher than 7 will be net positively charged (in cationic form), and therefore free in the solution. Those with p*I* values lower than 7 will either precipitate (if their p*I* is equal to the pH of the solution) or bind to the resin (Figure 2A). Centrifugation then allows for the precipitation of the resin, and for the decantation of the supernatant containing proteins with p*I* values higher than 7. In the second step, by adjusting the pH of the solution containing the resin and the remaining proteins, using buffer B to achieve a pH slightly lower than 4, all proteins with p*I*'s between 4 and 7 will be net positively charged, and therefore free in the solution. Those with a p*I* lower than 4 will either precipitate (if their p*I* is equal to the pH of the solution) or bind to the resin (Figure 2B). Centrifugation then allows for the precipitation of the resin, and for the decantation of the supernatant containing proteins with p*I* values between 4 and 7.

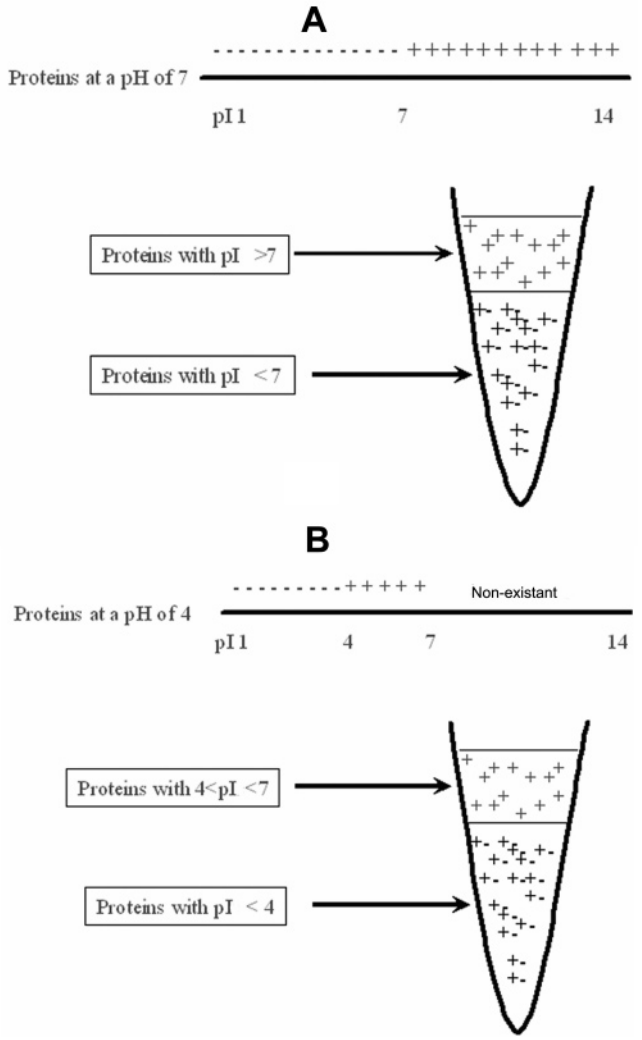

Figure 2. Protein prefractionation in a centrifuge tube: (A) at a pH slightly below 7, proteins with p*I*'s above 7 will be net positively charged and remain in the supernatant; those with p*I*'s below 7 will be net negatively charged and will therefore be bound to the resin; (B) at a pH slightly below 4, proteins with p*I*'s between 4 and 7 will be net positively charged and remain in the supernatant; those with a p*I* below 4 will be net negatively charged and will therefore be bound to the resin.

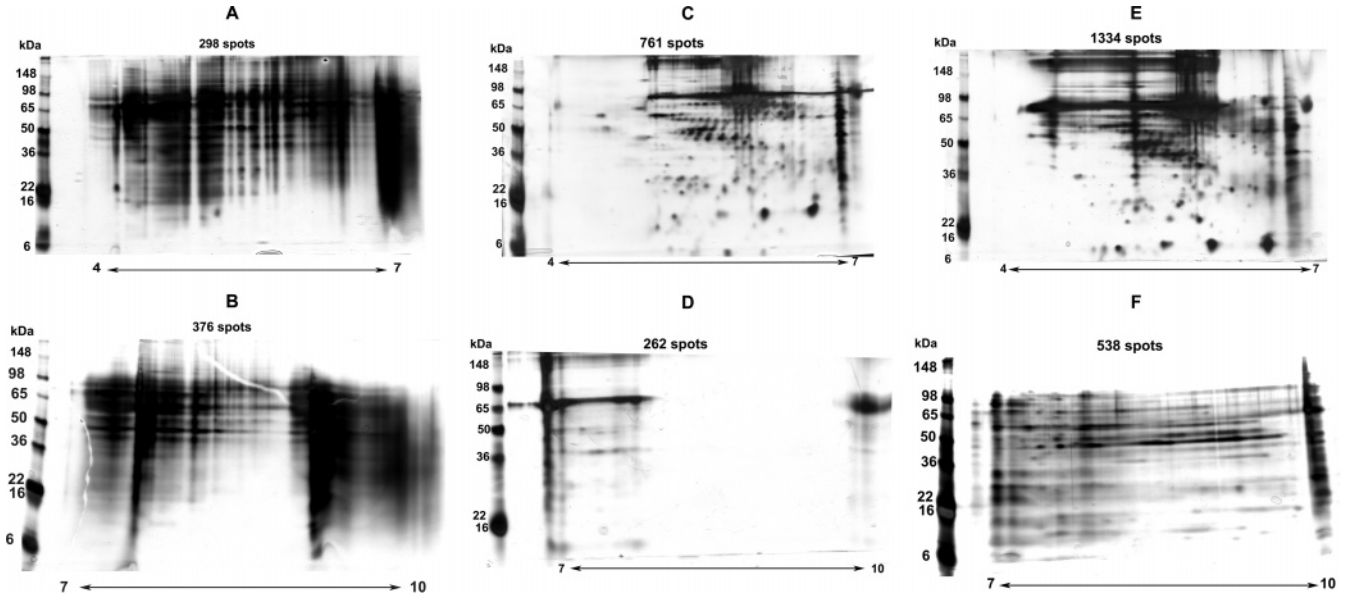

Figure 3. 2-DE of (A) 250 μg of whole LNCaP cell lysate using 11 cm, 4 to 7 pH range IPG strip in the first dimension; (B) 250 μg of whole LNCaP cell lysate using a 7 to 10 pH range IPG strip in the first dimension; (C) 100 μg of whole LNCaP cell lysate using 11 cm, 4 to 7 pH range IPG strip in the first dimension; (D) 100 μg of whole LNCaP cell lysate using a 7 to 10 pH range IPG strip in the first dimension; (E) 100 μg of a fraction of proteins with p*I* values between 4 and 7 after prefractionation of whole LNCaP cell lysate using an identical IPG strip in the first dimension; and (F) 100 μg of a fraction of proteins with p*I* values between 7 and 10 after prefractionation of whole LNCaP cell lysate using an identical IPG strip in the first dimension. A 10% polyacrylamide gel was used in the second dimension. All gels were silver stained.

After the adjustment of the resin pH to a value slightly below 7, an amount of 750 μg of total proteins in 330 μL LNCaP cell extracts were added to the resin and LNCaP cell lysates were prefractionated. Proteins with pIs above 7 were collected and a 100 μg (determined by the protein assay) were loaded onto a pH 7−10 IPG strip. The pH of the resin was then adjusted to a value slightly below 4 by washing the resin 4 times with 350 μL of buffer B, collecting the supernatant each time. These supernatants were then combined in one fraction and 100 μg of the proteins in that fraction (determined by the protein assay) were loaded onto a pH 4−7 IPG strip. 2-DE gels of the nonprefractionated LNCaP cell lysates using pH 4−7 or 7−10 IPG strips were then compared to those of the prefractionated protein extracts (Figure 3). The numbers of spots detected using Image Master 2D Platinum on the nonprefractionated pH 4−7 gel were 298 spots for a loading of 250 μg, 761 spots for a loading of 100 μg of total proteins, and 1334 spots for a loading of 100 μg of prefractionated proteins. As for the pH 7−10 gels, the numbers of spots detected were 376 spots for a loading of 250 μg, 262 spots for a loading of 100 μg of total proteins, and 538 spots for a loading of 100 μg of prefractionated proteins.

The prefractionated samples were then applied on wide pH 3−10 IPG strips to detect any cross-contamination between the fractions. The gels (Figure 4) clearly show that proteins having the p*I* range being prefractionated are enriched, and very little contamination is observed for the 4−7 fraction (Figure 4A). As for the 7−10 fraction, the gel shows some unresolved contamination (Figure 4B) primarily at the lower p*I* values, but the majority of proteins separated is in the 7−10 p*I* range.

After EGCG treatment, the number of spots detected on the nonprefractionated pH 4−7 gel is 811 spots versus 1458 spots for the prefractionated proteins. As for the pH 7−10 gel, the number of spots detected was 267 spots for the nonprefractionated sample versus 616 spots for the prefractionated proteins (Figure 5).

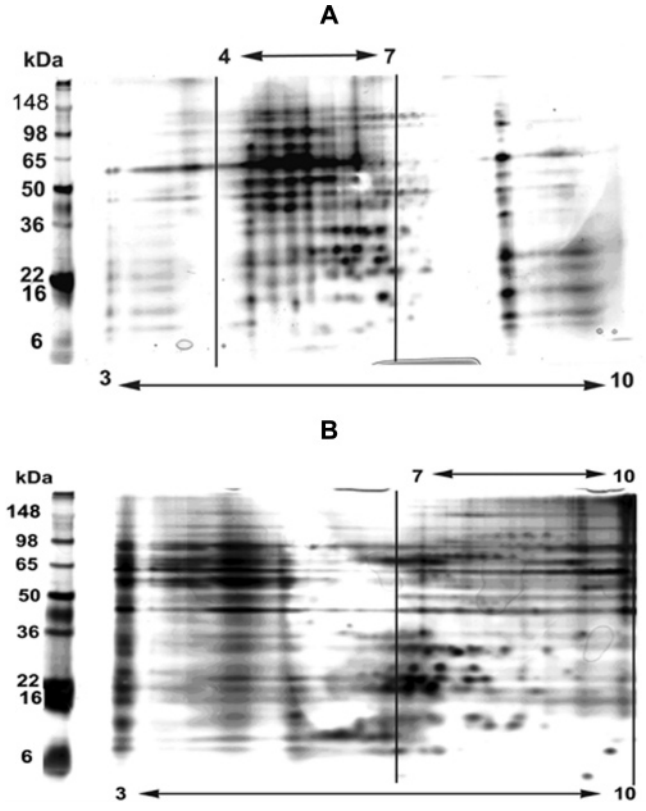

Figure 4. 2-DE of (A) 100 μg of a fraction of proteins with p*I* values between 4 and 7 after prefractionation of whole LNCaP cell lysate using a 3−10 IPG strip in the first dimension and (B) fraction of proteins with p*I* values above 7 after prefractionation of whole LNCaP cell lysate using a 3−10 IPG strip in the first dimension. A 10% polyacrylamide gel was used in the second dimension. Both gels were silver stained.

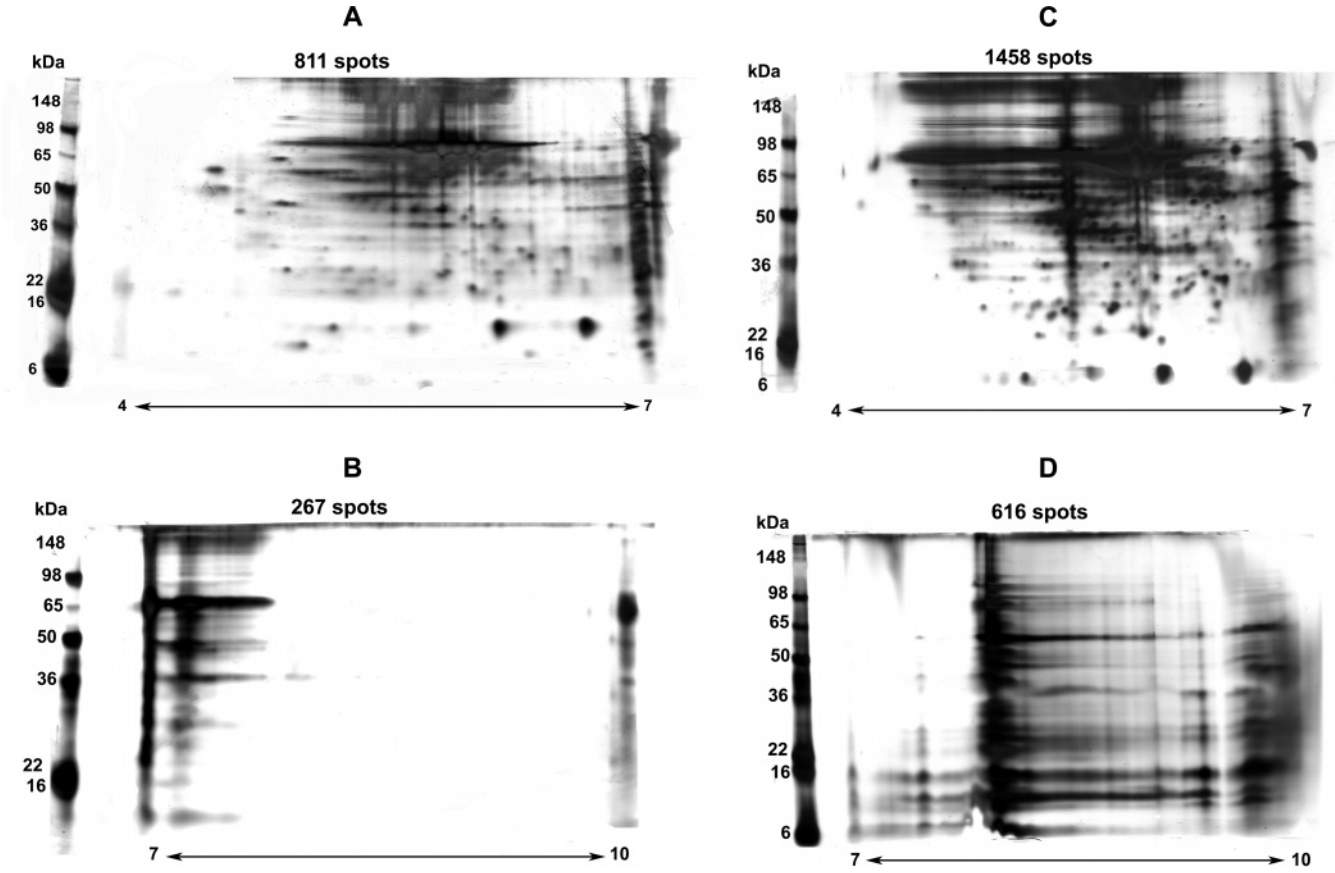

Figure 5. 2-DE of EGCG-treated LNCaP cell lysate (A) 100 μg of whole LNCaP cell lysate using 11 cm, 4 to 7 pH range IPG strip in the first dimension; (B) 100 μg of whole LNCaP cell lysate using a 7 to 10 pH range IPG strip in the first dimension; (C) 100 μg of a fraction of proteins with p*I* values between 4 and 7 after prefractionation of whole LNCaP cell lysate using an identical IPG strip in the first dimension. (D) 100 μg of a fraction of proteins with p*I* values between 7 and 10 after prefractionation of whole LNCaP cell lysate using an identical IPG strip in the first dimension. A 10% polyacrylamide gel was used in the second dimension. All gels were silver stained.

4. Discussion

PBE 94 resin functions as an anion exchanger. Net neutral and positively charged proteins will remain free in the solution, and only net negatively charged (anionic form) proteins will bind to this resin. Proteins are amphoteric molecules that can carry positive, negative, or zero net charge depending on the pH of their local environment. For every protein there is a specific pH at which its net charge is zero, defined as its p*I*.[17] If the pH of the solution is higher than the p*I* of a protein, this protein will be net negatively charged, and thus, bound to the resin. Conversely, if the pH of the solution is lower than the p*I* of a protein, this protein will be net positively charged and will remain free in solution.

On the basis of the pH gradient generated from the titration of the anion-exchange resin with buffers A and B (Figure 1), we performed the batch prefractionation in a centrifuge tube by washing the resin three times by a solution constituted of 50% buffer A and 50% buffer B before adding the cell lysate. Vortex mixing allows the proteins and the resin to disperse into the solution. Proteins having p*I* values below 7 will bind to the resin and those having p*I* values above 7 will remain free in solution and will be collected after centrifugation (Figure 2A). The isolation of proteins with p*I* values between 4 and 7 is carried out using similar rationale, as the pH of the solution was adjusted to a value lower than 4 by washing the resin 4 times with 350 μL of buffer B. Proteins with p*I* values between 4 and 7 were net positively charged, and thus free in the solution (Figure 2B). Precipitation and decantation allowed for the isolation of the solution containing these proteins.

2-DE obtained after loading 100 μg of prefractionated sample resulted in a 1.75-fold increase in the number of spots detected when compared to 2-DE obtained after loading 100 μg of total cell lysate (Figure 3C,E). In a similar fashion, 2-DE of 100 μg of proteins resulting from the use of pH 7 to 10 IPG strips was compared to the 2-DE of 100 μg of prefractionated proteins with p*I* values between 7 and 10 using an IPG strip with the same pH range. The increase in the number of spots detected was 2-fold (Figure 3D,F). In the nonprefractionated samples, many of the proteins loaded had pI values that did not fall within the narrow pH range of the IPG strip. This is particularly true when using 7−10 IPG strips as basic proteins are less abundant than acidic ones. Fractions isolated from LNCaP cell lysates containing proteins with p*I* values that fell primarily within the pH range of the IPG strip allowed for targeted loading, and as a result, better visualization of the less abundant proteins.

Overloading IPG strips with 250 μg of proteins resulted in poor focusing and as a result fewer proteins were detected (Figure 3A,B). Furthermore, loading the acidic fraction on wide pH 3−10 IPG strips revealed minimal cross-contamination (Figure 4A). Loading the basic fraction on wide 3−10 IPG strips revealed some contamination by acidic proteins (Figure 4B). This is mainly due to the fact that in any total cell lysate, basic proteins are less abundant than acidic ones.

In a similar fashion, prefractionation was performed on cell lysates of EGCG treated LNCaP cells. The prefractionation resulted in a 1.8-fold and 2.3-fold increase in the number of spots detected respectively for the acidic and basic proteome (Figure 5). Furthermore, comparing the EGCG-treated to nontreated samples, the differential number of spots detected after EGCG treatment increased from 55 spots for nonprefractionated samples to 202 spots for prefractionated ones, a 3.7-fold increase.

Prefractionation of samples based upon protein p*I* values prior to 2-DE helped us to overcome one of the primary limitations of 2-DE, as it allows for targeted protein loading, and results in improved resolution and visualization of the low-abundance proteins. When loading a prefractionated sample into an IPG strip, many of the proteins being focused during the isoelectric focusing (IEF) step are proteins with p*I* values consistent with the range of the IPG strip. When loading a crude biological sample, however, proteins with p*I* values higher or lower than the pH range of the IPG strip are usually dragged to the outermost extremes of the strip, and when strips with a narrow pH gradient are used, many of the proteins will be dragged to either end of the strip.

The prefractionation of crude biological samples prior to 2-DE, aiming to increase the resolution and the visualization of the proteins on the gel, is possible in a centrifuge tube using 1 mL of anion-exchange resin and two expressly designed buffers. Our prefractionation method is particularly advantageous, as it is carried out using common instrumentation, no electrophoretic force, and is performed in fewer than 30 min. In addition, the protein extract contains chaotropic agents (urea and thiourea) that make isoelectric prefractionation more efficient. As our prefractionation technique uses no voltage, no heating will occur. Therefore, carbamylation of proteins, usually caused by the combination of heat and urea, will not occur with this method. Furthermore, the prefractionation is carried out based on the p*I* values of the proteins, which is compatible to the first dimension of separation by 2-DE, so proteins of a certain p*I* can be targeted to match the p*I* range of the IPG strip. Our method prefractionates a protein sample into two fractions: one containing the acidic proteins with p*I* values between 4 and 7 that will be loaded on an acidic IPG strip (pH 4 to 7) and one containing a basic fraction with p*I* values above 7 that will be loaded on a basic IPG strip (pH 7 to 10), providing a perfect compatibility between the fractions obtained and the first dimension of the 2-DE separation.

The advantages of our prefractionation technique are as follows: first, 3.7-fold increase in the detection of differentially expressed proteins; second, up to 2.2-fold increase in the number of proteins detected; third, the prefractionation is carried out in 30 min; fourth, common instrumentation is used; fifth, carbamylation of the proteins is not induced; and finally, protein fractions obtained from the prefractionation are entirely compatible to the IPG strips of the isoelectric focusing gel. With the examples and results presented, we have demonstrated the simplicity, cost-effectiveness, and rapidity of our novel p*I*-based prefractionation procedure. This prefractionation method may be widely used for analyzing 10 or more samples efficiently for functional and quantitative proteomic comparisons of multiple systems such as subcellular, cellular, tissue, organ, and whole organism levels.

Abbreviations: 2-DE, Two-dimensional gel electrophoresis; DTT, Dithiothreitol; EGCG, epigallocatechin-3-gallate; IPG, immobilized pH gradient; MALDI, matrix-assisted laser desorption ionization; M_r, molecular mass; OG, octylglucopyranoside; PBE 94, polybuffer exchanger 94; p*I*, isoelectric point.

