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

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During the initial phases of carcinoma cell invasion, 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). Uria and López-Otin (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-repressed prostate cancer cell line designated as ARCaP, was selected 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.

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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/matriplysin; MMP-9, matrix metalloproteinase-9/gelatinase B; MMP-26, matrix metalloproteinase-26/endometase/matrilysin-2; MMPs, matrix metalloproteinases; CAPS, 3-cyclohexylamino-1-propanesulfonic acid.

2 This work has explored a putative biochemical mechanism by which endometase/matriplysin-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 Ala93–Met94 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.

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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, pancreatic, 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 (FN) and type IV collagen, with gelatinase activity. 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% CO2 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, 0.1% Triton X-100, 0.01% Brij-35) at 37 °C. For the Coomassie 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 FN (0.25 mg/ml) and MMP-9 (final concentration 0.05 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% bromophenol blue) and boiled for 5 min. Following electrophoresis on a 10% 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 ProBlottTM polyvinylidene difluoride membranes (Applied Biosystems) using CAPS buffer (10 mM CAPS, pH 11, 0.09% SDS). Proteins were visualized by staining with 20% Ponceau S followed by 20% 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 Transciptase-PCR Analysis—RNA was extracted from the original cells according to manufacturer protocols (Invitrogen, Carlsbad, CA), and 2 μg of total RNA were subjected to reverse transcrip-tase-PCR according to the standard protocol provided with the PCR kit (Invitrogen Corp., Carlsbad, CA). The MMP-26 forward primer was 5′-ACCATGGACCTGCTGATCACTAAGAG-3′; the reverse primer was 5′-AGGTATTCAGGACGAAATTCTTCC-3′; for glycolaldehyde-3-phosphate dehydrogenase, the forward primer was 5′-AGGAGAGTGGTGCTATGGGG-3′; and the reverse primer was 5′-TGTATTTGAGGAGATCTCGC-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 μl/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 a 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 Thr6-Glu-Thr-Glu-Thr-Glu-Leu-Leu-Glu-Glu-His-Arg-Asn-Gly-Thr-Asp20, and the sequence selected from the metalloproteinase domain was Asp58-Lys-Asn-Glu-His-Trp-Ser-Ala-Lys-Lys-Glu-Ser-Asp-Thr-Gly-Tyr-Aas260 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 matri lyasin (MMP-7), stromelysin (MMP-3), gelat inase A (MMP-2), gelatinase B (MMP-9), and some other proteins (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 using an alkaline phosphatase-conjugated secondary antibody (Jackson Immunolab, Plainsboro, NJ) diluted (1:2000) in the blocking buffer for 4 h at room temperature. The blot membranes were then scanned, and the signal intensities were measured by integrated morphometry analysis (IMA) (Metamorph System, version 4.65) 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% acetic acid 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 dehydrated 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 immunohistochemistry) or goat anti-human MMP-9 antibody (25 μg/ml for immunohistochemistry, R&D Systems, Minneapolis, MN) at 4 °C overnight and then incubated with an 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®/Blue-1 Uni with a FLAG tag at its C-terminal as described (22). Following confirmation of the expression, plasmids 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 (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 was 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 × 10⁵ 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. Cells were counted by IMA for MMP-26 and MMP-9 treated cells to preimmune IgG or parental cells, respectively, which 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 a negative control 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 intensity of MMP-26 staining was the highest in human prostate carcinoma (15 patient cases, Gleason grades 5–7). The immunostaining intensity of MMP-26 alone demonstrated weak cleavage of FN (Fig. 1C, lane 4). N-terminal sequencing showed that the 86-kDa protein had the sequence of MRTPR, which is the same N terminus as reported during activation of pro-MMP-9 by HgCl₂ (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. 1C, lane 6), whereas pro-MMP-9 exhibited no cleavage of FN (Fig. 1C, lane 7). Once activated by MMP-26, MMP-9 cleaved FN very effectively, generating at least 6 new bands (Fig. 1C, 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), and prostate cancer (p < 0.05), low in prostatitis (9 cases), and was very low in benign prostate hyperplasia (BPH) (12 cases) and normal prostate gland tissues (7 cases) (Fig. 2A). Densitometric and statistical analysis (Fig. 2B) 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. 2B).

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. 3A). 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. 3B). 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 N⁰⁰⁴GTD and N⁰⁰⁴QSS. 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

**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. 1C, lane 4). N-terminal sequencing showed that the 86-kDa protein had the sequence of MTTPRXG, which is the same N terminus as reported during activation of pro-MMP-9 by HgCl₂, human fibroblast-type collagenase (MMP-1), 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. 1C, lane 6), whereas pro-MMP-9 exhibited no cleavage of FN (Fig. 1C, lane 7). Once activated by MMP-26, MMP-9 cleaved FN very effectively, generating at least 6 new bands (Fig. 1C, lane 8).

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not detected in the DU145, LNCaP, or PC-3 cell lines (Fig. 3B), 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. 3C) 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 ($\mu$g/ml) (46.0%) for the MMP-26 antibody (Fig. 4A), and at concentrations of 10 (55.9%) and 25 ($\mu$g/ml) (53.1%) for the MMP-9 antibody (Fig. 4B), 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 ($\mu$g/ml) (18.8%) for the MMP-26 antibody (Fig. 4A), and at concentrations of 10 (52.2%) and 25 ($\mu$g/ml) (28.0%) for the MMP-9 antibody (Fig. 4B), when compared with the preimmune IgG. Antibody targeting the pro-domain of MMP-26 also significantly decreased the invasive potential of ARCaP cells through
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. 5A). 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. 5B).

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. 6A), but without a marked difference (\(< p > 0.05\)) in their invasive potentials (Fig. 6B). 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. 7A). 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. 7B).
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 colocalization between MMP-26 and MMP-9. Confocal laser scanning microscopic analysis revealed colocalization of both proteins in the cytoplasm of parental ARCaP (Fig. 8A, a–d) and sense-transfected cells (Fig. 8A, e–h), but not in the antisense-transfected cells (Fig. 8A, 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. 8A, m–p). MMP-26 and MMP-9 proteins were also found to be co-expressed in human prostate carcinoma tissue samples (Fig. 8B).

**DISCUSSION**

MMP-26 is able to activate MMP-9 by cleavage at the Ala96–Met45 site of the pro-MMP-9, which is the same cleavage site detected previously during activation with HgCl2 (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 (Cys99)-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 can activate MMP-9 but their cleavage sites in pro-MMP-9 are different. Matrilysin cleaved MMP-9 at two sites, Glu59–Met90 and Arg106–Phe107 of the propro-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
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 aaqueous 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\(^{89}\), which left the "cysteine-switch" sequence (PHC\(^{50-52}\)GVPD) intact (37), and suggests that Cys\(^{89}\) may not play a role in the latency of the zymogen form. Another group has demonstrated that autolytic activation of MMP-26 occurred at LIG\(^{29}\) \(\rightarrow\) Q\(^{30}\)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\(^{57}\) region of MMP-26, and the resultant antibody complex shields the autocoelavage 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 control, suggesting that this anti-sense 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 cascade suggest that MMP-26 may be a novel marker for certain types of prostate carcinomas, and perhaps a new therapeutic molecular target for prostate cancer.

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