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Matrix Metalloproteinase Inhibitors as Prospective Agents for the Prevention and Treatment of Cardiovascular and Neoplastic Diseases

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Abstract: Acting on a broad spectrum of extracellular, intracellular, and membrane-associated substrates, the matrix metalloproteinases (MMPs) are critical to the biological processes of organisms; when aberrantly expressed, many pathological conditions may be born or exacerbated. The prospect of MMP inhibition for therapeutic benefit in cancer, cardiovascular disease, and stroke is reviewed here. MMP inhibitor (MMPI) development constitutes an important branch of research in both academic and industrial settings and advances our knowledge on the structure-function relationship of MMPs. Targeting MMPs in disease treatment is complicated by the fact that MMPs are indispensable for normal development and physiology and by their multi-functionality, possible functional redundancy or contradiction, and context-dependent expression and activity. This complexity was revealed by previous efforts to inhibit MMP activity in the treatment of cancer patients that yielded unsatisfactory results. This review focuses on MMPI development since the late 90s, in terms of natural products and their derivatives, and synthetic compounds of low molecular mass incorporating specific zinc-binding groups (ZBGs). A few polyphenols and flavonoids that exhibit MMPI activities may have chemopreventive and neuro- and cardiovascular-protective effects. A new generation of potent and selective MMPIs with novel ZBGs and inhibition mechanisms have been designed, synthesized, and tested. Although only one collagenase inhibitor (Periostat, doxycycline hyclate) has been approved by the Food and Drug Administration as a drug for the treatment of periodontal disease, new hope is emerging in the form of natural and synthetic MMPIs for the prevention and treatment of stroke, cardiovascular disease, cancer, and other medical conditions.

INTRODUCTION

In the arena of basic sciences, numerous studies have confirmed a myriad of biological activities for the matrix metalloproteinases (MMPs), suggesting that this family of proteolytic enzymes may serve as key regulators for a plethora of functions, both physiological and pathological. A full understanding of their roles is an ongoing pursuit, as is their inhibition and the effects of their restraint, in terms of broad-spectrum inhibition, or through the specific inhibition of select MMPs. Owing to the complexity of their contributions to normal physiological processes, broad-spectrum inhibition of the MMPs has led to unintended side effects during clinical trials. Therefore, the development of MMP inhibitors (MMPIs) with increased specificity for unique MMP targets has been the focus of much recent research at the industrial level and in academia. The challenge is formidable, as are the potential benefits.

Here, following a brief discussion of MMP contributions to the progression of cancer and cardiovascular diseases inclusive of stroke, we address two divergent avenues of research working towards the common goal of MMP inhibition with enhanced specificity. We first explore new advances in the derivation of beneficial and potentially therapeutic MMPIs from natural sources, and then review current achievements and trends in the development of synthetic small molecule MMPIs.

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MMPS IN CANCER

Many studies incorporating human specimens have implicated matrix metalloproteinases in multiple types of cancer, including those of the lung, breast, skin, and colon, among many others [1]. The expression and activity of MMPs are elevated in and around human tumors, and are often associated with high tumor grades and stages. Moreover, excessive MMP expression has been correlated with cancer susceptibility and mortality, implicating MMPs in both early and late stages of the evolving disease.

In vivo experiments confirm that MMPs are potent players in the genesis and progression of cancer [2]. Mice that are engineered to overexpress MMP-1, -3, -7, or -14 develop hyperplastic lesions and, in some cases, malignant tumors [3-7]. MMP-3 or MMP-14 overexpression targeted to the mammary glands of mice leads to mammary hyperplasias and spontaneous adenocarcinomas [4, 6, 7]. Furthermore, tumor development is accelerated in MMP-overexpressing mice when they carry another oncogenic anomaly or are challenged with carcinogenic agents [3, 5, 8], while mice null for MMP-2, -7, -9, or -11 are resistant to cancer incidence triggered by a variety of oncogenic factors [9-13], with a surprising finding that the carcinomas that did arise in MMP-9 knockout mice appeared to be of a higher grade [10].

Studies have also shown that MMPs continue to contribute to tumorigenesis after the onset of tumor formation, and are key participants in processes such as invasion, angiogenesis, and metastasis. Malignant cell lines

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rely heavily on the activity of MMPs to traverse the basement membrane and extracellular milieu, with cell lines sometimes differing in the specific MMPs employed to accomplish this [14-23]. MMP-9, in particular, seems to be critical for intravasation *in vivo* [24]. MMP-2 and MMP-9 are also important for the formation of new blood vessels in animal models of tumor-associated angiogenesis [9, 11, 15, 25], and MMP-9 has been shown to foster tumor growth at metastatic sites [26]. In keeping with these observations, MMP inhibitors have been found to hinder tumor development in various animal models of cancer, suppressing tumor growth, invasion, angiogenesis, and metastasis [27-30].

The variety of roles that MMPs play in cancer development can be explained, at least in part, by the broad scope of substrates digested by members of the MMP family. In general, proteolytic activity by the MMPs leads to the destruction of physical barriers, the liberation of growth factors, and the generation of bioactive molecules, allowing cells to flourish in a flexible microenvironment enriched with nourishing factors. Numerous proteins have been shown to be substrates of the MMPs *in vitro*, including virtually every structural component of the extracellular matrix, secreted growth factors, and many membrane-associated proteins [2]. Several of these substrates are solidly established as factors important in tumorigenesis, and experiments utilizing a variety of cell lines have offered mechanistic insight into how MMPs promote key features of neoplastic progression.

Compromise of the rigid extracellular matrix and a concomitant adaptation of cells to their new microenvironment are necessary for malignant cells to grow and disseminate. MMPs are largely responsible for degrading components of the extracellular matrix and cell surface to accommodate cellular functions. For example, membrane-type 1 matrix metalloproteinase (MT1-MMP, MMP-14) cleavage of extracellular proteins, such as laminin-5 and collagen, is critical for cell growth and migration [31, 32], and MMP-14, -15, and -16 are utilized for cell migration through fibrin [17]. MMPs also assist in cellular migration through the cleavage of cell adhesion molecules. MMP-14 has been shown to cleave the adhesion molecules syndecan-1 and CD44 to stimulate cell movement [33, 34]. MMP-2 and MMP-9 can cleave galectin-3, a protein that serves in adhesion and the mediation of other cellular processes [35]. MMP-3 and MMP-7 have both been reported to cleave E-cadherin, a transmembrane protein crucial for the establishment of cell-cell contacts among epithelial cells [20, 36]. Disruption of these E-cadherin junctions by down-regulation of E-cadherin gene expression or proteolytic cleavage of this protein, either alone or in tandem with additional mechanisms leading to E-cadherin gene and protein inactivation, appears to be a prerequisite for the spread of carcinoma cells [20, 36].

Cleavage products of MMP proteolysis may then feed back to cells, influencing cellular activity. For instance, it was found that the E-cadherin fragment generated by MMP-3 and MMP-7 was necessary to promote cell invasion [36], while MT1-MMP and MMP-2 cleavage of laminin-5 produces a fragment that induces cell migration [32, 37]. A cryptic site in collagen IV, exposed upon proteolysis and required for angiogenesis and tumor growth *in vivo*, is presumably revealed by MMP activity [38].

MMP proteolytic activity also exerts significant impact on secreted and membrane-associated growth factors and their signaling pathways. The extracellular matrix is a rich storehouse of growth factors, and disrupting this highly structured network results in increased bioavailability of many different growth factors. For example, MMP-2, -3, or -7 cleavage of the proteoglycan decorin may release transforming growth factor-beta (TGF-β) [39]. MMPs may directly activate growth factors, as well. Several MMPs can activate tumor necrosis factor-α (TNF-α), a potent cytokine that has strong implications in tumorigenesis [40]. MMP-2, -9, -13, and -14 may activate TGF-β to promote growth, survival, and angiogenesis [41-43]. MMPs may also indirectly activate growth factors through the inactivation of negative regulators. For example, MMP-1, -2, and -3 all may cleave insulin-like growth factor (IGF) binding protein-3, which acts as a key modulator of IGF signaling [44]. Finally, MMP activity may alter other signaling proteins. MMP-7 cleaves the pro-apoptotic protein Fas ligand, thus promoting cellular survival [45, 46]. MMP-14 activates a tyrosine kinase pathway that leads to the upregulation of vascular endothelial growth factor (VEGF), which in turn stimulates cell growth and angiogenesis [47, 48].

Despite the volumes of experimental evidence validating MMPs as therapeutic targets for the treatment of cancer, several studies have actually exposed a dual nature for some MMPs, demonstrating that MMPs may also exert anti-tumorigenic effects. For instance, male MMP-8-deficient mice are more susceptible to skin tumors [49]. MMP-9 cleavage of basement membrane collagen generates an anti-angiogenic fragment [50], and MMP-2, -7, -9, and -12 may generate the angiogenic inhibitor angiostatin [51-53]. For example, in integrin alpha1-deficient mice, in which the synthesis of MMP-7 and MMP-9 is markedly increased, a concomitant increase in circulating plasma levels of angiostatin results in the decreased vascularization of implanted tumors, suggesting that MMP inhibition might actually result in increased tumor angiogenesis [54]. In another study incorporating an orthotopic model of non-small cell lung cancer (NSCLC) in integrin alpha1-null mice, a genetic model for increased MMP-9, pharmacological inhibition of MMPs at the time of tumor cell injection resulted in an increase in the number of both primary and metastatic lung cancers, suggesting that primary growth and metastases of NSCLC are worsened by the early inhibition of MMPs [55]. Indeed, pro-tumorigenic effects were observed in some previous clinical trials that utilized MMP inhibitors for the treatment of human cancers [1, 27], however, the mechanisms of action of those MMP inhibitors in human patients are still unclarified. Consequently, there are no clear and direct answers on what types of MMP inhibitors with which functional groups should be used, which MMPs should be targeted for which subset of human tumors or which sub-group of patients, and at what particular stage of tumor progression those tumors should best be targeted. Much more basic science, preclinic, and clinic research are crucial to provide answers to these questions to

achieve "customized medicine" to meet individual patient needs in the future.

Extensive data have been gathered since the first MMP inhibitor clinical trials, shedding new light on the complexity of the MMP family. Today, it is known that there are a multitude of MMPs, each with potent capabilities, and that MMPs participate in all stages of tumorigenesis in varying, sometimes even paradoxical, ways. Thus, new therapeutic agents targeting specific subgroups of MMPs, while sparing the remainder, are now being explored so that medicines specific to individual genotypes and other conditions may be offered. Such controlled and individualized treatment is critical for a disease state as heterogeneous as cancer.

MMPS IN CARDIOVASCULAR DISEASE AND STROKE

Cardiovascular diseases are now the leading causes of death in Western society, at a rate that exceeds even that of cancer, and as with cancer, MMPs have been implicated in multiple cardiovascular and related neurological pathologies. Circulating levels of MMPs are elevated in patients with acute myocardial infarction and unstable angina. Enhanced MMP expression has also been detected in atherosclerotic plaques, and activation of MMPs appears to be involved in the vulnerability of the plaque to rupture. Increased MMP expression is also observed after coronary angioplasty, with or without stent placement. In addition, MMPs are likely to play important roles during the repair phases of cerebral ischemia in terms of their contributions to angiogenesis and the reestablishment of cerebral blood flow. All of these findings combine to suggest that MMP inhibitors are likely to be useful in the development of pharmacological approaches to reduce mortality rates associated with cardiovascular diseases [56] and neurodegenerative disorders [57].

In a murine model of cardiac infarction, utilizing fluorescent probes activated upon proteolytic cleavage by MMP-2 and MMP-9, the *in vivo* upregulation of these enzymes was demonstrated, with maximal expression at 1 to 2 weeks, persisting to 4 weeks following induction of the infarct [58]. Increased levels of MMP-3 in circulating plasma are an independent predictor of adverse events in patients with coronary artery disease, suggesting a potential role for MMP-3 in the risk stratification and clinical management of these patients [59]. MMP inhibitors may be useful in the treatment of degenerative aortic disease associated with thinning of the medial aortic wall, as increased levels of proteolytic activity attributed to MMPs have been identified in patients with aortic aneurisms and aortic stenosis [60]. In addition to these effects, the administration of synthetic MMP inhibitors in experimental animal models of cardiovascular disease significantly inhibits the progression of atherosclerotic lesion formation, neointima formation, left ventricular remodeling, pump dysfunction, and infarct healing, indicating that MMP inhibitors might serve as potential therapeutic agents for the prevention and treatment of heart failure [61]. Early MMP inhibition after myocardial infarction yields a preservation of left ventricle structure, supporting the concept that preserving the original extracellular matrix (ECM) early after coronary occlusion lessens ventricular remodeling. Preserving the original ECM of infarcted left ventricles by use of early short-term doxycycline (DOX) treatment protects cardiac structure and function [62], although recent studies in an *in vitro* rat model of myocardial ischemia suggest that the benefits of DOX treatment might also be derived through the inhibition of plasmin [63].

Atherosclerotic plaque rupture, in which the fibrous cap overlying an atheroma undergoes catastrophic mechanical breakdown, is the most prevalent cause of premature death in advanced societies, and increased levels of MMP expression or activation are likely to contribute to pathological matrix destruction and plaque rupture [64]. Significantly increased concentrations of the active form of MMP-8 have been detected in atherosclerotic plaques obtained from human patients, suggesting that active MMP-8 may contribute to the degradation of the collagen cap of atherosclerotic plaques [65]. Additional studies report a high expression of active MMP-7 in human atherosclerotic plaques, indicating a potential role for this enzyme in the weakening of fibrous caps, predisposing them to rupture [66]. In addition, MMP-9 levels were found to be slightly increased with advancing age in plaques obtained from patients suffering from advanced atherosclerotic lesions [67]. MMP-14, with a broad substrate specificity and the ability to activate other matrix metalloproteinases, has also been detected at high levels in atherosclerotic plaques [68].

MMP expression may be related to the formation of restenotic lesions following therapeutic interventions for stenosed arteries, such as percutaneous transluminal coronary angioplasty, in the presence or absence of stent placement. In a murine carotid artery model of blood flow cessation, MMP-2 was shown to contribute to neointimal hyperplasia through degradation of ECM proteins and the internal elastic lamina, facilitating the migration of medial smooth muscle cells from the arterial media to the intima [69]. In another study of patients undergoing angiography procedures, high serum levels of MMP-1 were found to be associated with the presence of complex coronary lesions [70]. For the treatment of vascular injury secondary to such procedures, water-soluble MMP inhibitors have been designed and developed as intra-arterial infusion drugs for vascular injury [71], and MMP inhibitors have also been considered for stent coatings [72], facilitating the timely release of MMP inhibitors in a site-directed fashion. The implantation of stents results in a more vigorous ECM and MMP response than balloon angioplasty alone, and when compared to a placebo, a potent and broad-spectrum MMP inhibitor, GM6001, significantly inhibited intimal hyperplasia and intimal collagen content in a rabbit model of neointimal hyperplasia, increasing lumen area in stented arteries without effecting proliferation rates, affording a novel approach to the prevention of in-stent restenoses [73]. However, in a study to determine if the nonspecific MMP inhibitor batimastat could inhibit neointimal thickening following stent placement in a porcine atherosclerotic model, no significant influence was shown at 6 weeks following stenting [74], and broad-spectrum MMP inhibition also failed to prevent intimal hyperplasia following angioplasty and stenting in an atherosclerotic primate model [75].

Such conflicting reports, arising from the use of different inhibitors in differing animal models with a variety of

endpoints, has proven to be a source of great complexity to this avenue of study, and provides a continuing challenge to investigators. Further complicating the issue is evidence for protective roles exerted by the MMPs. MMP-2 gene ablation was found to reduce survival rates and exacerbate cardiac failure in association with increased levels of myocardial inflammation in mice, suggesting a cardioprotective role for this MMP in the pathogenesis of cytokine-induced cardiomyopathy [76]. Nevertheless, the development of therapeutic drugs specifically targeting MMPs shows continued promise, provided that specific targets might be identified, then targeted with MMP inhibitors that exhibit enhanced specificity for these unique targets. The development of natural or synthetic pharmaceuticals inhibiting specific MMP targets might ultimately prove quite beneficial in the prevention of atherosclerotic lesion development, plaque rupture, and restenosis [77], either in the form of stand-alone therapies, or in combination with other therapeutic agents as part of a multi-pronged approach.

MMPs have been linked to neuronal cell death that occurs during the course of many neurodegenerative disorders, including stroke. The aberrant upregulation of MMPs, particularly of MMP-9, has been linked to both neuron apoptosis and brain damage [57], although MMP-13 has also been implicated as playing an early role in brain infarct volumes in patients suffering from middle cerebral artery occlusions [78]. In a murine model of transient focal cerebral ischemia, MMP-9 was found to degrade the ECM protein laminin, inducing neuronal apoptosis, and furthermore, the inhibition of MMP-9 activity rescued neurons from apoptosis [79]. It has also been demonstrated, utilizing MMP inhibitors and mice null for MMP-9, that the gelatinolytic activity of MMP-9 contributes to disruption of the blood-brain barrier in a variety of disorders arising from neurovascular dysregulation including stroke, head trauma, and migraine [80]. Preservation of the blood-brain barrier utilizing MMP inhibition has also been shown in a rat model of middle cerebral artery occlusion [81]. In a murine model of permanent middle cerebral artery occlusion inhibition of MMP-9 resulted in a reduction of infarct size when treatment was started prior to the insult, and this protection was not afforded to MMP-9 knockout mice, where enhanced expression of the proform of MMP-2 was observed [82]. It is interesting to note that leukocytes, most likely neutrophils, have been implicated as a key cellular source of MMP-9 following neuronal injury, promoting leukocyte recruitment and blood-brain barrier breakdown secondary to microvascular basal lamina proteolysis [83].

NATURAL MMP INHIBITORS

Perhaps the most thoroughly studied class of natural MMP inhibitors are the endogenous tissue inhibitors of metalloproteinases (TIMPs), of which four are currently known, designated as TIMP-1 through -4. It is assumed that the natural ratio of MMPs to TIMPs is tightly regulated, and a disruption in the natural balance between these two families is often associated with the progression of multiple disease states. Each of the four TIMPs forms a complex with the MMPs in a 1:1 stoichiometry, exhibiting high affinity, but varying degrees of selectivity. This family of inhibitors has been extensively reviewed in multiple venues [84-86].

The primary focus of this section will be the numerous attempts that have been made to find, or develop, matrix metalloproteinase inhibitors (MMPIs) that may be derived from natural resources such as herbs, plants, fruits, and other agriculture products. New and potentially beneficial compounds isolated from these sources have been shown to exhibit some degree of MMPI activities, but they are far less potent and specific than the TIMP family. These natural compounds include long chain fatty acids, epigallocatechin gallate (EGCG) and other polyphenols, flavonoids, and a variety of other natural compounds (Fig. (**1**)).

Long chain fatty acids

Long-chain fatty acids (*e.g.* oleic acid, elaidic acid, and *cis*- and *trans*-parinaric acids) inhibit MMP-2 and MMP-9 with K_i values in the micromolar range [87], although they demonstrated weak inhibitory effects against collagenase-1 (MMP-1). The fatty acid chain length and its degree of saturation is related to the level of inhibition, as C18 fatty acid showed stronger inhibition than C16, C14, and C10, and the degree of nonsaturation was shown to correspond to and enhance the overall inhibitory capacity of the chains [87]. Experiments with human skin tissue sections revealed that micromolar concentrations of elaidic acid, a long-chain unsaturated fatty acid, protected collagen and elastin fibers against degradation by gelatinases A (MMP-2) and B (MMP-9), respectively [87]. The fibronectin (FN)-like domain of MMP-2 is apparently critical for this inhibition, as a deletion of FN-II modules markedly decreased the inhibitory capacity of oleic acid toward MMP-2, suggesting an interaction between long-chain fatty acids and the fibronectin-like domain of this protease [87].

Long-chain unsaturated fatty acids have also been reported to inhibit both the expression and activity of a member of a different metalloproteinase family, aggrecanases [87, 88]. Oleic acid, an 18-carbon fatty acid with one double carbon bond in the *cis* position, inhibited hydrolysis of a fluorogenic peptide substrate by MMP-2 in a dose-dependent manner [87, 89, 90]. Oleic acid partially inhibits the formation of lung metastases following subcutaneous implantation of colon carcinoma cells in athymic mice [87, 91]. Fatty acids also bind to serine proteinases, such as neutrophil elastase [92, 93] and plasmin [94, 95], and modulate their catalytic activities.

Fujita *et al*. reported Callysponginol Sulfate A, **1**, a new sulfated C_{24} acetylenic fatty acid isolated from the Marine Sponge Callyspongia truncate, as a MT1-MMP inhibitor [96]. This compound inhibited recombinant MT1-MMP with an IC_{50} of 15.0 μg/mL, although the desulfated form did not show any inhibitory activities.

Epigallocatechin Gallate (EGCG)

Epigallocatechin gallate (EGCG), **2**, a polyphenol catechin isolated from green tea, has drawn great attention over the past few years for its therapeutic potential in numerous diseases. EGCG can act as a potent inhibitor of matrix metalloproteinases [97, 98], targeting multiple MMP-mediated cellular events in cancer cells, thereby providing a new mechanism for the anticancer properties of this molecule [97]. Other polyphenol catechins have also shown

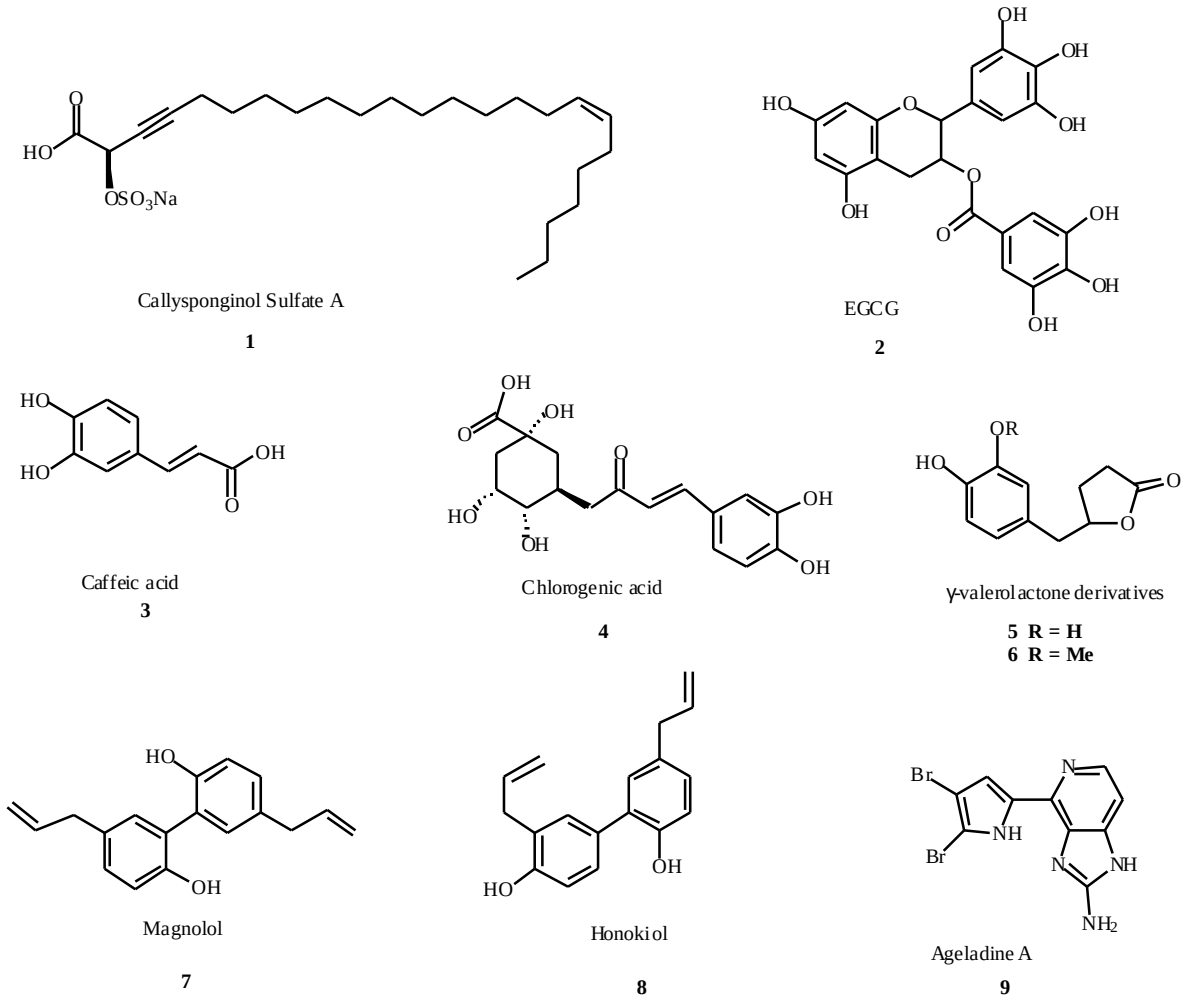


Fig. (1). Natural MMP Inhibitors.

various degrees of MMP inhibition, although their inhibitory effects are generally less potent than that of EGCG.

MT1-MMP, which degrades type I collagen and activates MMP-2, plays a significant role in angiogenesis, as well as in tumor cell invasion and metastasis [99]. EGCG has a potent and distinct inhibitory activity against MT1-MMP [99]. In U-87 glioblastoma cells, the addition of EGCG strongly inhibited MT1-MMP-dependent proMMP-2 activation [97]. The inhibitory effects of EGCG on MT1-MMP were also demonstrated by the down-regulation of MT1-MMP transcript levels, and by the inhibition of cell migration by MT1-MMP transfected COS-7 cells, suggesting that this catechin may act at both the gene expression and protein activity levels [97]. Treatment of cells with non-cytotoxic doses of EGCG significantly reduced the amount of secreted proMMP-2, with a concomitant increase in intracellular levels of this protein, suggesting that EGCG might also act upon intracellular secretory pathways [97].

EGCG repressed the invasion of lung carcinoma 95-D cells in invasion assays [100], and reverse transcriptase-polymerase chain reaction (RT-PCR) analyses showed that EGCG down-regulated the expression of MMP-9 [100]. Also in EGCG-treated 95-D cells, the levels of nuclear factor-κB (NF-κB) localized in the nucleus were diminished in a dose-dependent manner, while feeding the cells with EGCG resulted in a decrease of intracellular oxidants [100]. The inhibition of tumor invasion by EGCG was therefore attributed to decreased expression of MMP-9 and NF-κB [100].

In human prostate carcinoma DU145 cells, the inhibition of MMP-2 and MMP-9 by EGCG is mediated *via* inhibition of phosphorylation of ERK1/2 and p38, and inhibition of activation of the transcription factors c-jun and NF-κB [101]. EGCG may play a role in the prevention of invasive metastatic processes of both androgen-dependent and androgen-independent prostate carcinomas [101]. EGCG forms a reversible complex with MMP-2, resulting in inhibition of its gelatinolytic activity [102]. EGCG had no effect on MMP-2 binding to immobilized native and denatured type I collagen, but significantly enhanced pro- and activated MMP-2 binding to TIMP-2 [102]. However, the molecular mechanisms by which EGCG blocks these gelatinolytic activities remain unknown [102].

EGCG inhibited the interleukin-1-induced mRNA and protein expression of MMP-1 and MMP-13 in human chondrocytes [103]. Importantly, EGCG showed a differential, dose-dependent inhibitory effect on the expression and activity of MMP-13 and MMP-1, indicating that low doses of EGCG have a marked selective inhibitory effect on the activity of MMP-13 as compared to MMP-1 [103]. A similar differential, dose-dependent inhibition of the transcription factors NF-κB and AP-1 by EGCG was also noted [103]. Jun protein is also related to the expression of MMP-1, and EGCG decreased the transcription activity of Jun in fibroblasts following irradiation with ultraviolet A (UVA) [104]. Both the mRNA and protein levels of MMP-1 were increased by UVA irradiation, while no significant changes were observed in TIMP-1 levels, so the ratio of MMP-1 to TIMP-1 showed statistically significant differen-ces when compared with cells not exposed to radiation damage [104]. EGCG decreased the ratio of MMP-1 to TIMP-1 by inhibiting UVA-induced MMP-1 expression, so it is presumed that EGCG can protect human fibroblasts against UVA damage by down regulating the transcription activity of the Jun protein and the subsequent expression of MMP-1 [104].

It is believed the anticancer and apoptosis-inducing properties of green tea are mediated by its polyphenolic constituents, particularly the catechins [105]. A number of reports have shown that the green tea polyphenol EGCG is among the most effective chemopreventive and apoptosis-inducing agents [105], and EGCG has also been shown to inhibit the growth of various cancer cell lines [106].

In cell-culture systems employing the human prostate cancer cell lines DU145 (androgen insensitive) and LNCaP (androgen sensitive), EGCG was found to induce apoptosis, cell-growth inhibition, and cyclin kinase inhibitor WAF-1/p21-mediated cell-cycle dysregulation [107]. A cDNA microarray analysis followed by EGCG treatment of LNCaP cells revealed the induction of genes that functionally exhibit growth-inhibitory effects, and the repression of genes belonging to the G-protein signaling network [107]. In animal studies employing a transgenic adenocarcinoma of the mouse prostate (TRAMP), a model that mimics progressive forms of human prostatic disease, oral infusion of a polyphenolic fraction isolated from green tea at a human-achievable dose (equivalent to 6 cups of green tea/day) was found to significantly inhibit prostate cancer development and metastasis [107]. The oral administration of this polyphenolic fraction resulted in significant inhibition of VEGF, MMP-2, and MMP-9 [107]. These data suggest multiple targets for prostate cancer chemoprevention by green tea, or more broadly, by its polyphenolic constituents [107].

EGCG inhibits ovarian cancer cell growth through the induction of apoptosis, cell cycle arrest, and the regulation of cell cycle-related proteins [108]. EGCG also prevents the carcinogenesis of cervical cancer through the induction of apoptosis and the inhibition of telomerase activity [109]. Thus, biological activities of EGCG are not limited to MMP inhibition and EGCG may have other functions independent of MMP inhibition.

EGCG treatment was found to result in a dose-dependent decrease in the viability and growth of two human melanoma cell lines, A-375 (amelanotic malignant melanoma) and Hs-294T (metastatic melanoma), while normal human melanocytes were not affected [110]. EGCG treatment of these melanoma cell lines resulted in decreased cell proliferation and the induction of apoptosis [110]. EGCG also significantly inhibited the colony formation ability of the melanoma cells studied, with a significant induction of cell cycle arrest and apoptosis [110].

EGCG also exerts neuroprotective effects, inhibiting lipopolysaccharide-induced microglial activation and protecting against inflammation-mediated dopaminergic neuronal injury [111]. Microglial activation is believed to play a pivotal role in the selective neuronal injury associated with several neurodegenerative disorders, including Parkinson's disease and Alzheimer's disease [111]. EGCG is a potent inhibitor of microglial activation, and therefore might serve as a useful candidate for therapeutic approaches seeking to alleviate microglia-mediated dopaminergic neuronal injury in Parkinson's disease [111].

Nitric oxide (NO) plays an important role in the neuropathogenesis induced by brain ischemia/reperfusion and hypoxia, but NADPH-d/nNOS expression was significantly depressed in hypoxic rats treated with high dosages of EGCG (25 or 50 mg/kg) [112], suggesting that EGCG may attenuate oxidative stress following acute hypoxia [112], and thus, may have future possibilities as a neuroprotective agent against excitotoxicity-related neurologic disorders such as brain ischemia [113]. EGCG is now being considered as a therapeutic agent in epidemiological studies aimed to alter brain aging processes, and as a possible neuroprotective agent in progressive neurodegenerative diseases [114].

EGCG may also help to protect renal function. Nephrotoxicity is a clinically important side effect of cyclosporine (CsA), and CsA-induced nephrotoxicity results from increased production of free radical species in the kidney [115]. EGCG acts as an antioxidant, exerting a protective effect from alterations in renal function that result from oxygen free radicals, and EGCG treatment has been shown to afford significant protection from free radical–mediated injury in the kidney resulting from CsA-induced changes [115].

Finally, EGCG may also serve as an anti-inflammatory and antibacterial agent. Monocytes are the main effector cells of the immune system, and the regulation of their survival and apoptosis is essential for monocyte-involved immune responses [116]. As EGCG has been shown to induce the apoptosis of monocytes, EGCG may have potential benefits as a new anti-inflammatory agent [116].

Other Polyphenols

Caffeic acid (CA), **3**, found in fruits, vegetables, wine, olive oil, and coffee [117, 118], has demonstrated inhibitory activities towards a number of enzymes such as lipoxygenases, cyclooxygenase, glutathione S-transferase (GST) isozymes (such as GST 1-1, GST 2-2, GST 3-3, GST 4-4, and GST 7-7), and xanthine oxidase [117, 119-123]. In addition to its reported antioxidant activity [117, 124], anti-

tumor activity [117, 125, 126], and anti-inflammatory properties [117, 121], CA has also been shown to inhibit the activity of MMP-9 [117].

The stems of *Euonymus alatus* have been utilized as a traditional medicine for cancer treatment [117, 127], and a CA derivative, 5-caffeoylquinic acid (chlorogenic acid; CHA), **4**, has been isolated from the stem barks of *Euonymus alatus* [128]. CHA also exerts a strong inhibitory effect against MMP-9 activity in a concentration-dependent manner as determined by zymography [128].

Ipomoea pes-caprae is used in some parts of the world to treat fatigue, strain, arthritis, and rheumatism [129, 130], and quinic acid esters and related caffetannins isolated from this herb have been shown to possess collagenase inhibitory activities [130].

Pine bark extract has been used in Europe and North America for the treatment of wound healing and for inflammatory diseases [131-133], and the procyanidin-rich maritime pine bark extract Pycnogenol has well-documented antioxidant and anti-inflammatory activity [132]. Pycnogenol and its two major metabolites, y-(3,4-dihydroxy-phenyl)-g-valerolactone and y-(3-methoxy-4-hydroxy-phenyl)-g-valerolactone, **5** and **6**, respectively, exhibit strong inhibitory effects towards the activity of MMP-1, MMP-2, and MMP-9, and both metabolites appeared more active than Pycnogenol [132]. It was also demonstrated that the inhibition of MMP-9 by both metabolites, but not Pycnogenol, were reversed in the presence of Zn^{2+}, suggesting that these metabolites might be capable of interacting with Zn^{2+} and bind directly at the active site of MMP-9. In addition, both metabolites inhibit MMP-9 expression by human monocytes in a dose-dependent manner, and only sub-micromolar concentrations of both compounds were required to achieve 50% inhibition of MMP-9 secretion [132].

Two biphenol compounds, Magnolol and Honokiol, **7** and **8**, respectively, from *Magnolia obovata* bark extract, have been found to inhibit the activity of MMP-9 [134]. These compounds inhibited the migration of HT-1080 cells at a concentration of 100 μM, as well as their invasion through a *Matrigel* barrier [134]. Magnolol also exhibited significant *in vivo* antimetastatic effects in a murine liver and spleen metastasis model utilizing a lymphoma line, as well as a murine lung metastasis model utilizing a melanoma line [135].

Flavonoids

Flavonoids have also been found to have inhibitory activities toward MMPs. Ende *et al.* tested 8 flavonoids for their inhibitory effects on the recombinant catalytic domains of MMP-2 and -9, and found that these flavonoids inhibit MMP-2 and MMP-9 in the micromolar range [136]. Kinetic analyses using luteolin revealed that this inhibition is non-competitive [136]. Interestingly, flavonoids inhibit the activities of other zinc metalloproteinases such as leucine aminopeptidase, aminopeptidase M, and carboxypeptidase [136, 137]. This inhibition may not be the result of coordination between the hydroxyl group and the active site catalytic zinc, and the molecular mechanism of this inhibition remains uncertain.

Flavonoids and polyphenols down-regulate the expression of MMPs in different cell types, inhibiting cancer cell invasion. The reactive oxygen species generated by oxidative stress can induce the expression of MMPs, and because flavonoids and polyphenols are well known anti-oxidants and radical scavengers, the down-regulation of MMPs by these compounds may be the result of their ability to interfere with the pathways of oxidative stress response. Their inhibition of MMPs at the levels of expression and enzymatic activity suggest that flavonoids and polyphenols might also influence the processes of extracellular matrix degradation and remodeling, which may in turn contribute to the antimetastatic and antiarteriosclerotic effects of these compounds.

Other Natural Compounds

Fujita *et al*. have reported that ageladine A, **9**, a fluorescent alkaloid isolated from the marine sponge *Agelas nakamurai*, inhibits MMP-1, -8, -9, -12, and -13 with IC_{50} values of 1.2, 0.39, 0.79, 0.33, and 0.47 μg/mL, respectively [138]. This compound could also inhibit MMP-2, with an IC_{50} of 2.0 μg/mL, but N-methylated derivatives did not inhibit MMP-2 [138]. The inhibition is not due to Zn^{2+} chelation, as ageladine is not capable of chelating to Zn^{2+}, and a kinetic analysis indicated that the inhibition was not competitive [138]. In addition, bovine aortic endothelial cell migration and vascular formation by murine ES cells were significantly inhibited by this compound [138].

SYNTHETIC MMP INHIBITORS

The development of matrix metalloproteinase inhibitors (MMPIs) has come a long way since the search for small molecule MMPIs with oral bioavailability was first initiated in the late 1970's for the treatment of arthritis [139], and it remains a challenging endeavor for investigators [140]. Here we focus primarily on the development of small molecule MMPIs reported since the late 1990's, while discussing new achievements and general trends.

The early design of MMPIs was based on mimicking the peptide structures of substrate cleavage sites, grafting a zinc-binding group (ZBG) at the scissile site to generate peptidomimetic MMPIs. These initial design efforts were later rationalized by x-ray crystallographic studies [141]. The active sites of the MMPs all have a shallow cleft with a flat non-prime side and a narrow prime side centered around a well-defined S_1' principal specificity pocket. All of the MMP catalytic domains share a marked sequence similarity and a conserved topology [142]. Two significant distinguishing features, the depth of the S_1' pocket, and the length and composition of the loop constituting the outside wall of the S_1' pocket, explain why most efforts were dedicated to the development of inhibitors providing special interactions with the S_1' pocket [143]. The nature of one key residue at the S_1' pocket plays a decisive role in determining the depth of the S_1' pocket; for MMP-1, this is residue 214, located in helix B of the catalytic domain [144]. In some cases, however, MMPs with a shallow S_1' pocket can still accommodate a long side chain through conformational changes [145-147]. Thus, this S_1' variability can be exploited to design inhibitors with P_1' groups able to establish specific interactions with residues in the lower part of the S_1' loop as a means to obtain

highly selective inhibitors of the MMPs, although the inherent flexibility of the S_1' loop makes it difficult to predict the exact binding mode of a particular P_1' group. As a consequence, selective inhibition for a small subset of the MMP family has been achieved [140, 148, 149], but the specific inhibition of a single MMP has not yet been reported. Computer modeling now plays an important role in the process of designing potent and selective MMPIs, although accuracy problems still remain because many factors need to be taken into account during the energy minimization process, such as conformational changes of the target and ligand, in addition to solvent effects. It should be kept in mind that inhibitory selectivity by a factor of 1000 means the free energy difference between two enzyme-inhibitor complexes is about 4 kcal/mol at 300K. Therefore, to be helpful in the development of selective inhibitors, modeling methods should ideally have accuracies on the order of 1 kcal/mol, which remains below the capacities of current computational methods used to estimate *in silico* binding free energies [150].

Most MMPIs incorporate a ZBG, a backbone that forms hydrogen bonds with the enzyme, and side chains appended to the backbone that interact with specific subsites within the active site. MMPIs are broadly classified according to their ZBG. Four major ZBGs have been exploited for the development of zinc metalloproteinase inhibitors: carboxylates, thiolates, phosphinyls, and hydroxamates [140]. Interestingly, while the first three ZBGs have been widely applied in the design of therapeutic agents targeting other enzymes, like those of the angiotensin converting enzyme (ACE) inhibitors used to combat cardiovascular diseases, the vast majority of MMPIs belong to the hydroxamate category [140]. A hydroxamate ZBG was first introduced in the design of an inhibitor for zinc metalloproteinase-thermolysin in 1978 by Nishino and Power [151], much later than the first three ZBGs, but close to the time when the design of MMP inhibitors was first initiated, providing the impetuous for development of a new class of MMPIs incorporating a hydroxamate ZBG [152, 153].

The hydroxamate ZBG can chelate to the active site zinc ion in a bidentate fashion, and it can also form hydrogen bonds with the enzyme backbone, conferring upon this type of MMPI the strongest binding properties of the four ZBGs. This binding strength may overwhelm the contribution to protease binding by other groups in the inhibitor structure, however, leading to a reduction in the selectivity of hydroxamate inhibitors. The first generation of hydroxamate MMPIs was developed using succinate as a template, leading to nanomolar inhibition of selected MMPs [140]. There were several drawbacks for inhibitors of this type, however, including poor *in vivo* stability, rapid and extensive clearance by biliary excretion, and poor oral absorption [148].

Nonpeptidic hydroxamates and sulfonamide hydroxamates were designed to solve these problems, and were quite successful in the development of potent and selective MMPIs with favorable biological properties. Robinson *et al.* at Pfizer designed pyrrolidinone-based hydroxamates that showed good selectivity for MMP-13 (*e.g.* **10)** (Fig. (**2**)) [154]. Structural modification of the pyrrolidinone scaffold then led to the discovery of a novel series of imidazolidine-based MMPIs with strong inhibitory activities for MMP-13, as exemplified by **11** [155]. *N*-Hydroxy-α-sulfonyl acetamides with the structure of **12** were developed for the treatment of osteoarthritis [156, 157]. Structure-activity relationship (SAR) studies showed that aromatic sulfonyl compounds were more potent than aliphatic or heteroaromatic sulfonyl derivatives, and that compounds disubstituted at the α-position exhibited higher inhibitory activities. It was also found that an aliphatic basic amine was essential for *in vivo* activity. Further structural modifications indicated that the oxidation states of sulfur tended to steer the selectivity and potency either toward MMPs or tumor necrosis factor-α converting enzyme (TACE, or disintegrin and metalloproteinase17/ADAM17) [158]. Noe *et al.* at Pfizer designed tetrahydropyran-centered sulfone hydroxamate inhibitors selective for MMP-13, as exemplified by **13** [159]. These inhibitors possessed favorable physiochemical properties and low metabolic clearance. Solid-phase synthesis of β-(arylsulfonyl) hydroxamates was carried out to facilitate optimization of this series, resulting in sub-nanomolar MMPIs specific for MMP-2 (*e.g.* **14**) [160]. 3,4-dialkoxylation at the arylsulfonyl moiety drastically curtailed the inhibitory activities for MMPs (the IC_{50} values were all >10,000 nM), while retaining inhibitory activities for phosphodiesterase 4 (PDE4), as exemplified by **15**. A trifluoromethyl group was incorporated at the β-carbon of β-(arylsulfonyl) hydroxamates to investigate the "fluorine effect" on inhibitory potency [161], and inhibitor **16** (Fig. (**3**)), synthesized in racemic and chiral forms, exhibited low nanomolar inhibitory activities for MMP-3 and MMP-9.

Significant efforts have been devoted to the development of α-amino acid-based sulfonamide hydroxamates as a new type of MMPI following the identification of CGS-27023A, **17**, as an orally available broad-spectrum MMPI [140]. Scozzafava *et al.* reported that inhibitory activities could be still retained for MMPs and clostridium histolyticum collagenase (ChC) when the arylsulfonyl was replaced by perfluoroalkylsulfonyl (*e.g.* **18**), arylsulfenyl (*e.g.* **19**), arylureido (*e.g.* **20**), benzoylthioureido (*e.g.* **21**), or arylsulfonyluriedosulfonyl moieties (*e.g.* **22**) (Fig. (**4**)) [162]. Simple *D*-valine-based biphenylsulfonamide hydroxamate (**23)** was prepared and found to be a good MMPI, but with poor bioavailability [163]. Arylsulfonylureido-glycine hydroxamates were designed to study the effects of bulky *N*-substituents on inhibitory potency (*e.g.* **24**) [164]. The kinetic data indicated that these hydroxamates and their corresponding carboxylates all exhibited high inhibitory potency, and the bulky *N*-substituent moderately increased inhibitory activities, possibly through interaction with the enzyme S_2' subsite. *N*-Isopropoxysulfonamide hydroxamates (**25**) were found to be potent and selective inhibitors of MMP-2 and MT1-MMP, while sparing MMPs-1, 3, and 9 [165, 166]. An alkyl substituent at the α-position to the hydroxamate apparently has a hydrophobic interaction with the S_1 subsite, resulting in higher inhibitory activities (*e.g.* **25**, R_2 = *i*-Pr). Compound **25** was proven to be effective in an *in vitro* model of invasion through a *Matrigel* barrier by fibrosarcoma HT1080 cells, and was also shown to be effective in an *in vitro* model of angiogenesis. Alanine-based sulfonamide hydroxamates were designed as bacterial collagenase ChC inhibitors [167], and the best ChC

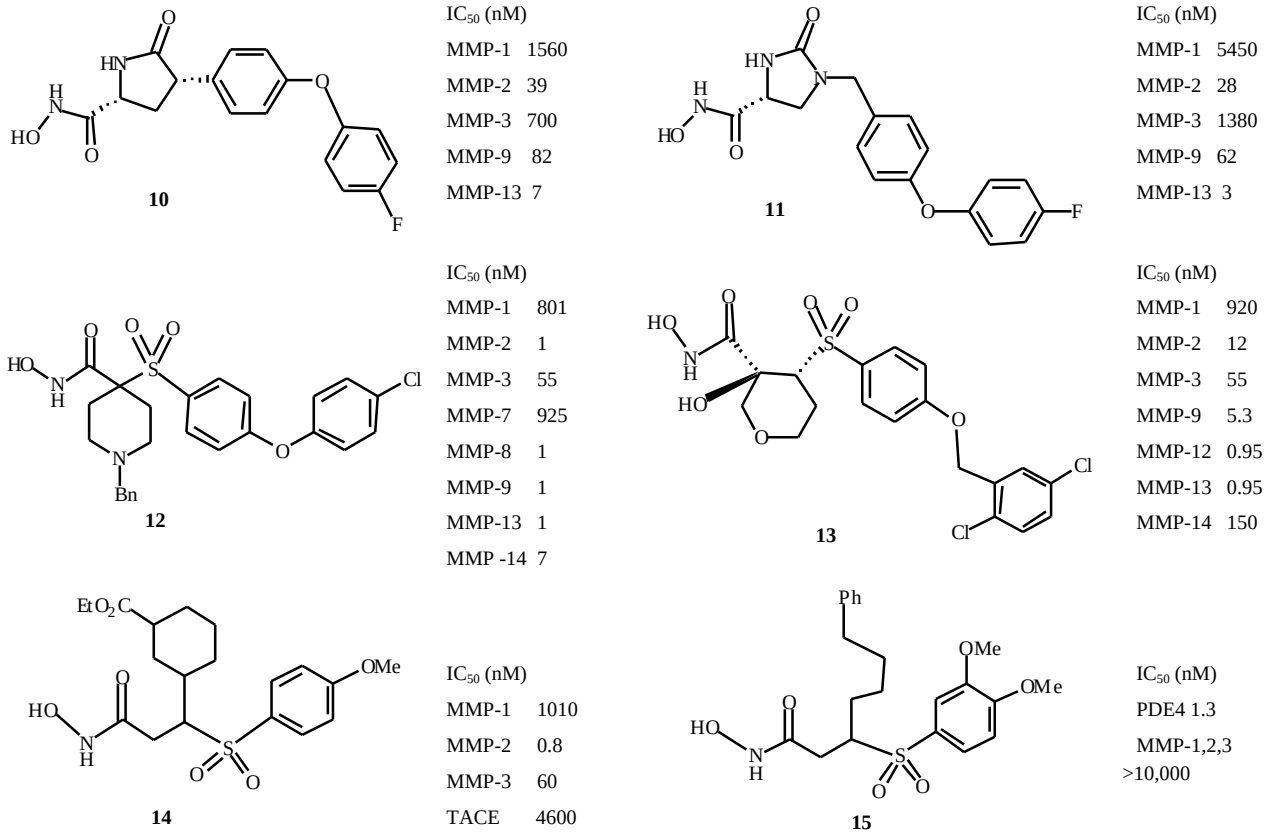
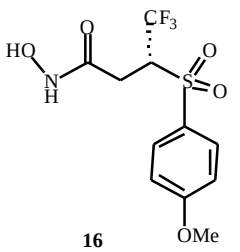


Fig. (2). Inhibitory activity of compounds **10** to **15**.

inhibitors were found to be those containing pentafluorophenylsulfonyl and 3- and 4-substituted phenylsulfonyl P_1' groups, as exemplified by **26**. A series of homocysteine-based analogues of CGS-27023A were also prepared, with variations at the three subsites corresponding to P_1, P_1', and P_2', yielding MMPIs with low nanomolar inhibitory potency and enhanced specificity for MMP-9 (*e.g.* **27**) [168]. A series of α-amino acid-based hydroxamates with α,α-disubstitution were shown to be potent and selective MMPIs for MMP-13, while sparing MMP-1 (*e.g.* **28**) [169]. For potent MMP-13 inhibition, it was found that a substituent on the sulfonamide nitrogen was not necessary. The α,α-disubstitution increased metabolic stability, but reduced inhibitory activities. Increasing the size of the P_1' group could offset this negative effect and at the same time increase the selectivity over MMP-1. Unfortunately, preclinical and clinical studies of the leading compound, pyran derivative **28,** resulted in fibrosis and musculoskeletal syndrome (MSS) in humans.

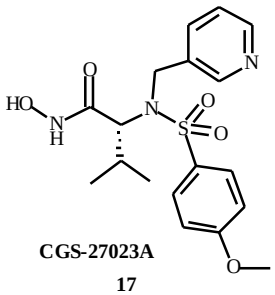
It was hypothesized that the conformational rigidity induced by a ring incorporated into the inhibitor backbone could lead to increased *in vitro* potency and enhanced pharmacological properties, as exemplified by AG-3340, **29** [140]. Thiazine and thiazepine-based MMPIs were prepared, and the most potent series of inhibitors was obtained by modification of the amino acid *D*-penicillamine [170]. The representative compound (**30**) was shown to be a potent, broad-spectrum inhibitor. Based on this result, another series of heterocycle-based MMPIs were designed to introduce a P_2' substituent that could be modified, as with **31** [171]. The P_2' substituent in this series was believed to contribute to high inhibitory potency against stromelysin-1 (MMP-3), collagenase-3 (MMP-13) and gelatinases A and B (MMP-2 and MMP-9, respectively), and also to provide selectivity against collagenase-1 (MMP-1) and matrilysin (MMP-7). An x-ray crystallographic structure of a compound **31** and stromelysin-1 complex validated the design rationale and provided insights into the SAR and selectivity trends observed for the series [171]. The P_2' benzyl group was poised to have good van der Waals contacts with the Leu-222 of stromelysin. The flexibility of the benzyl group may allow alternative conformations for its favorable interaction with neighboring residues (*e.g.* Pro-221 or Val-163), avoiding possible undesirable interactions with the S_2' pocket of MMP-1. As a result, inhibitor **31** exhibited relatively low selectivity.

Potent and selective TACE and MMP inhibitors utilizing the 1,4-diazepine, and thiazepine ring systems were synthesized and evaluated *in vitro* and in an *in vivo* model of TNF-α release [172]. Diazepine **32** (X=NH) showed excellent *in vivo* activity in a murine study of TNF-α release, as well as oral efficacy in a collagen-induced arthritis model. Benzodiazepine-based inhibitors with the structure of **33** were found to exhibit potent inhibitory activity and display interesting selectivity profiles against MMPs and TACE [173, 174]. Changes of the R_1 group showed no significant effects on potency against TACE, MMP-9, and MMP-13,



16

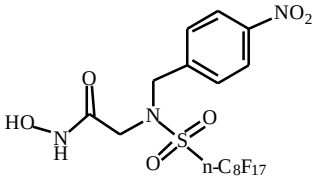
IC_{50} (nM)	
MMP-3	3.2
MMP-9	209



CGS-27023A

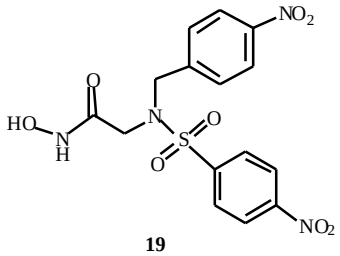
17

IC_{50} (nM)	
MMP-1	33
MMP-2	20
MMP-3	43
MMP-9	8



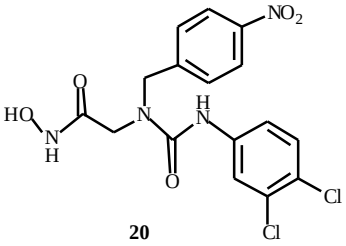
18

IC_{50} (nM)	
MMP-1	79
MMP-2	0.9
MMP-8	1.3
MMP-9	1.3
ChC	8



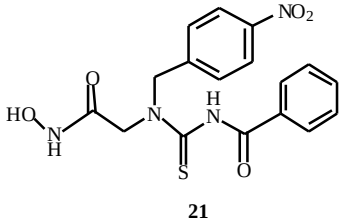
19

IC_{50} (nM)	
MMP-1	18
MMP-2	10
MMP-8	8
MMP-9	11
ChC	13



20

IC_{50} (nM)	
MMP-1	31
MMP-2	6
MMP-8	5
MMP-9	7
ChC	11



21

IC_{50} (nM)	
MMP-1	37
MMP-2	2
MMP-8	1.5
MMP-9	1.4
ChC	6

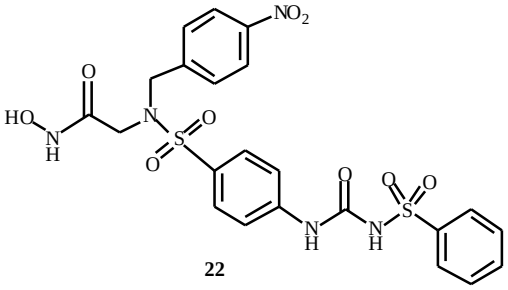
Fig. (3). Inhibitory activity of compounds **16** to **21.**

but alterations in the R_2 group, from a methoxy to a 2-ynyloxy group, drastically steered the preference towards TACE inhibition.

Proline-templated sulfonamide hydroxamate MMPIs showed potent inhibitory activities, and a potency enhancement was observed when C-4 in proline possessed sp^2 character (*e.g.* **34**) [175]. An x-ray crystal structure of a stromelysin-inhibitor **34** complex revealed that the oxime moiety extended to the S_2' pocket, which is a very hydrophobic environment. However, the flat SAR of the oxime supported the idea that enhanced inhibitory potency for inhibitors with sp^2 character at C-4 should be attributed to the favorable conformational change of the proline ring. 4-Aminoproline-based MMPIs exhibited high potency for most MMPs, excepting MMP-1 [176, 177]. An x-ray study revealed that inhibitor **35** (Fig. (**6**)) binds to the active site of stromelysin in a manner similar to its analogue, **34** [177], but these inhibitors showed low to moderate bioavailability, with short half-lives. Piperazine-based sulfonamide hydroxamate MMPIs were designed and detailed SARs were conducted, producing a series of potent inhibitors with varying degrees of selectivity, as exemplified by **36,** which showed high absorption and desirable efficacy in a bovine nasal cartilage assay [178]. Pipecolic acid-based hydroxamates were developed as MMP-13 and aggrecanase inhibitors, and detailed SAR studies were obtained [179-181]. Substitution at C-3 of the piperidine ring increased aggrecanase activity, with markedly improved bioavailability and lower metabolic clearance (*e.g.* **37**). Azasugar was also found to be a good scaffold for the design of sulfonamide hydroxamate MMPIs and TACE inhibitors (*e.g.* **38)** [182].

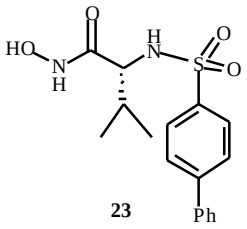
Ma *et al.* reported a series of tetrahydroisoquinoline based sulfonamide hydroxamates that showed potent inhibitory activities exclusive of MMP-7, as exemplified by **39** [183]. Cyclic sulfonamide sultam hydroxamates have been found to be potent inhibitors for MMP-2, -9, and -13, with high selectivity over MMP-1 and good oral bioavailability (*e.g.* **40**) [184].Phosphonamide-based inhibitors with a tetrahydroisoquinoline scaffold have been reported to be broad-spectrum inhibitors for MMP-1, -3, -9, and TACE, with IC_{50} values in the nanomolar range (*e.g.* **41**) [185, 186]. Cyclic phosphinamides and phosphonamides were also reported to be potent MMPIs demonstrating *in vivo* efficacy (*e.g.* **42**) [187].

Levin *et al.* reported anthranilic acid-based MMPIs with potent inhibitory activities and excellent selectivities [188-190]. Initial screening indicated that only *ortho*-anthranilic acid-based compounds exhibit good inhibition, and SAR studies showed that C-3 substitution could boost inhibition potency up to 2 orders of magnitude, giving IC_{50} values in the single nanomolar range. Modification of the arylsulfonyl moiety could provide inhibitors selective for MMP-9, MMP-13, or TACE. Representative compound **43** showed not only high inhibitory activity, but also better *in vivo* efficacy than CGS-27023A. A new series of MMPIs was created by



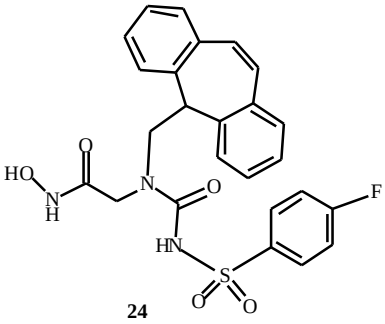
22

IC_{50} (nM)	
MMP-1	61
MMP-2	0.6
MMP-8	0.7
MMP-9	0.9
ChC	15



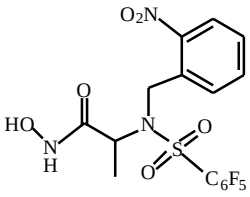
23

IC_{50} (nM)	
MMP-1	110
MMP-2	1
MMP-3	5
MMP-7	140
MMP-9	18
MMP-13	2



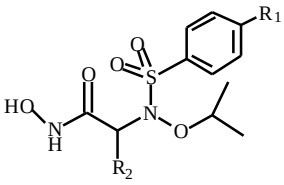
24

IC_{50} (nM)	
MMP-1	11
MMP-2	1.5
MMP-8	2.0
MMP-9	2.3
ChC	6



26

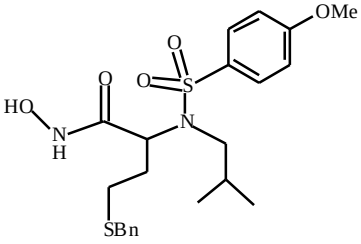
IC_{50} (nM)	
ChC	0.313



25

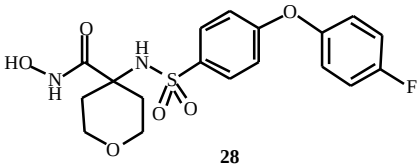
R_1=Ph; R_2=H	
IC_{50} (nM)	
MMP-1	>50,000
MMP-2	12
MMP-3	4,500
MMP-7	>50,000
MMP-9	200

R_1=Ph; R_2=i-Pr	
IC_{50} (nM)	
MMP-1	147
MMP-2	0.09
MMP-3	50
MMP-7	>1,000
MMP-9	6.7
MMP-14	9.8



27

IC_{50} (nM)	
MMP-1	104
MMP-2	0.7
MMP-3	0.7
MMP-9	<0.1
MMP-13	12



28

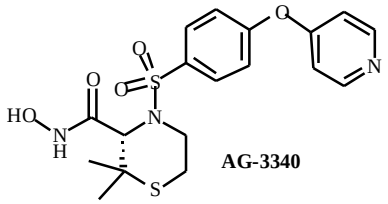
IC_{50} (nM)	
MMP-1	420
MMP-13	0.75

Fig. (4). Inhibitory activity of compounds **22** to **28.**

replacing the phenyl ring of anthranilic acid with heteroaryl rings and simple cyclohexyl moieties [191], and SAR studies showed that pyridine derivative **44** exhibited the best inhibitory activities.

The second generation of hydroxamate MMPIs successfully overcame the drawbacks exhibited by the first generation, exhibiting strong inhibition potency and good *in vivo* stability. Nevertheless, many hydroxamate MMPIs developed to treat arthritis or other conditions often induced a tendonitis-like fibromyalgia or MSS in humans. Thus far, the pharmacological causes for these side effects remain unknown [192]. Due to the intensive competition in the area of hydroxamate MMPIs, and the apparent drawbacks mentioned earlier, MMPIs with alternative ZBGs have been actively pursued.

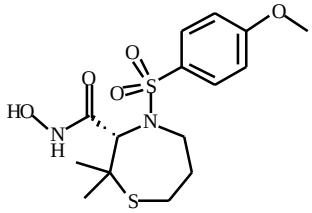
Except for a few hydroxamates made by solid-phase syntheses, most hydroxamate inhibitors are obtained from the corresponding carboxylic acids. Consequently, carboxylate compounds have been tested collaterally for their MMP inhibitory activities. Although carboxylates exhibit weaker zinc-binding properties than hydroxamates, they are known to show better oral bioavailability and are less prone to metabolic inactivation. The expected loss of binding affinity after replacement of hydroxamates with carboxylates can be compensated to some degree by adequate structural modifications. Valine-based biarylsulfonamide carboxylates



AG-3340

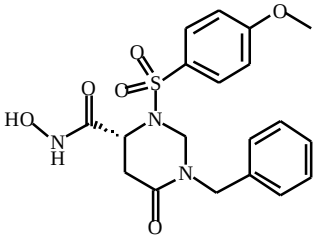
29

IC_{50} (nM)	
MMP-1	8.2
MMP-2	0.083
MMP-3	0.27
MMP-7	54
MMP-13	0.038



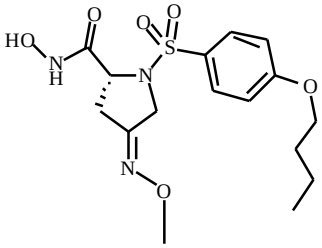
30

IC_{50} (nM)	
MMP-1	0.8
MMP-2	2.7
MMP-3	0.7
MMP-7	30
MMP-8	1.4
MMP-9	0.9
MMP-13	0.9



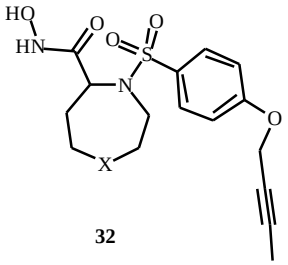
31

IC_{50} (nM)	
MMP-1	22.2
MMP-2	1.0
MMP-3	3.1
MMP-7	10,000
MMP-8	6.4
MMP-9	6.8
MMP-13	2.56



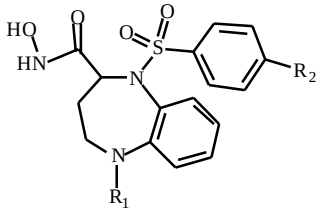
34

IC_{50} (nM)	
MMP-1	26
MMP-2	<1
MMP-3	1
MMP-7	89
MMP-13	0.3



32

IC_{50} (nM)		IC_{50} (nM)
X=NH		X=S
MMP-1	274	408
MMP-13	32	36
TACE	24	20



33 $R_1 = COCH_3$

IC_{50} (nM)		IC_{50} (nM)
R_2=OMe,		R_2=OCH_2-CC-Me
MMP-1	18	835
MMP-9	1.4	228
MMP-13	1.0	77
TACE	103	16

Fig. (5). Inhibitory activity of compounds **29** to **34**.

were evaluated, and it was found that *p*-halogenated derivatives exhibited high potencies against MMP-2, -3, and -13, while sparing MMP-1, -7, and -9 (*e.g.* **45**) [163]. Pharmacokinetic studies revealed that compound **45** showed better metabolic stability than its corresponding hydroxamate inhibitor. Hetereoarylphenylsulphonamide carboxylates were prepared and evaluated as potential MMPIs [193], the thiazole **46** was found to a potent MMP-2 and MMP-9 inhibitor. Propargylglycine-based carboxylate MMPIs (*e.g.* **47**) [194] and cyclohexylglycine-based carboxylates (*e.g.* **48**) [195] exhibited selectivity for MMP-2 and MMP-13, with low nanomolar potency, while sparing MMP-1 and MMP-3. Piperidinylglycine-based carboxylates were found to inhibit multiple target MMPs (MMP-2, -3, -8, -9, and -13), while at the same time sparing MMP-1 and MMP-7 (*e.g.* **49**) [196]. Tetrahydroisoquinoline-3-carboxylate based MMPIs have also been designed [197], and structural modifications, especially at the P_1' residue, resulted in potent and orally bioavailable MMPIs, as exemplified by **50**.

Phosphorus-based ZBGs have been one of the major choices for the design of zinc metalloproteinase inhibitors [198], even though their weaker affinities for zinc when compared to the hydroxamates limited their application in MMPI design. As a result, non-optimized phosphinic peptides exhibit poor inhibitory activities for MMPs, but extremely potent phosphinic MMPIs can be prepared through several rounds of structural modification (*e.g.* **51**) [147, 199]. P_1' modification led to the discovery of highly selective inhibitors of MMP-11, as exemplified by **52,** which showed metabolic stability and anti-tumor properties [200, 201]. A series of carbamoylphosphonates were prepared and found to be selective for MMP-2, as exemplified by **53** [202], a water-soluble inhibitor that showed no toxic effects

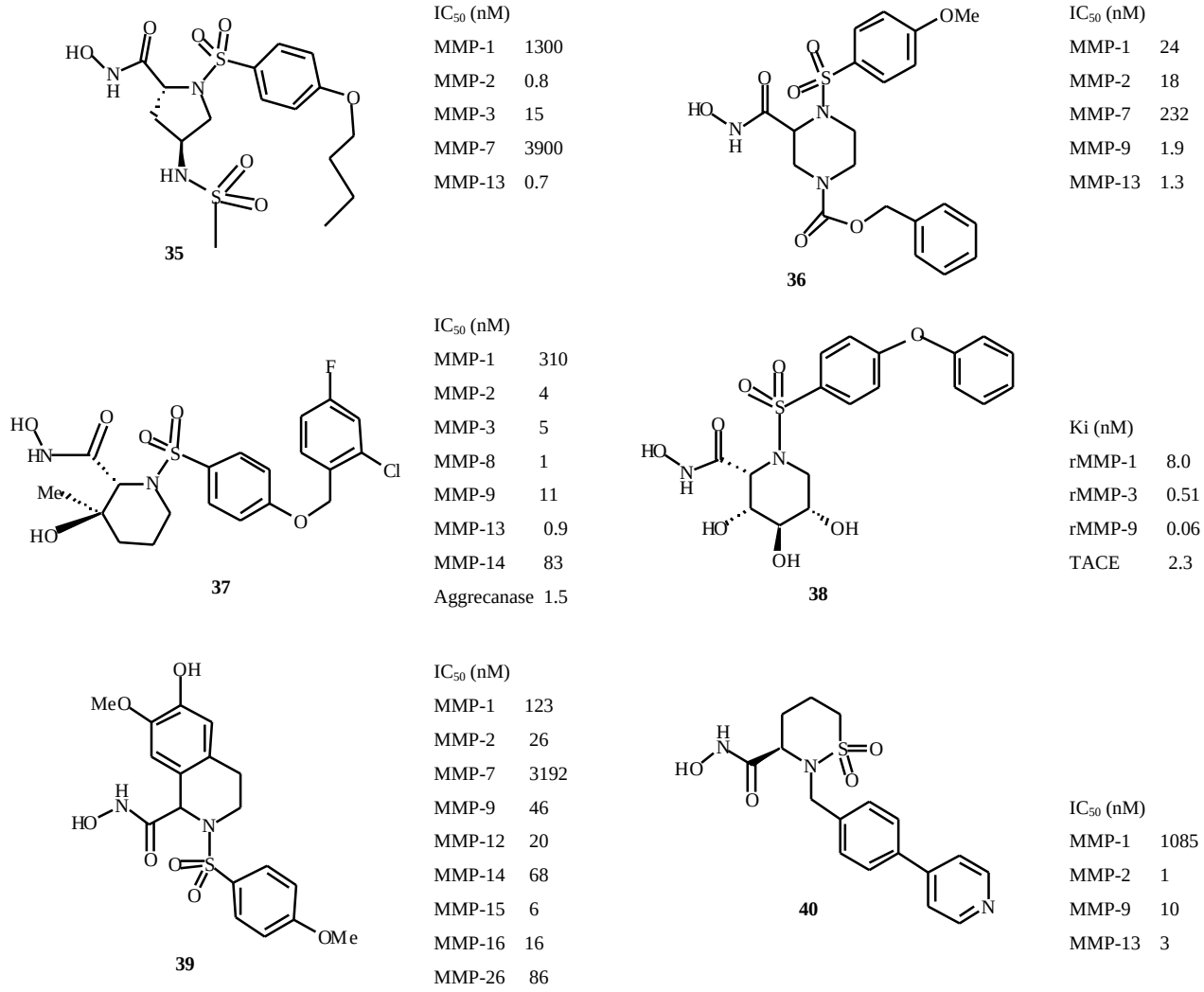
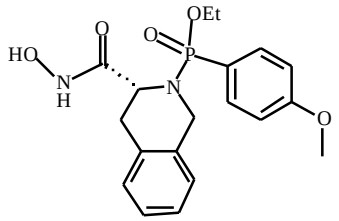


Fig. (6). Inhibitory activity of compounds **35** to **40**.

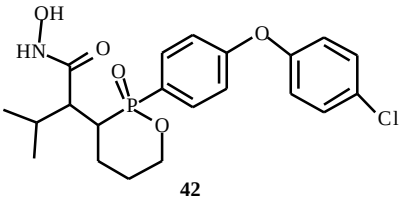
in vitro or *in vivo*. Both boronate and phosphonate groups were incorporated into the same molecule to test the possible synergistic inhibition of MMP-2, and most of these compounds showed moderate activity (IC_{50} ~50 μM) (*e.g.* **54**) [203]. Peptidyl biphenylalkylphosphinate MMPIs were designed and evaluated as inhibitors of MMP-2 and MMP-8 [204], and these inhibitors exhibited moderate inhibitory activities with IC_{50} values in the micromolar range (*e.g.* **55**).

The mercaptan (thiol) functionality is a well-known metal chelating ligand that has been widely explored in the design of potential therapeutic agents. One successful case is Captopril, a thiol inhibitor of the angiotensin-converting enzyme (ACE) [205]. While MMP inhibitors with a thiol ZBG generally have less binding affinity compared with hydroxamate inhibitors [140], structural optimizations, such as P_1' alteration and introduction of extra binding moieties, is a common practice to address this problem. Another approach is to introduce an extra zinc-chelating ligand to improve the binding affinity, such as a carbonyl or hydroxy group [140]. Ethers, amines, and sulfides as metal-chelating ligands have found wide applications in coordination chemistry [206]. It was theorized by our group that these heteroatoms, if properly located to connect the thiol ZBG and the peptidomimetic segment, could act as a second zinc-chelating ligand to afford a novel type of MMP inhibitors, as schematized in Fig. (**9**) [207]. Based on this rationale, a series of thiol compounds were designed and synthesized as probes to examine the validity of the design rationale and their utility as MMP inhibitors. As shown in Table **1**, compound **MAG-42**, in which the mercaptosulfide was linked by an ethylene unit and the sulfide sulfur attached to the P_1' α-carbon directly, exhibited the strongest potency. However, the ether and amine analogues (**DAO** series) of **MAG-42** showed much less potency. The insertion of a methylene unit between the P_1' α-carbon and sulfide sulfur caused a dramatic decrease in inhibitory potency (**MAG-21** and **MAG-32**). If the ethylene linker of the mercaptosulfide moiety was changed into a propylene linker, the effects on inhibitory activity were negative, regardless of whether the sulfide sulfur was connected to the P_1' α-carbon directly (**MAG-96** and **MAG-167**) or through a methylene unit (**MAG-46** and **MAG-47**). One surprising feature of the most



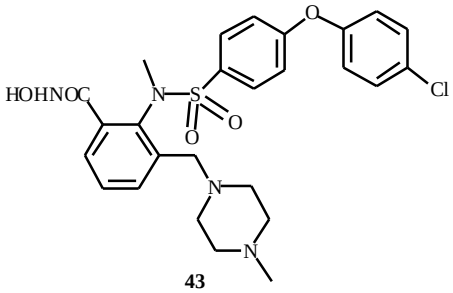
41

Ki (nM)	
MMP-1	4.58
MMP-3	5.20
MMP-9	5.05
TACE	7.15



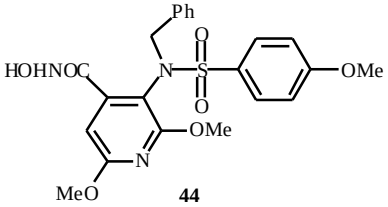
42

IC_{50} (nM)	
MMP-1	130
MMP-3	16
MMP-9	0.63



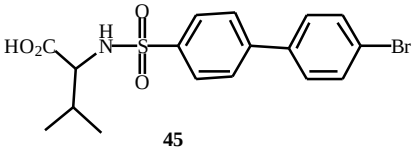
43

IC_{50} (nM)	
MMP-1	155
MMP-9	1
MMP-13	0.8
TACE	122



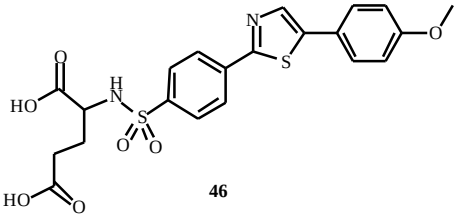
44

IC_{50} (nM)	
MMP-1	1227
MMP-9	15
MMP-13	47



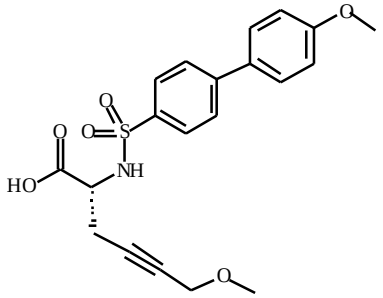
45

IC_{50} (nM)	
MMP-1	6000
MMP-2	4
MMP-3	7
MMP-7	7200
MMP-9	7900
MMP-13	8



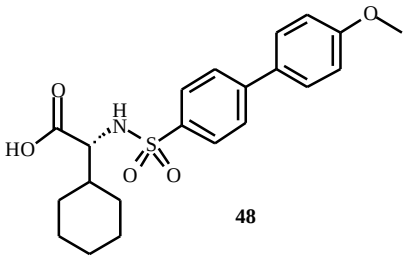
46

IC_{50} (nM)	
MMP-2	50
MMP-3	50



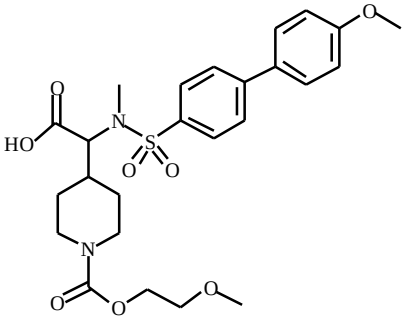
47

IC_{50} (nM)	
MMP-1	6,150
MMP-2	20
MMP-3	4090
MMP-13	76



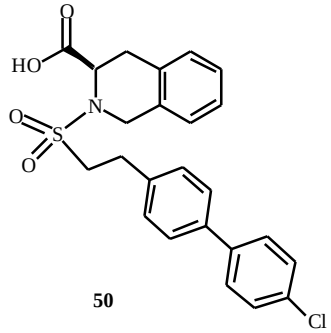
48

IC_{50} (nM)	
MMP-1	4350
MMP-2	8
MMP-3	2150
MMP-7	15147
MMP-13	43



49

IC_{50} (nM)	
MMP-1	1850
MMP-2	0.9
MMP-3	69.6
MMP-7	3950
MMP-9	13.9
MMP-13	2.4



50

IC_{50} (nM)	
MMP-1	>10,000
MMP-3	20
MMP-13	20

Fig. (7). Inhibitory activity of compounds **41** to **50**.

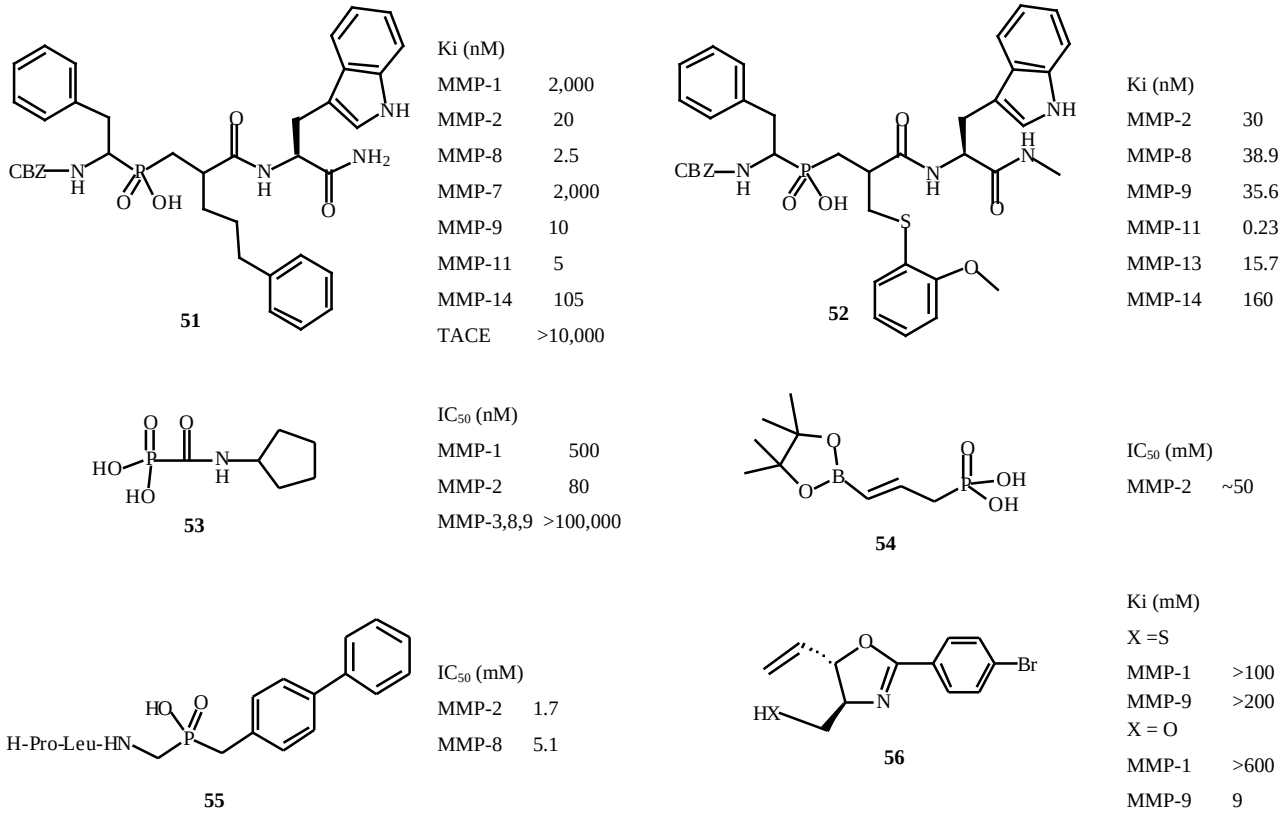


Fig. (8). Inhibitory activity of compounds **51** to **56**.

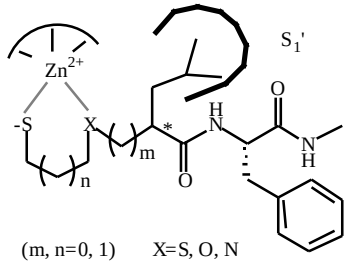


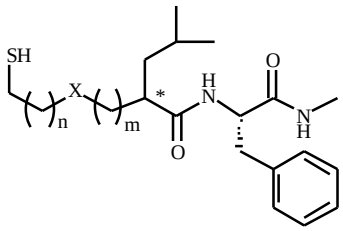
Fig. (9). Schematic representation of design rationale for MMP inhibitors with new thiol-based zinc chelating groups [207].

potent mercaptosulfide, **MAG-42**, was its stereochemistry: the (*R*) configuration at the P_1' α-carbon, corresponding to a *D*-amino acid, afforded better inhibitory activity than the diastereomer with the (*S*) configuration at the P_1' α-carbon (**MAG-92**) by about one order of magnitude. Stability in the presence of air is a crucial factor for thiol inhibitors because they may be readily air-oxidized in buffer and lose their effectiveness over time, and unfortunately, **MAG-42** in buffer showed a complete loss of inhibitory activity within 45 min at 25 °C [208].

The inhibitor **MAG-42** was then used as a prototype to probe the prime and non-prime sites to derive enhanced inhibitory activities and biological properties [207]. The effects of substitution at the ethylene linker of the mercaptosulfide were studied, and these results are shown in Table **2** [207, 209]. Of the two diastereomers generated by substitution α to the thiol, the (*S*)-diastereomer showed inhibitory potency that was generally superior to that of **MAG-42**, while the (*R*)-diastereomer had decreased potency (**MAG-133** *vs* **MAG-128** and **MAG-254** *vs* **MAG-245**). The inhibitory activities were not significantly changed when the α-substituent was changed from a methyl group to a phthalimidylethyl or a phthalimidylbutyl group, but substitution by methyl or isobutyl groups β- to the thiol elicited a moderate loss of potency (**MAG-143**, **MAG-80**, **MAG-161** and **MAG-189**). There were no consistent inhibitor potency trends correlated to the stereochemistry at the β-position.

Ring systems were introduced into the mercaptosulfide ZBG in an effort to enhance potency and to increase bioavailability (Table **3** & Fig. (**10**)) [207, 208, 210-213]. Both cyclopentyl and cyclohexyl scaffolds were explored, with the former appearing more effective. Among cyclopentane derivatives, with the two sulfurs being either *cis* or *trans*, **MAG-182** showed the best inhibitory activities against MMP-2, MMP-8, and MMP-9, with IC_{50} values in low nanomolar or subnanomolar range, rendering this a leading compound for further elaboration. Incorporation of a cyclopentyl ring at the mercaptosulfide functionality increased the stability of the ZBG, enhancing its resistance towards oxidation, as **MAG-182** retained its potency in buffer for 6 hours [210]. The mercaptosulfide MMPIs with cyclopentane or pyrrolidine rings exhibited significantly higher inhibition potency when the two chiral centers at the sulfur-attached carbons had β-H stereochemistry [214].

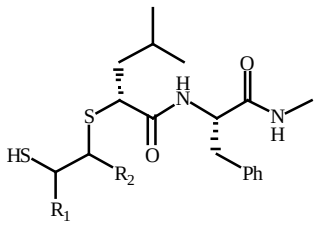
Table 1. The Enzyme Inhibitory Activities of Thiol Compounds with Another Possible Zinc Binding Group. IC_{50} Values are Expressed in nM



Compound	m	n	X	* Stereochemistry	MMP-8	MMP-1	MMP-9	MMP-2	MMP-3	MMP-7
DAO-30	0	1	O	D_{lp}	910	1,700	260	330	29,000	4,800
DAO-32	0	1	O	D_{mp}	480	1,700	720	690	38,000	1,700
DAO-294 HCl salt	0	1	N	*R*	60	420	54	7.6	6,500	150
DAO-296 HCl salt	0	1	N	*S*	180	760	210	180	17,000	490
MAG-42	0	1	S	*R*	3.6	52	1.2	1.4	250	33
MAG-92	0	1	S	*S*	11	400	17	34	3,600	180
MAG-32	1	1	S	*R*	2,200	4,000	1,900	1,300	110	
MAG-21	1	1	S	*S*	800	1,300	890	300	18,000	
MAG-167	0	2	S	*R*	760	7,600	1,300	1,500	23,000	38,000
MAG-96	0	2	S	*S*	360	13,000	1,600	5,300	76,000	130,000
MAG-46	1	2	S	*R*	7,000	32,000				
MAG-47	1	2	S	*S*	500	7,000				

*D_{lp}, less polar diastereomer eluted first in column chromatography; D_{mp}, more polar diastereomer eluted second in column chromatography.

Table 2. Enzyme Inhibitory Activities of Mercaptosulfide Inhibitors with α- or β-Substitution at the Ethylene Linker. IC_{50} Values are Expressed in nM

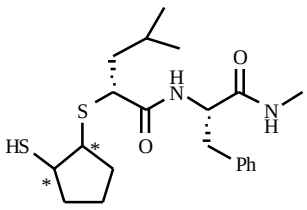


Compound	R_1	R_2	MMP-8	MMP-1	MMP-9	MMP-2	MMP-3	MMP-7
MAG-128	(*R*) Me	H	19	200	18	110	400	100
MAG-133	(*S*) Me	H	0.52	13	0.53	0.92	46	3.6
MAG-245	(*R*) $CH_2CH_2CH_2CH_2NPht$	H	5.5	360	1.3	8.3	56	20
MAG-254	(*S*) $CH_2CH_2CH_2CH_2NPht$,	H	0.55	20	0.17	0.65	10	3.3
MAG-292	(*S*) CH_2CH_2NPht,	H	0.63	4.2	0.14	0.77	22	3.3
MAG-143	H	(*R*) Me	6.6	210	4.5	12	1000	340
MAG-80	H	(*S*) Me	19	240	10	12	870	170

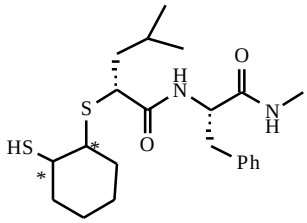
(Table 2) Contd….

Compound	R_1	R_2	MMP-8	MMP-1	MMP-9	MMP-2	MMP-3	MMP-7
MAG-161	H	(*R*) *i*-Bu	7.9	690	140	19	2000	1100
MAG-189	H	(*S*) *i*-Bu	110	250	7.6	12	1100	550
MAG-42	H	H	3.6	52	1.2	1.4	250	33

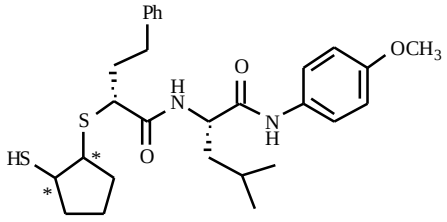
* NPht; Phthalimidyl



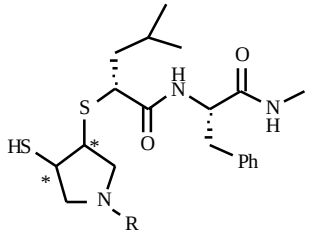
MAG-299 *trans*-D_{lp}
MAG-300 *trans*-D_{mp}
MAG-181 *cis*-α-H
MAG-182 *cis*-β-H



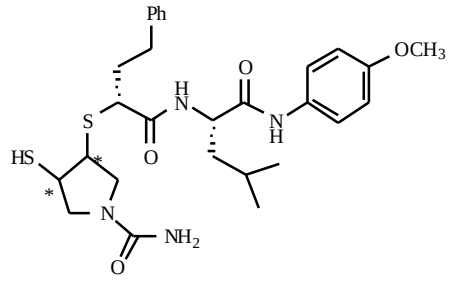
MAG-289 *trans*-D_{lp}
MAG-288 *trans*-D_{mp}
MAG-291 *cis*-D_{lp}
MAG-293 *cis*-D_{mp}



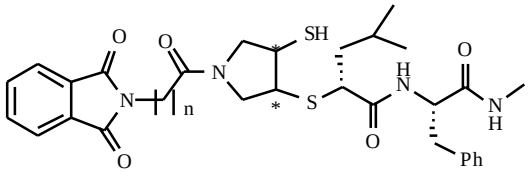
YHJ-72 *cis*-α-H
YHJ-73 *cis*-β-H



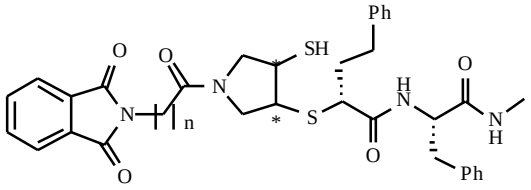
YHJ-176 *cis* (1:1), R=H
YHJ-282 *cis* (1:1), R=CH_3CO-
YHJ-294 *cis*-α-H, R=CO-NH_2
YHJ-294 *cis*-β-H, R=CO-NH_2



YHJ-74 *cis*-α-H
YHJ-75 *cis*-β-H



YHJ-96 *cis* (1:1), n=1
YHJ-97 *cis* (1:1), n=3
YHJ-132 *cis*-α-H, n=2
YHJ-132 *cis*-β-H, n=2



YHJ-233 *cis* (1:1), n=2

Fig. (10). Chemical structures of MMP inhibitors with cyclic zinc binding groups.

Inhibitors with a Leu side chain were more potent against the shallow pocket MMPs (MMP-1 and MMP-7) than were those with a homoPhe side chain. Inhibitors with a homoPhe side chain were more potent against the known deep pocket MMPs, such as MMP-3, MMP-12, and MMP-14, than were their Leu-analogues. Among the MMPs with an intermediate pocket (MMP-2, MMP-8, MMP-9, and MMP-26), there were no significant and consistent differences in the inhibitory potencies for Leu and homoPhe analogues.

A nitrogen was then introduced into the cyclopentane system to allow for the attachment of additional sidechains that might interact with the non-prime side of the active sites. Compound **YHJ-176**, with only a free amine, was an

Table 3. MMP Inhibitors with Cyclic Mercaptosulfide Zinc Binding Groups. IC_{50} or (K_i) Values are Expressed in nM. The Chemical Structures are Listed in Fig. (10)

Compound	MMP-8	MMP-1	MMP-9	MMP-2	MMP-3	MMP-7
MAG-299 *trans*-D_{lp}	2.6	91	11	14	280	39
MAG-300 *trans*-D_{mp}	1.9	36	25	29	220	34
MAG-181 *cis*-α-H	4.1	680	44	85	2500	(710)
MAG-182 *cis*-β-H	0.89	49	(0.57)	1.1	470	(40)
MAG-289 *trans*-D_{lp}		320	6.5			
MAG-288 *trans*-D_{mp}		100	16	49	390	50
MAG-291 *cis*-D_{lp}		800	22			830
MAG-293 *cis*-D_{mp}		230	6.0			210
YHJ-72 *cis*-α-H	(530)	>12K	(180)	(930)	(150)	(5,500)
YHJ-73 *cis*-β-H	(70)	>12K	(8.6)	(20)	(100)	(1000)
YHJ-176 *cis* (1:1)	32	260	5.3	200	4,100	230
YHJ-282 *cis* (1:1)	2.1	53	4.2	12	370	34
YHJ-96 *cis* (1:1)	(1.4)	(110)	(4.9)	(17)	(300)	(50)
YHJ-97 *cis* (1:1)	(10)	(75)	(3.9)	(8.5)	(31)	(12)
YHJ-294-1 *cis*-α-H	130	5,200	550	430	40K	3,500
YHJ-294-2 *cis*-β-H	1.2	100	1.2	6.1	360	26
YHJ-133 *cis*-α-H	(110)	(1,900)	(122)	(90)	(1,100)	(670)
YHJ-132 *cis*-β-H	(0.57)	(8.8)	(1.1)	(0.7)	(6.0)	(6.5)
YHJ-74 *cis*-α-H	(300)		(220)	(88)	(270)	(3,600)
YHJ-75 *cis*-β-H	(44)	(2400)	(3.0)	(6.9)	(21)	(300)
YHJ-223 *cis* (1:1)		(190)	(0.35)	(1.8)	(13)	(250)

*D_{lp}, less polar diastereomer eluted first in column chromatography; D_{mp}, more polar diastereomer eluted second in column chromatography; (1:1), equal mixture of the two diastereomers.

MMP-9-specific inhibitor, with good selectivity over MMP-2 (a 40-fold potency difference). This property made it unusual because the other mercaptosulfide MMPIs did not discriminate between these two biologically important gelatinases. The inhibitors **YHJ-282**, **YHJ-96**, and **YHJ-97**, with pyrrolidine *N*-acyl substituents of various lengths, had comparable potencies in most cases, indicating few specific interactions arising from the sidechains. The introduction of simple urea functionality (**YHJ-294** and **YHJ-75),** or phthalimidoethyl-substituted ureas (**YHJ-132** and **YHJ-223)**, at the pyrrolidine nitrogen enhanced the water solubility of these inhibitors, while also somewhat improving their potency when compared to the cyclopentane analogues (**MAG-182** and **YHJ-73**). The incorporation of a pyrrolidine ring at the mercaptosulfide ZBG further increased the air oxidation stability of this series of MMPIs, and the inhibitor **YHJ-132** was stable for two days in calf serum at 37 °C [210].

A series of oxazoline-based MMPIs designed with a similar strategy has also been reported [215]. It was theorized that the nitrogen of the oxazoline moiety and another ZBG could bind to the catalytic zinc in a bidentate fashion, and the substituents at C-2 and C-4 could extend into the S_1' and S_2' pockets. However, the oxazoline thiols were surprisingly less potent than the oxazoline alcohols (*e.g.* **56**), suggesting that these inhibitors bound to the MMPs in unexpected ways.

In one way or another, MMP inhibitors with the different ZBGs described here all have properties that have hampered their development into therapeutic agents, but efforts to design new ZBGs have not been suspended, as this strategy provides a vital platform for the successful development of new generations of MMP inhibitors with better biological activities.

Cohen *et al.* introduced 11 new ZBGs for the design of MMPIs, as shown in Fig. (**11**) [216], and these ZBGs possess enhanced hydrolytic stability that stems from their cyclic structures, as well as biological tolerance and binding affinities that are superior to those of the hydroxamate ZBGs. The x-ray crystal structures of model complexes

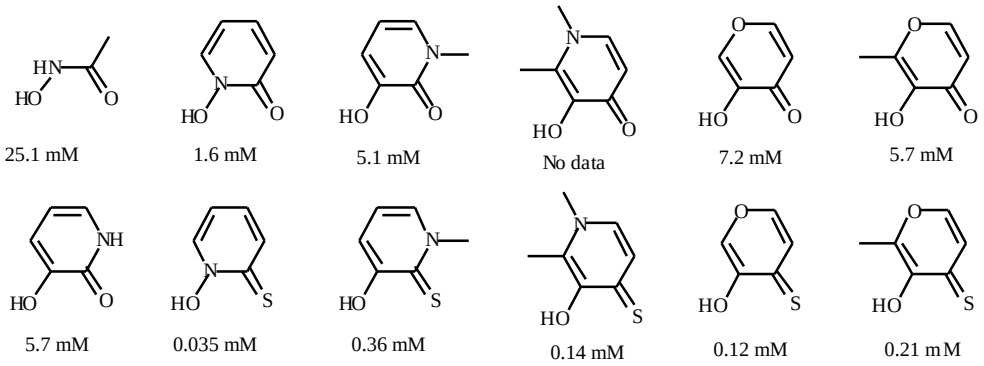


Fig. (11). Structures of new ZBGs identified by Cohen *et al.* and their IC_{50} values for MMP-3 (acetohydroxamic acid listed for comparsion) [216].

indicate that these ZBGs chelate to zinc in a bidentate fashion, as expected [216].

Pyrimidin-2,4,6-triones, or barbiturates, were first discovered to be novel ZBGs appropriate for MMPI design by Grams *et al.* [217]. The x-ray crystal structure of MMP-8 and RO200-1770 (**57**) (Fig. (**12**)) revealed that the barbiturate moiety binds to the active-site zinc and forms several hydrogen bonds with the enzyme [218, 219], with the two C-5 substitutents binding to the S_1' and S_2' pockets of MMP-8. Modification of these two C-5 substituents also led to the discovery of MMPIs showing selectivity for MMP-2, MMP-9, and MMP-3, in the absence of toxic or sedative effects, as exemplified by **58** [220]. Blagg and co-workers also reported barbiturate MMPIs with potencies similar to that of the hydroxamates, and with high selectivity for MMP-13 over MMP-14 (*e.g.* **59**) [221]. A new series of spiro-barbiturates with the structure of **60**, designed by computer modeling, showed low nanomolar potency for MMP-2, MMP-9, and MMP-13, while sparing MMP-1 and MMP-3 [222].

Novel inhibitors with a hydrazide ZBG showed good potency and selectivity for MMP-2 over MMP-1 (*e.g.* **61**) (Fig. (**13**)) [223]. Among potential ZBGs, Wang *et al.* selected aminomethylbenzimidazole as a novel ZBG to develop nonpeptidic MMP-9 inhibitors, and reported the development of an MMP-9 inhibitor (**62**) of moderate potency [224].

The Mobashery group has described thiirane-based irreversible MMPIs illustrated by Fig. (**14**) [225-227]. These mechanism-based MMPIs were found to be highly potent, comparable to the tissue inhibitors of metalloproteinases (TIMPs), and to selectively inhibit gelatinases A and B (MMP-2 and MMP-9, respectively) in an irreversible fashion while sparing other MMPs.

The introduction of novel non-zinc binding MMPIs has opened up new avenues in design rationale. A structurally distinct lead compound with drug-like properties, CL-82198 (**63**), was identified from high throughput screening [228]. This lead compound was a moderate inhibitor (10 μM) for MMP-13, exhibiting no activity against MMP-1, MMP-9, and TACE. NMR studies indicated that inhibitor **63** bound entirely within the S_1' pocket of MMP-13 [228]. In a similar fashion, Morals *et al.* identified two non-zinc chelating

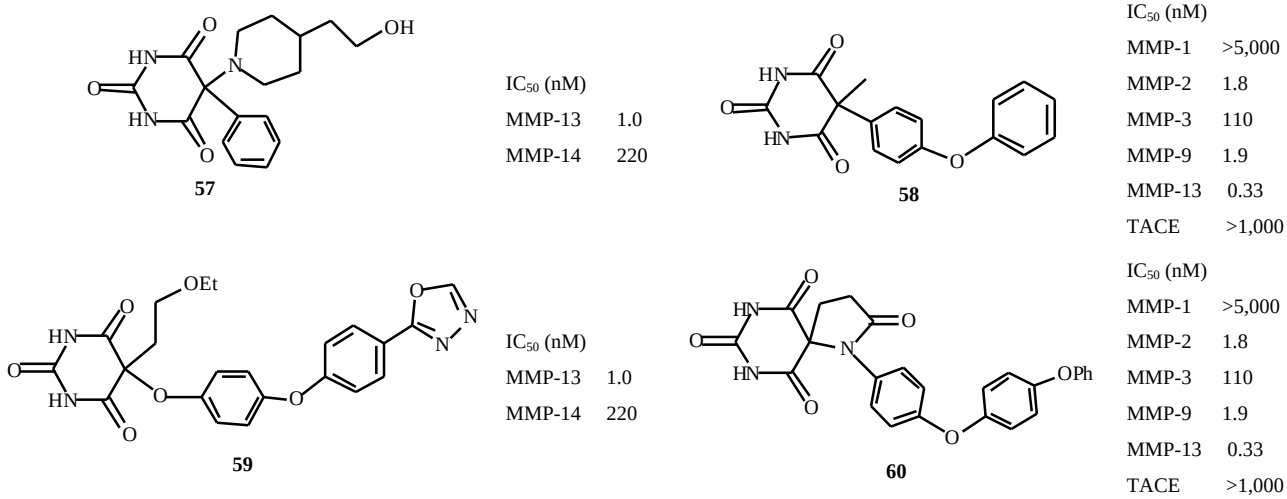


Fig. (12). Inhibitory activity of compounds **57** to **60**.

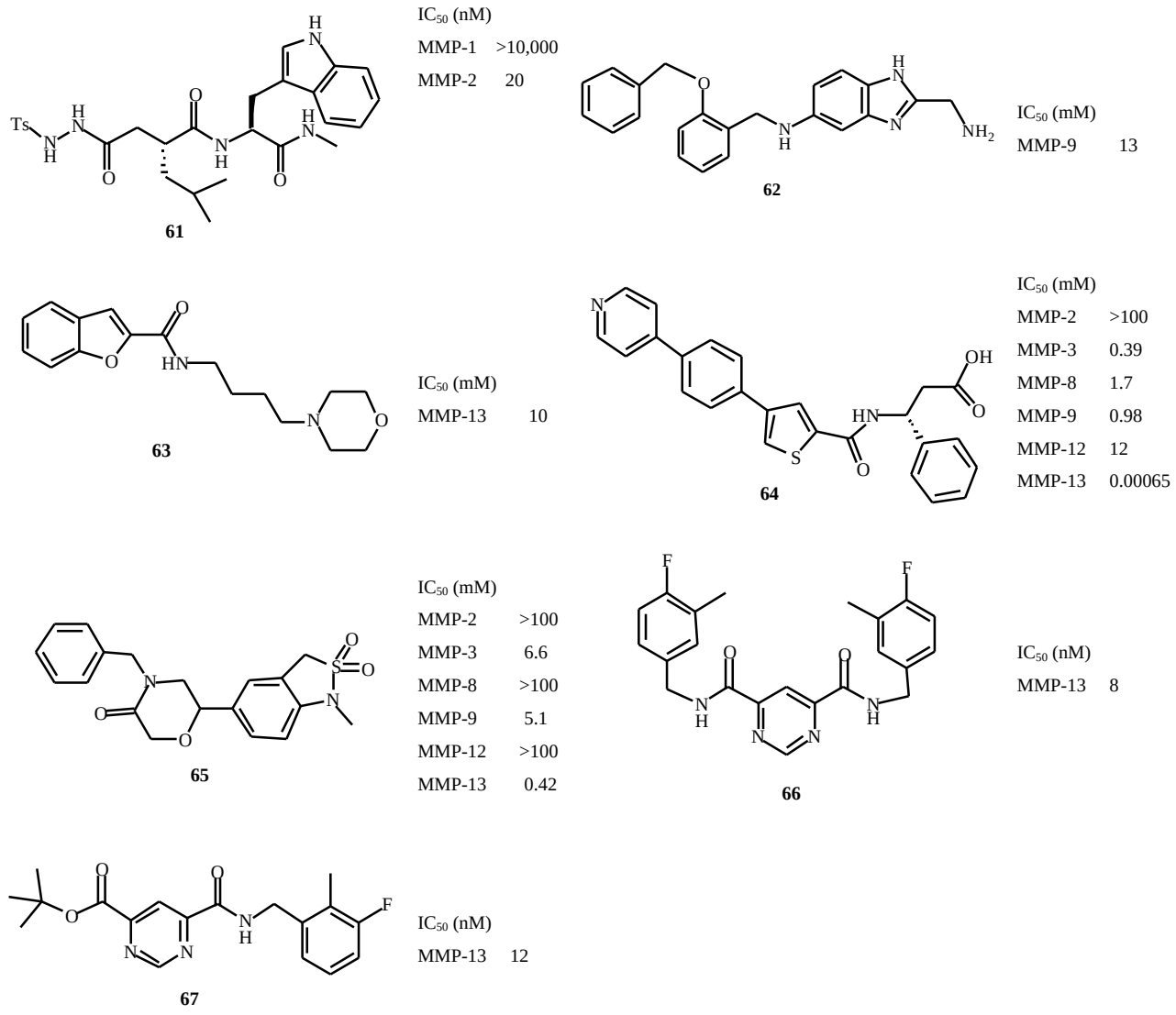


Fig. (13). Inhibitory activity of compounds **61** to **67**.

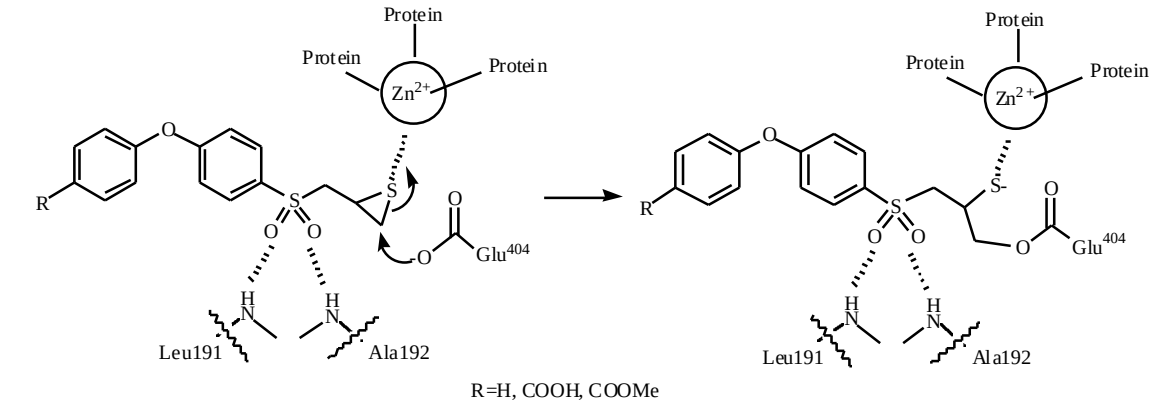


Fig. (14). Mechanism-based gelatinase inactivation by thiirane inhibitors [225-227].

MMP inhibitors, **64** and **65** [229]. The x-ray crystal structures of the MMP-12 - inhibitor complexes revealed that both inhibitors were bound to the active site, partially anchored in the S_1' pocket, while their central morpholinone and thiophene rings were sitting over the catalytic zinc [229]. Pyrimidinedicarboxamides have been reported as highly selective MMP-13 inhibitors, exhibiting no detectable activity against other MMPs (*e.g.* **66**) [230], and high-resolution x-ray structures revealed that their novel binding mode is characterized by the absence of interactions between the inhibitors and the catalytic zinc. These inhibitors bind in the S_1' pocket, and extend into an additional S_1' side pocket that is unique to MMP-13 [230]. Other pyrimidine-dicarboxamides have also been reported to be potent MMP-13 inhibitors (*e.g.* **67**) [231-233].

Cyclic peptides containing the sequence HWGF, isolated from phage display peptide libraries, exhibited some inhibitory activity for MMP-2 and MMP-9, but not for several other MMP family members [234]. One prototype cyclic peptide, CTTHWGFTLC (IC_{50} 5 μM for MMP-2 and 10 μM for MMP-9), inhibited the migration of human endothelial and tumor cell lines. Moreover, it prevented tumor growth and invasion in animal models and improved survival of mice bearing human tumors [234].

CONCLUDING REMARKS

Considerable enthusiasm remains for the promise of clinically beneficial matrix metalloproteinase inhibitors, though it has been somewhat dampened by clinical trials that ultimately yielded disappointing results. While protease inhibition remains fertile ground for continued study, it has become clear that significant barriers remain to be felled, including the identification of specific MMP targets unique to each disease state where MMP inhibition might be advantageous, and the development of orally available MMPIs with good *in vivo* stability, minimal side effects, and a specific inhibition profile. These benefits might also extend to many disease states beyond the scope of this review, particularly in the cases of rheumatoid arthritis and osteoarthritis [235, 236]. An increased understanding of the structure, regulation, and function of the individual MMPs will likely lead to more effective strategies in the development of highly selective inhibitors for any given MMP, combining sophisticated theoretical and experimental approaches to uncover the specific structural and dynamic features that can then be exploited to achieve the desired level of inhibition. Therapeutic benefits derived from proteolytic inhibition remains a viable scheme well worth the investment of time and resources, particularly when this inhibition is paired in rational combinations with other targeted pathway inhibitors. Future medicine may afford effective, personalized therapy targeting a subgroup of MMPs unique to a particular disease condition in a spatial- and temporal-specific manner, while minimizing adverse side effects in patients.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ACE	=	Angiotensin-converting enzyme
CA	=	Caffeic acid
CHA	=	Chlorogenic acid (5-caffeoylquinic acid)
CsA	=	Cyclosporine
DOX	=	Doxycycline
EGCG	=	Epigallocatechin gallate
ECM	=	Extracellular matrix
FN	=	Fibronectin
GST	=	Glutathione S-transferase
IGF	=	Insulin-like growth factor
(MMPs)	=	Matrix metalloproteinases
(MMPI)	=	MMP inhibitor
MMP-1	=	Matrix metalloproteinase-1 (collagenase-1)
MMP-2	=	Matrix metalloproteinase-2 (gelatinase A)
MMP-7	=	Matrix metalloproteinase-7 (matrilysin)
MMP-9	=	Matrix metalloproteinase-9 (gelatinase B)
MT1-MMP	=	Membrane-type 1 matrix metalloproteinase (MMP-14)
MSS	=	Musculoskeletal syndrome
NSCLC	=	Non-small cell lung carcinoma
PDE4	=	Phosphodiesterase 4
SAR	=	Structure-activity relationship
TACE	=	Tumor necrosis factor-alpha converting enzyme (ADAM17, disintegrin and metalloproteinase17)
TGF-β	=	Transforming growth factor-beta
TIMPs	=	Tissue inhibitors of metalloproteinases
TNF-α	=	Tumor necrosis factor-α
UVA	=	Ultraviolet A
VEGF	=	Vascular endothelial growth factor
ZBGs	=	Zinc-binding groups

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ORIGINAL ARTICLE

Coordinated peak expression of MMP-26 and TIMP-4 in preinvasive human prostate tumor

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The identification of novel biomarkers for early prostate cancer diagnosis is highly important because early detection and treatment are critical for the medical management of patients. Disruption in the continuity of both the basal cell layer and basement membrane is essential for the progression of high-grade prostatic intraepithelial neoplasia (HGPIN) to invasive adenocarcinoma in human prostate. The molecules involved in the conversion to an invasive phenotype are the subject of intense scrutiny. We have previously reported that matrix metalloproteinase-26 (MMP-26) promotes the invasion of human prostate cancer cells via the cleavage of basement membrane proteins and by activating the zymogen form of MMP-9. Furthermore, we have found that tissue inhibitor of metalloproteinases-4 (TIMP-4) is the most potent endogenous inhibitor of MMP-26. Here we demonstrate higher (p<0.0001) MMP-26 and TIMP-4 expression in HGPIN and cancer, compared to non-neoplastic acini. Their expression levels are highest in HGPIN, but decline in invasive cancer (p<0.001 for each) in the same tissues. Immunohistochemical staining of serial prostate cancer tissue sections suggests colocalization of MMP-26 and TIMP-4. The present study indicates that MMP-26 and TIMP-4 may play an integral role during the conversion of HGPIN to invasive cancer and may also serve as markers for early prostate cancer diagnosis.

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***Keywords*:** matrix metalloproteinase-26, tissue inhibitor of metalloproteinases-4, high-grade prostatic intraepithelial neoplasia, prostate cancer biomarkers, early diagnosis, immunohistochemistry, co-immunoprecipitation

Introduction

The defining phenotype of malignancy is the cells' ability to invade the surrounding stroma and metastasize. This requires the degradation of several components of the extracellular matrix (ECM). Matrix metalloproteinases (MMPs) are a family of zinc-catalyzed proteolytic enzymes known to digest the basement membrane, ECM components, and cell surface proteins [1]. MMPs have been linked with cancer cell invasion, growth, angiogenesis, inflammation, and metastasis [1-5]. In addition, MMPs have been shown to play a role in the release of growth factors that can in turn contribute to the invasiveness and growth of tumors [6].

Endometase/matrilysin-2 (MMP-26) is a member of the MMP family recently cloned by our group and others [7-10]. MMP-26 is one of the two smallest members of this family, exhibiting minimal domain structure consisting of a catalytic domain and a prodomain, which maintains the enzyme in a latent form prior to its activation. Once active, MMP-26 has been shown to cleave multiple components

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Abbreviations: matrix metalloproteinase (MMP); tissue inhibitor of metalloproteinases-4 (TIMP-4); high-grade prostatic intraepithelial neoplasia (HGPIN); co-immunoprecipitation (co-IP); extracellular matrix (ECM); ductal carcinoma *in situ* (DCIS); infiltrating ductal carcinoma (IDC); atypical intraductal hyperplasia (AIDH); Tris-buffered saline (TBS)

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of the ECM, including fibronectin, type IV collagen, vitronectin, gelatins, and fibrinogen, as well as non-ECM proteins such as insulin-like growth factor-binding protein-1 and α-1 protease inhibitor [7-11]. MMP-26 is also able to activate progelatinase B (pro-MMP-9), an enzyme that plays a critical role in ECM remodeling [12].

MMP-26 mRNA is primarily expressed in epithelial cancers, such as lung, breast, endometrial, and prostate carcinomas [7-10]. Our previous studies have shown that MMP-26 expression in human prostate carcinoma is significantly higher than that in prostatitis, benign prostate hyperplasia (BPH), and normal prostate tissue [12]. In addition, our group has shown that the expression of MMP-26 in human breast tissue is significantly higher during preinvasive ductal carcinoma *in situ* (DCIS) when compared to infiltrating ductal carcinoma (IDC), atypical intraductal hyperplasia (AIDH), and normal breast epithelia adjacent to DCIS and IDC [13]. These results suggest that MMP-26 plays an important role in the early stage prior to the development of invasive breast and prostate cancers.

The activity of MMPs is regulated locally by specific tissue inhibitors of metalloproteinases (TIMPs). Four members of the human TIMP family have been identified as follows: TIMP-1, TIMP-2, TIMP-3, and TIMP-4 [14-17]. The cleavage of synthetic peptides *in vitro* by MMP-26 is inhibited by TIMP-1, TIMP-2, and TIMP-4, with TIMP-4 displaying the greatest inhibitory potency [7, 13]. TIMP-4 is a tight-binding and slow-binding inhibitor of MMP-26, with an apparent K_i value of 0.62 nM [13]. TIMP-4 mRNA has been detected in a variety of normal tissues, including those of the heart, kidney, pancreas, colon, testis, endometrium, and placenta [17-19]. Under normal conditions, MMPs and TIMPs are expressed at low levels in most adult tissues, but may become upregulated in pathophysiologic conditions such as wound healing and tumor progression. Mimicking the expression pattern of MMP-26, TIMP-4 expression in human breast DCIS is significantly higher than that detected in IDC, AIDH, and in normal breast epithelium adjacent to DCIS and IDC [13]. The therapeutic potential of TIMP-4 in the treatment of malignant progression has been examined, and the transfection of TIMP-4 cDNA into human MDA-MB-435 breast cancer cells inhibited tumor cell invasion across *Matrigel*, a barrier of reconstituted basement membrane components [20]. Incubation of these same cells with recombinant TIMP-4 protein displayed a similar inhibitory effect [21].

Much of the cumulative data relating to TIMPs and MMPs in prostate cancer indicate that TIMP expression decreases in cancer, while the ratio of MMPs to TIMPs increases. TIMP-1, -2, and -3 mRNA molecules and proteins were detected in human prostate cancer specimens [22, 23], and TIMP-1 has been shown in various studies to be downregulated in prostate cancer as compared to normal prostate [22, 24]. A study of TIMP-1, -2, and -3 mRNA showed significant reduction of TIMP-2 and -3 in prostate cancer, as well as significant increases in the ratios of MMP-9 to TIMP-1, -2, and -3, and in the ratio of MMP-14 to TIMP-3 [23].

Here, we explore the expression of MMP-26 and TIMP-4 in human prostate tissue. We show that the expression of MMP-26 and its most potent endogenous inhibitor, TIMP-4, are highest in preinvasive high-grade prostatic intraepithelial neoplasia (HGPIN), and that levels of the two proteins decline significantly in adjacent areas of cancer in the same tissues. Moreover, immunohistochemical staining of serially sectioned prostate cancer tissues suggests colocalization of MMP-26 and TIMP-4. These results are analogous to our previous findings in human breast cancer showing that MMP-26 is closely coordinated with TIMP-4, with both proteins showing maximal expression in preinvasive DCIS. The combined data from these investigations indicate that MMP-26 and TIMP-4 may be involved in early carcinoma development and the transformation to an invasive phenotype.

Materials and Methods

Specificity of antibodies

The two MMP-26 antibodies utilized for this study are directed against either the human MMP-26 prodomain sequence of Thr^{50}-Gln-Glu-Thr-Gln-Thr-Gln-Leu-Leu-Gln-Gln-Phe-His-Arg-Asn-Gly-Thr-Asp^{67} or the C-terminal sequence of Gln^{246}-Arg-Ile-Gln-His-Leu-Tyr-Gly-Glu-Lys-Cys-Ser-Ser-Asp^{259}. No peptide with >45% level of identity to these selected sequences was found as determined using the BLAST search method at the National Center for Biotechnology Information website (http://ncbi.nih.gov/BLAST/). These rabbit polyclonal and mono-specific antibodies were tested and verified to be highly specific for MMP-26 [12]. A goat polyclonal antibody against the C-terminal sequence region of MMP-26 (E-14) was obtained from Santa Cruz Biotechnology, and was utilized for the detection of MMP-26 following immunoprecipitation. A mouse monoclonal antibody against MMP-9 (Ab-1) was obtained from Calbiochem (San Diego, CA, USA). The TIMP-4 antibody was directed against the sequence Ser^{61}-Ala-Asp-Pro-Ala-Asp-Thr-Glu-Lys-Met-Leu-Arg-Tyr-Glu^{74}-NH_2. This sequence also shares less than 45% homology with any other known peptide. This rabbit polyclonal and mono-specific antibody was purified, characterized, and its specificity validated as described previously [25].

Western blot

Proteins were extracted from human malignant prostate tissue using T-PER (Pierce, Rockford, IL, USA). Tissue samples were homogenized with T-PER (2 ml per 0.1 g of tissue), and after centrifugation (10 000 × *g*, 5 min), the supernatant was collected. Tissue extracts were subjected to SDS-PAGE, and then blotted onto nitrocellulose blotting membranes (Pall Life Sciences, Pensacola, FL, USA). Membranes were blocked in Tris-buffered saline (TBS) solution containing 0.25% Tween 20 and 5% bovine serum albumin prior

to incubation with primary antibodies. For the detection of MMP-26 directly from tissue extracts, rabbit anti-MMP-26 antibodies were used. For the detection of MMP-26 following immunoprecipitation, a goat anti-MMP-26 antibody (E-14) was used. Membranes were incubated with alkaline phosphatase-conjugated secondary antibodies for 30 min at room temperature, and positive bands were visualized using NBT/BCIP substrates (Fisher Biotech, Fairlawn, NJ, USA).

Co-immunoprecipitation (co-IP)

To examine the interaction between MMP-26 and TIMP-4, prior to treatment of prostate cancer tissue extract with anti-TIMP-4 antibody, preimmune IgG was treated with prostate cancer tissue extract to minimize nonspecific interactions as follows. One microliter of tissue extract was incubated with 1 mg of rabbit preimmune IgG and 20 ml of resuspended protein A/G plus agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C for 1 h. After centrifugation (1000 × *g*, 5 min), the precleared supernatant was collected and the precipitate was used for the detection of MMP-26 as a negative control. Collected supernatant was mixed with 1.5 mg of rabbit anti-TIMP-4 antibody and 20 ml of resuspended protein A/G plus agarose beads at 4°C for 4 h. After centrifugation (1 000 × *g*, 5 min), the supernatant was collected and this precipitate was also used for the detection of MMP-26. Each precipitate was washed four times with phosphate-buffered saline (pH 7.4) followed by centrifugation (1000 × *g*, 5 min). Separated samples were subjected to further analyses by Western blot and zymography. To examine the interaction between TIMP-4 and MMP-9 or MMP-26 and MMP-9, complexes of rabbit anti-TIMP-4 antibody and protein A/G agarose beads or complexes of rabbit anti-MMP-26 antibody and protein A/G agarose beads were used to draw down TIMP-4 or MMP-26, respectively.

Gelatin zymography

Gelatin zymography was performed for MMP-9 detection. For this, 0.5 mg/ml gelatin was co-polymerized into a 10% polyacrylamide resolving gel at the time of casting. Complexes of rabbit anti-TIMP-4 antibody and protein A/G agarose beads or complexes of rabbit anti-MMP-26 antibody and protein A/G agarose beads or supernatant during immunoprecipitation were then subjected to separation by gelatin-SDS-PAGE. Following electrophoresis, the gel was immersed in buffer containing 0.01% Brij-35, 10 mM HEPES/NaOH, 10 mM NaCl, 10 mM $CaCl_2$, 0.1 mM $ZnSO_4$, pH 7.5, at 4 °C for 12 h six times to remove SDS and allow enzymatic gelatin substrate hydrolysis. The gel was stained in 0.025% Coomassie blue in methanol:acetic acid:water (volume ratio of 5:1:4) for 4 h. Finally, the gel was destained with ethanol:acetic acid:water (volume ratio of 1:1:8) for 2 days.

Immunohistochemistry

Formalin-fixed, paraffin-embedded human prostate cancer tissues serially sectioned and fixed on slides were obtained from Mayo Clinic (Jacksonville, FL, USA). All the tissue sections are of the same thickness of 4 mm. The sections were dewaxed with xylene and rehydrated successively in 100% ethanol, 95% ethanol, and distilled water. The slides were then subjected to antigen retrieval by boiling in 0.1 mM citric acid (pH 6.0) for 12 min. The samples were then incubated with primary antibody diluted to 25 μg/ml in blocking buffer (0.2% Triton X-100, 5% normal goat serum, and 3% bovine serum albumin in TBS) for 1 h at room temperature. The primary antibodies used were affinity-purified polyclonal rabbit anti-human pro-MMP-26 and TIMP-4 [12, 25], or were obtained from commercial sources and derived from different species. Purified preimmune IgG from rabbit was used as a negative control. After the primary antibody was rinsed off with TBS, the tissue sections were incubated with an alkaline phosphatase-conjugated secondary antibody (Sigma) diluted 1:1000 in blocking buffer. Signals were detected by adding Fast Red (Sigma). The sections were counterstained lightly with hematoxylin for viewing of nonreactive cells.

Table 1. Study population and staining analysis

Pathology	*n*	Mean intensity	Standard deviation	Standard error
(a) Statistical analysis for MMP-26 reactivity				
Benign	32	34.92	9.85	1.74
HGPIN	27	82.01	14.81	2.85
Cancer	33	61.47	12.61	2.19
(b) Statistical analysis for TIMP-4 reactivity				
Benign	36	31.25	11.11	1.85
HGPIN	28	79.48	15.53	2.93
Cancer	38	57.72	10.39	1.69

The mean color intensity and variation in immunohistochemical staining from a population of 41 patient specimens as described in Materials and Methods (see Immunohistochemistry and evaluation and statistical analysis). *n*, sample size (number of patient samples, out of the original 41, in which the designated pathology was observed). Mean intensity represents the mean of the means, while the standard deviation and standard error values are representative of the mean intensity derived from each individual patient sample for any given pathology/category. Please see Results (Study population) for more detailed description.

Evaluation and statistical analysis

Each slide was examined at 400× using a Nikon Eclipse 50i microscope with a QImaging 1394 QICAM camera system. With the QcapturePro program v. 5.0, images of the most representative areas of non-neoplastic acini, HGPIN (if present), and cancer were photographed, obtaining three separate JPEG images of each. The images were analyzed on a Windows 2000 PC using the public domain NIH Image program, ImageJ, developed at the National Institutes of Health and available on the Internet (http://rsb.info.nih.gov/nih-image/). For each picture, three spatially separate epithelial cytoplasmic areas, excluding the nuclei, were measured. After an outline had been traced around the area using the freehand spline, the Analyze/Measure function was used to record the mean color intensity within the selected area. The mean intensity of at least three separate areas from each patient sample (*n*; Table 1) was then calculated and normalized to correct for background intensity, and one-way between-subjects analysis of variance was performed for each sample type (Benign, HGPIN, Cancer). Statistical analysis of low-grade prostatic intraepithelial neoplasia (LGPIN) was not feasible because of its insufficient occurrence in the examined tissues.

Results

Study population

The initial study population consisted of 41 patient samples of prostate cancer that had been serially sectioned. The specimens had Gleason scores ranging from 2 to 10, exclusive of Gleason score 3. As only one patient sample representative of Gleason scores 2 or 10 was available, these samples were excluded from the study and subsequent statistical analyses. Some slides had cancer but not benign tissue, or vice versa. Not every case had HGPIN. Some pathology was not clearly interpretable. In addition, during the immunostaining process some of the tissue sections were dislodged from the slides, and not every sample contained all three types of epithelium, resulting in small fluctuations in the total number of patient samples evaluated in each category. After immunostaining of the tissues with antibodies against MMP-26 or TIMP-4, three categories of epithelium were scored based upon staining intensity, showing significant differences ($p<0.0001$) in paired comparisons of non-neoplastic (benign), HGPIN, and cancer. The characteristics of the specimens evaluated, the resultant mean scores of their normalized values, and their deviations are detailed in Table 1.

Detection of pro- and active forms of MMP-26 by Western blotting

Unlike other MMPs, pro-MMP-26 proceeds to auto-activation via cleavage sites that are upstream of the cysteine-switch motif [11, 26], and N-terminal sequencing of auto-activated MMP-26 reveals several cleavage sites at $Leu^{49}\downarrow Thr^{50}$ [11], $Gln^{59}\downarrow Gln^{60}$ [26], and $Ala^{75}\downarrow Leu^{76}$ [11]. Comparison of the antigen peptide sequence designed for the prodomain of MMP-26 (Thr^{50}-Gln-Glu-Thr-Gln-Thr-Gln-Leu-Leu-Gln-Gln-Phe-His-Arg-Asn-Gly-Thr-Asp^{67}) with the N-terminal sequences of auto-activated MMP-26 reveals that the anti-prodomain MMP-26 antibody can detect both the zymogen and activated forms of MMP-26. Figure 1 demonstrates that both the anti-prodomain and anti-C-terminal MMP-26 antibodies can recognize both the proform and the activated form of bacterially expressed MMP-26. For reasons that are not clear, the anti-C-terminal MMP-26 antibody is more sensitive than the anti-prodomain antibody. Prostate cancer tissue extracts reveal the presence of both pro- and activated MMP-26. The anti-prodomain MMP-26 antibody is capable of detecting many forms of activated MMP-26 in the prostate cancer tissue extracts, whereas the anti-C-terminal MMP-26 antibody is able to detect only one form of activated MMP-26. The molecular weights of the activated forms in the tissue extract show little variation from the positive bands for bacterially expressed MMP-26.

Detection of MMP-26 and TIMP-4 complex by co-IP

Complexes of the rabbit anti-TIMP-4 antibody and protein A/G agarose beads were used to pull down TIMP-4 from prostate cancer tissue extracts. To examine the possibility of complex formation between MMP-26 and TIMP-4 in these extracts, a goat anti-MMP-26 antibody was used to probe for MMP-26 within the immunoprecipitates and in the retained supernatant. Figure 2 shows positive signals for MMP-26 in the supernatant fraction and perhaps in the anti-TIMP-4 immunoprecipitate. The majority of MMP-26 was found in the supernatant, and a very small fraction of MMP-26 was complexed with TIMP-4, as a very faint MMP-26 band was detected in the anti-TIMP-4 fraction (IP_anti-TIMP-4) when compared to the immunoprecipitates recovered from the preclearing phase (IP_rabbit IgG). A goat polyclonal antibody (E-14, Santa Cruz Biotechnology) against near the C-terminal sequence region of MMP-26 reveals a band similar to that observed with the rabbit anti-C-terminal MMP-26 antibody. Although antibodies from different species were utilized, a mouse alkaline phosphatase-conjugated anti-goat secondary antibody interacted with the rabbit antibody, resulting in the detection of the heavy (above 48 kDa) and light (27 kDa) chains of rabbit IgG.

Detection of pro- and active MMP-9 by gelatin zymography

Expression of MMP-9 from prostate cancer tissue ex-

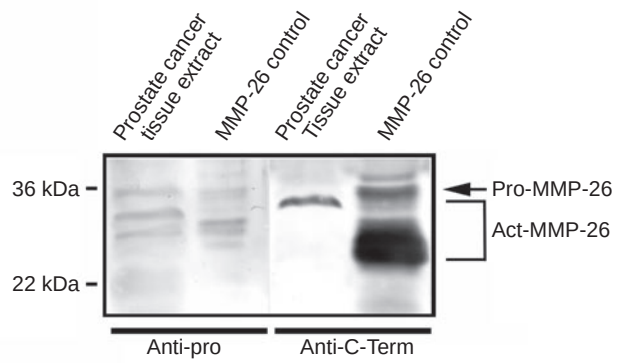

Figure 1 Western blot MMP-26 detection from human prostate cancer tissue extracts using rabbit anti-prodomain and anti-C-terminal MMP-26 antibodies. Bacterially expressed MMP-26 was used as a control. Sequence comparisons between the MMP-26 prodomain epitope and N-terminal analyses of activated MMP-26 shows overlap of those peptide sequences. As a result, the anti-pro-MMP-26 antibody recognizes both the pro- and activated (act-) forms of MMP. The anti-C-terminal MMP-26 antibody demonstrates a higher sensitivity for MMP-26 than the anti-pro-MMP-26 antibody, but recognizes only a single active form in the tissue extract.

tracts was not detected by Western blot analysis perhaps due to its low concentration or low antibody sensitivity (data not shown). Precipitated TIMP-4/antibody/protein A/G complexes or MMP-26/antibody/protein A/G were used for zymography. MMP-9 was not detected in either the TIMP-4 precipitate (IP_anti-TIMP-4) or the MMP-26 precipitate (IP_anti-MMP-26). Pro- and active MMP-9 remained in the supernatants (TIMP-4 supernatant, MMP-26 supernatant) (Figure 3). Expression levels of MMP-9 were very low in these prostate cancer tissues. The majority of MMP-9 presented as the proform with very little activated MMP-9 detected.

Immunohistochemistry to detect MMP-26, TIMP-4, and MMP-9

Immunohistochemical analysis revealed that MMP-26 was present in the prostatic epithelium as well as in the surrounding stroma (Figures 4A–C). The data showed that MMP-26 expression in HGPIN and cancer was significantly higher than that seen in non-neoplastic epithelia ($p<0.0001$). Furthermore, the expression of MMP-26 was maximal in HGPIN, and was found to be expressed at significantly lower levels ($p<0.001$) in cancer within the same tissue specimens (Figure 5A). MMP-26 expression in cancer displayed no correlation with Gleason score during this study (data not shown). The expression of TIMP-4 was also detected in the prostatic epithelium as well as in the surrounding stroma (Figures 4D–F), in a pattern that mimicked that of MMP-26. TIMP-4 expression in HGPIN and cancer was again significantly higher than that seen in non-neoplastic epithelium ($p<0.0001$), and furthermore, the expression of TIMP-4 was highest in HGPIN, with significantly reduced expression ($p<0.0001$) in cancer within the same tissue specimen (Figure 5B). As before, the expression of TIMP-4 in HGPIN and cancer was not correlated with Gleason score (data not shown). Prostate cancer tissue revealed no positive signals for the expression of MMP-9 using a mouse monoclonal antibody against MMP-9 (Ab-1) from Calbiochem (data not shown), in accordance with the low levels of MMP-9 expression seen during the gelatin zymography assays.

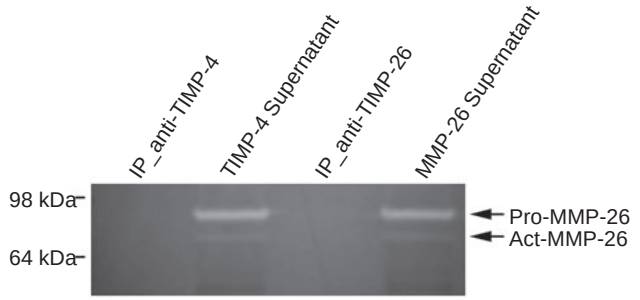

Figure 3 Gelatin zymography of TIMP-4 immunoprecipitate from prostate cancer tissue extracts to detect MMP-9. Gelatin (0.5 mg/mL) was co-polymerized into a 10 % polyacrylamide resolving gel. IP_anti-TIMP-4 and IP_anti-pro-MMP-26 designate the precipitates following immunoprecipitation with anti-TIMP-4 and anti-pro-MMP-26 antibodies, respectively, in the presence of protein A/G agarose beads. TIMP-4 supernatant and MMP-26 supernatant designate the retained supernatant following the procedures detailed above. MMP-9 was detected only in the supernatant, and failed to form a complex with either the TIMP-4 or MMP-26 antibodies.

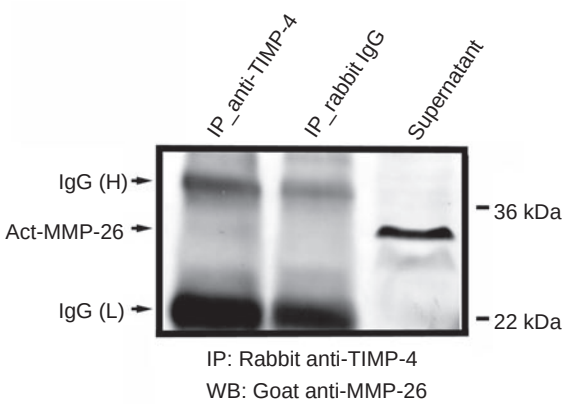

Figure 2 Western blot (WB) of immunoprecipitates (IP). TIMP-4 was drawn down using rabbit anti-TIMP-4 antibodies, and the precipitates were then probed with a goat anti-MMP-26 antibody against the C-terminal. A strong MMP-26 band was detected in the retained supernatant fraction (supernatant), while a very weak band was detected in the precipitate (IP_anti-TIMP-4). Blotting of the precipitates from preclearing with rabbit preimmune IgG were completely clear (IP_rabbit IgG). In both cases, light (27 kDa) and heavy (48 kDa) IgG chains, i.e. IgG (L) and IgG (H), were also visualized.

Semi-colocalization between TIMP-4 and MMP-26

Serial sections of prostate cancer tissue were used to determine if any areas of colocalization exist between MMP-26 and TIMP-4. Figure 6 shows areas of high TIMP-4 expression that correspond to areas of high MMP-26 expression, suggesting colocalization between these enzymes, while adjacent control sections stained with rabbit preimmune IgG produced no positive signals.

Discussion

MMPs and TIMPs have been associated with invasion and metastasis in many human cancers, and evidence is growing that MMP-26 and TIMP-4 are important factors

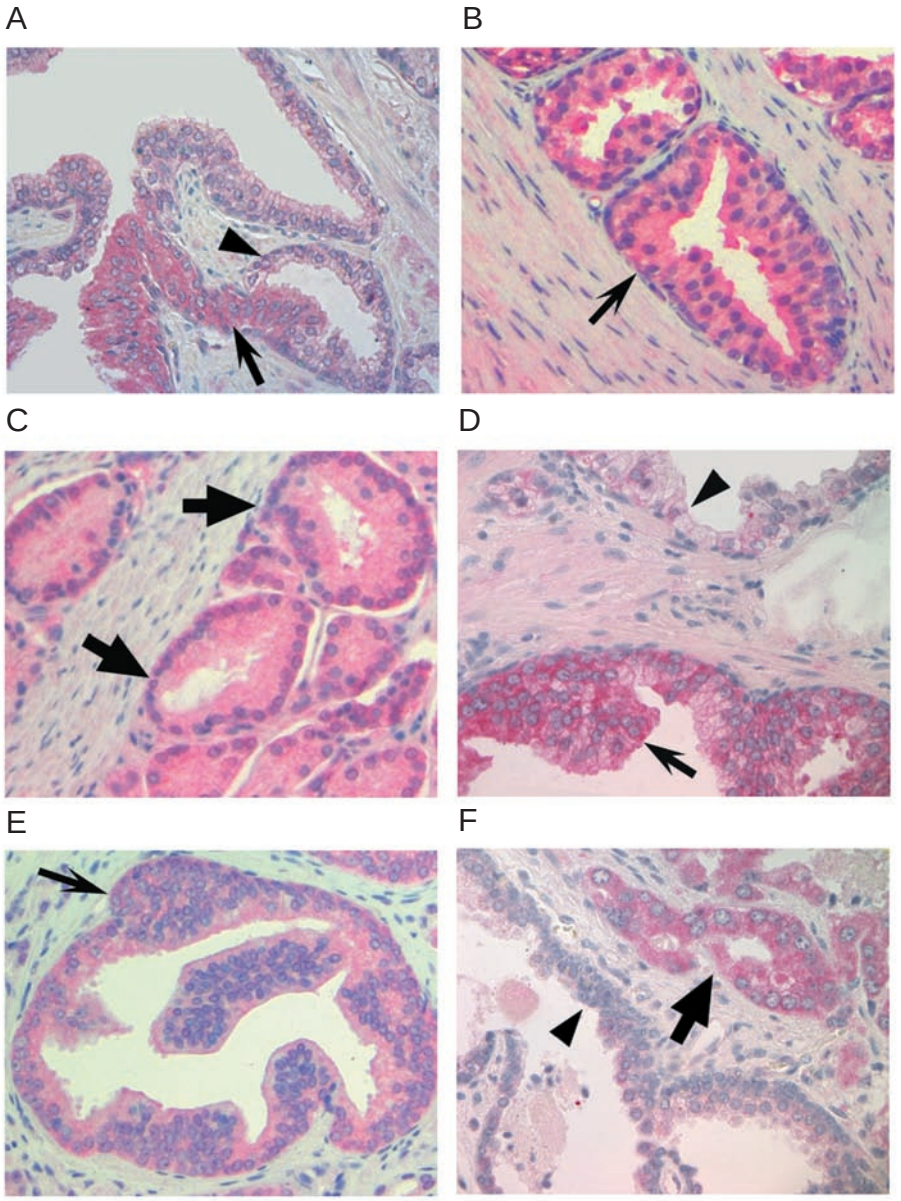

Figure 4 MMP-26 and TIMP-4 immunohistochemical staining in non-neoplastic epithelium (arrowhead), HGPIN (thin arrow), and cancer (thick arrow). **(A–C)** MMP-26 immunostaining. **(A)** HGPIN is stained more intensely than the adjacent non-neoplastic epithelium. The areas left and above the region of HGPIN are non-neoplastic tissues representing partial glandular involvement of a benign acinus; **(B)** another example of intense HGPIN staining; and **(C)** positive staining in cancer tissues. **(D–F)** TIMP-4 immunostaining. **(D)** HGPIN is stained more intensely than non-neoplastic epithelium; **(E)** a second example of intense HGPIN staining; and **(F)** regions of cancer exhibit more intense staining than non-neoplastic epithelium. All pathologic diagnoses were performed by KAI in accordance with established criteria [27].

during prostate cancer progression. The epithelium of normal and preinvasive human prostate tissues is physically separated from the stroma by two continuous layers: first by basal cells, and second, by a basement membrane. Disruption of both the basal cell layer and the basement membrane are prerequisites for carcinoma cell invasion. In this study, we show that the levels of MMP-26 and TIMP-4 in human prostate tissues are significantly higher in HGPIN than either non-neoplastic epithelium or cancer. In addition, we show that the levels of MMP-26 and TIMP-4 reach their

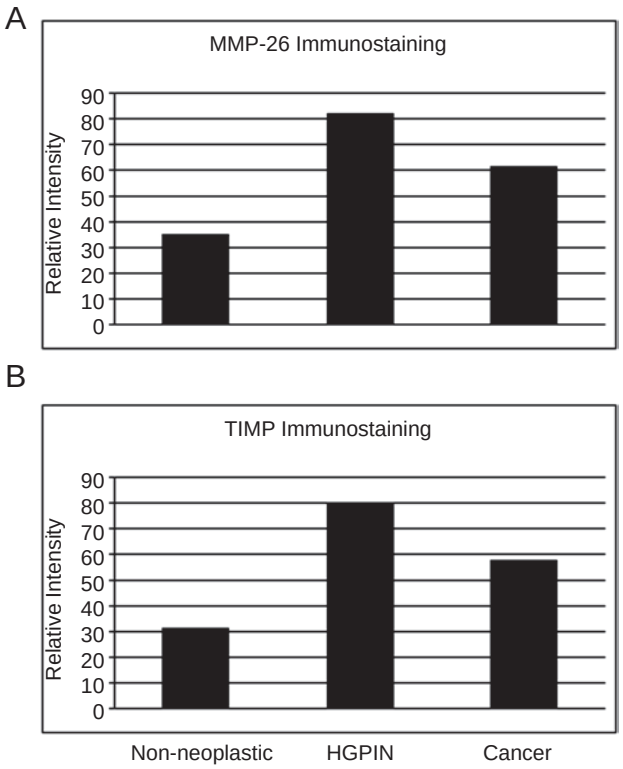

Figure 5 Analyses of MMP-26 and TIMP-4 immunostaining in non-neoplastic ducts and acini, HGPIN, and cancer. **(A)** MMP-26 immunostaining. HGPIN stained more intensely than non-neoplastic epithelium ($p<0.0001$) or cancer ($p<0.001$) in the same tissues, with the least intense staining observed in benign acini. **(B)** TIMP-4 immunostaining. Findings mimicked those of MMP-26, with HGPIN staining more intensely than non-neoplastic epithelium ($p<0.0001$) or cancer ($p<0.0001$), and the least intense staining again found in benign acini. Notably, significant differences ($p<0.0001$) were found between every pair of tissue categories analyzed. Standard error values were of such small magnitude as to warrant their omission (see Table 1).

maximum in HGPIN, then decline significantly as a duct or acinus progresses from HGPIN to cancer. Furthermore, the parallel expression patterns of MMP-26 and TIMP-4 indicate that they are highly coordinated throughout malignant transformation.

MMP-26 is a multifaceted enzyme with many direct ECM targets as well as non-ECM protein targets including insulin-like growth factor-binding protein-1 and α-1 protease inhibitor [7-10]. Previous results from our group revealed that MMP-26 promoted the invasion of human prostate cancer cells through both type IV collagen and fibronectin, not only through the direct proteolytic cleavage of these proteins but also through the activation of pro-MMP-9, a highly efficient enzyme with multiple targets in the ECM [12]. While these current results show that the expression of MMP-9 in prostate cancer tissues was not detectable by Western blot, low levels of MMP-9 expression were detected by zymography, primarily in the zymogen form.

TIMP-4 is a potent inhibitor of MMP-26 [7, 13], and transfection of human breast cancer cells with TIMP-4 cDNA showed that TIMP-4 could significantly inhibit cancer cell invasion across a *Matrigel* barrier [20]. Furthermore, our group has shown that MMP-26 and TIMP-4 are colocalized in human breast carcinoma tissue [13]. While our current co-IP studies show only scant evidence of TIMP-4/MMP-26 complex formation, immunostaining of serially sectioned prostate cancer tissues does reveal areas of possible colocalization between MMP-26 and TIMP-4. TIMP-4 is a tight-binding and slow-binding inhibitor of MMP-26 *in vitro*, with an apparent K_i value of 0.62 nM [13], but *in vivo*, and during the co-IP analyses, the presence of additional proteins might interfere with the formation of a TIMP-4/MMP-26 complex. Taken as a whole, these findings combine to suggest that MMP-26 and TIMP-4 might

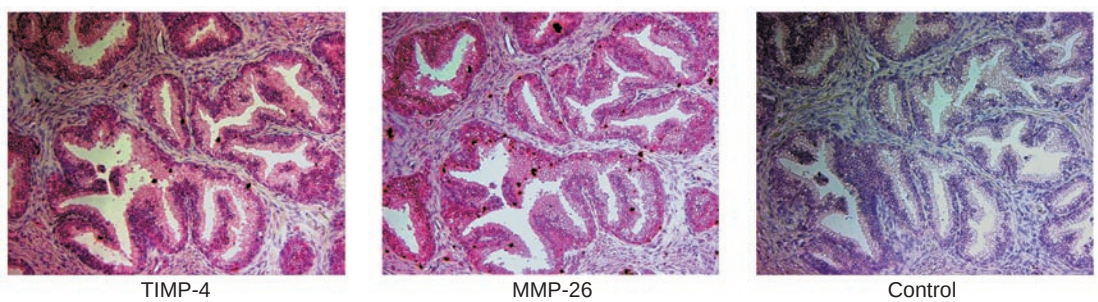

Figure 6 Immunohistochemical staining of serially sectioned human prostate cancer tissues containing HGPIN reveals similar patterns of staining for TIMP-4 and MMP-26 on adjacent sections. Rabbit preimmune IgG control using another adjacent section is negative.

participate in interactions that ultimately affect both the surrounding ECM and the activity of other proteins critical to the progression of *in vivo* tumor growth.

The finding that the levels of MMP-26 and TIMP-4 are highest in HGPIN is significant because HGPIN is considered the preinvasive precursor of prostate cancer. HGPIN shares malignant cytologic features with cancer, but lacks the stromal invasion that characterizes cancer. The progression of HGPIN to cancer is associated with progressive disruption of the basal cell layer and focal loss of the basement membrane [27]. Our current findings are analogous to our previous studies in breast cancer that show the highest expression of MMP-26 and TIMP-4 in DCIS, with lower expression present in normal breast epithelium and invasive cancer [13]. Prostatic HGPIN is the preinvasive analog of DCIS. These data also correlate with studies of endometrial cancer, where MMP-26 and TIMP-4 expression was shown to decrease with the loss of histologic differentiation [18, 28]. Furthermore, our study can be correlated with an investigation of squamous cell cancer (SCC), which found the highest expression of MMP-26 in low-grade SCC and an absence of MMP-26 expression in dedifferentiated high-grade SCC [29]. Taken together, these data indicate that MMP-26 and TIMP-4 may play important roles in facilitating the initial conversion to an invasive phenotype.

Disruption of the basement membrane is one of the final steps that allow pathologically transformed epithelial cells to become invasive. The basement membrane or basal lamina that encircles a normal or preinvasive human prostate duct or acinus is composed of proteins that include laminin, type IV collagen, entactin, glycosaminoglycans, and heparin sulfate proteoglycans. MMP-26, in conjunction with one of its cleavage products, active MMP-9, efficiently cleaves many components of the basement membrane [12]. MMP-26 may be able to gain access to cleavage sites within the basement membrane through focal disruptions in the basal cell layer. The maximal expression of MMP-26 in HGPIN, with subsequent reduced levels in cancer, indicates that this enzyme may be temporally regulated. As MMP-26 expression is lost, invasive tumors may then begin expressing other enzymes better suited for late-stage invasion and metastasis.

The observed trend in TIMP-4 expression mimicked that of MMP-26, with its highest expression in HGPIN and significantly decreased expression in cancer. This parallel protein regulation differs from many previous studies that showed differential expression patterns between MMPs and TIMPs in human prostate cancer, and may indicate the existence of different regulatory mechanisms among the TIMPs. One hypothesis for the coordinated expression of MMP-26 and TIMP-4 is that as the expression of the weaker inhibitors of MMP-26 decline, TIMP-4 may be left as the sole compensatory mechanism to overcome the increased activity of MMP-26. The increase in the levels of MMP-26 may correlate with the increased expression of TIMP-4 in a positive feedback manner so that the pathologically upregulated activity of MMP-26 might require a higher set-point of TIMP-4. We have recently reported a similar coordinated expression pattern of MMP-26 and TIMP-4 in human breast cancer [13], and co-regulation between MMP-26 and TIMP-4 in cycling endometrium and in endometrial cancer has also been reported [18, 28, 30]. Progression to invasive cancer may occur when the upregulation of TIMP-4 in response to increased levels MMP-26 is no longer able to counteract the proinvasive influence of MMP-26. Once the basement membrane has been ruptured, however, cancer cells may become less dependent upon the activity of MMP-26, resulting in a decline in the expression of this enzyme along with that of its highly coordinated inhibitor, TIMP-4.

The present study indicates that MMP-26 and TIMP-4 may play important roles in the initial conversion of prostate cancer cells to an invasive phenotype, and that these two proteins are highly coordinated throughout this transformation. The high levels of MMP-26 and TIMP-4 in HGPIN suggest their use as biomarkers for the early diagnosis of a subset of patients with HGPIN before there is invasive tumor and metastasis. The timely identification of early-stage prostate tumor might lead to novel strategies for treatment that would halt or delay the progression of the disease. Although the implications of the coordinately high expression of MMP-26 and TIMP-4 in human HGPIN are not completely understood, this study reveals the importance and necessity of future studies into the complex roles and interactions of MMP-26 and TIMP-4 in human prostate cancer.

Acknowledgments

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