Expression of the Metalloproteinase Matrilysin in DU-145 Cells Increases Their Invasive Potential in Severe Combined Immunodeficient Mice

William C. Powell, J. David Knox, Marc Navre, Tom M. Grogan, John Kittelson, Raymond B. Nagle, and G. Tim Bowden


ABSTRACT

Human prostate cancer displays a high degree of variability in its rate of spread, which could be due largely to differences in the invasive potential of the tumor cells. The degradation of the basal lamina and stromal extracellular matrix is mediated in part by the secretion of matrix metalloproteinases (MMPs). Matrilysin (PUMP-1, MMP-7) and gelatinase A (M,, 72,000 type IV collagenase, MMP-2) have been shown to be overexpressed in prostate carcinoma. We have expressed the single MMP matrilysin in the tumorigenic but nonmetastatic human prostate tumor cell line DU-145 to determine if matrilysin has a functional role in prostate tumor cell invasion. DU-145 cells expressing matrilysin were significantly more invasive than vector-only transfected cell lines as assayed by a severe combined immunodeficient mouse model of tumor cell invasion. Vector-only transfected DU-145 cells injected i.p. into severe combined immunodeficient mice invaded the diaphragm in only 1 of 9 mice (11%), whereas matrilysin-transfected DU-145 cells invaded the diaphragm in 12 of 18 mice (66%). The difference between the controls and matrilysin-transfected cells was statistically significant (P < 0.006). These results suggest a functional role for matrilysin in the initial invasion of prostate cancer through the epithelial basal lamina and into the surrounding stroma.

INTRODUCTION

Prostate cancer is now the most common cancer afflicting U.S. males, exceeding lung cancer for the first time (1). It is estimated that 122,000 new cases of prostate cancer will have been diagnosed in 1991, and mortality from prostate cancer (32,000 cases in 1991) is the second leading cause of cancer-related deaths in U.S. males (2). These statistics are important because a large segment of the male population is about to enter the age group that prostate cancer afflicts. Prostate cancer is very rare until age 50, but then its rate abruptly rises (2). It is estimated that the percentage of men over age 55 will increase from 18% to 25% of the total male population by the year 2010 (3). This increase in the number of men will cause prostate cancer to become a large public health problem (4). Prostate cancer displays a high degree of biological variability, ranging from occult disease incidentally found during transurethral resection of the prostate or autopsy to highly invasive, aggressive cancer which metastasizes to the regional lymph nodes and bone and is followed rapidly by death. When adenocarcinomas are found by routine rectal examination, needle biopsy in patients with elevated prostate-specific antigen, or incidentally in transurethral resection of prostate specimens there is no current method for determining whether this tumor will remain latent for the patient’s lifetime or invade and metastasize within 5 years. It is apparent that this variability manifests itself in the extent of tumor cell invasion through the epithelial BL (5).

The process by which neoplasms metastasize is composed of a complex series of events (6, 7). One of the initial steps in tumor cell invasion is the degradation of the BL and local invasion of the surrounding tissue. To move through the BL, cells must secrete proteinases that are able to degrade the components of the BL. The matrix metalloproteinases are a multigene family of secreted proenzymes, the substrates of which are the proteins that make up the BL and the extracellular matrix (8). The family is divided into three subclasses based on their homology and substrate specificity (9). The three subclasses are the collagenases, the gelatinases, and the stromelysins. The activated form of these MMPs can be inhibited by proteins known as tissue inhibitors of metalloproteinases (6-8). Currently there are two known human TIMPs, TIMP-1 and TIMP-2. TIMP-1 inhibits all of the MMPs but preferentially inhibits gelatinase B (M, 92,000 type IV collagenase, MMP-9), TIMP-2 inhibits gelatinase B, preferentially inhibits gelatinase A, and may inhibit other members of the MMP family (10, 11). Tight regulation of the expression and activity of both MMPs and TIMPs maintains cells in a noninvasive phenotype. A change altering the balance in favor of MMP activity may be one of the initial steps in tumor cell invasion.

A study by Fuchs et al. (5) showed that there was a progressive loss of BL with increasing grade of human prostate carcinoma. These results support the hypothesis that MMP expression may be indicative of the progression of prostate cancer from a benign to a malignant state. Two members of the MMP family of enzymes, matrilysin (PUMP-1, MMP-7) and the gelatinase A (M, 72,000 type IV collagenase, MMP-2), have recently been shown to be expressed at the messenger RNA level in 76% and 60% of primary prostate carcinomas, respectively (12). This compares to an incidence of 27% for matrilysin and less than 25% for the gelatinase A expression in normal human prostate (12).

Matrilysin was originally cloned and described as a putative metalloproteinase (PUMP-1) by Muller et al. The cDNA clone was isolated from a cDNA library made from a pool of mRNAs from human tumors that hybridized to the rat stromelysin cDNA probe (13). Matrilysin has since been shown to be a true metalloproteinase with a substrate specificity that includes casein; gelatins of types I, III, IV, and V; fibronec-tin; elastin; and proteoglycans (14-16). Matrilysin has also been shown to be expressed in human breast and colon cancers (17, 18). To further understand the molecular basis for biological variability in human prostate cancer and the functional role of MMPs in prostate tumor cell invasion we transfected the matrilysin cDNA into DU-145, a prostate tumor cell line which does not constitutively express the endogenous matrilysin gene. The DU-145 cell lines overexpressing this metalloproteinase were tested for their in vivo invasive potential by i.p. injection into SCID mice.

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1 Supported by American Cancer Society Grant PDT-388 (R. B. N.) and NIH Grant CA40584 (G. T. B.). This work is also supported in part by the Arizona Disease Control Commission.

2 To whom requests for reprints should be addressed, at Department of Radiation Oncology, University of Arizona Medical School, 1501 N. Campbell Ave., Tucson, AZ 85724.

3 Present address: Affymax Research Institute, 4001 Miranda Ave., Palo Alto, CA 94304.
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MATERIALS AND METHODS

Cells and Cell Culture. The human prostate tumor cell line DU-145 was obtained from the American Type Culture Collection (Rockville, MD). DU-145 cells were cultured in MEM (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Gibco), penicillin (50 units/ml), and streptomycin sulfate (500 µg/ml) in 10% CO2. All cell lines tested negative for the presence of Mycoplasma. The DU-145 cell line was derived from a central nervous system metastasis of a primary prostate adenocarcinoma (19). DU-145 cells have been shown to be tumorigenic but not metastatic by heterotransplantations into nude mice (20, 21).

Plasmid Constructions. To express the matrilysin metalloproteinase in DU-145 cells the full-length cDNA for matrilysin was blunt end cloned in the sense direction into the BamHI site of the eukaryotic expression vector pH-ß-APr-neo-1, which contains the neomycin selectable marker gene and the human β-actin promotor driving the transcription of the inserted cDNA (22). The construction of this plasmid was such that human β-actin 5' noncoding sequences were left attached to the matrilysin transcript. Therefore, the transfected and endogenous transcripts could be distinguished from one another by differential lengths. This construct, named pH-ß-matrilysin, and the parental plasmid pH-ß-APr-neo-1 were used in the transfection of DU-145 cells.

Transfections. Calcium phosphate-mediated DNA transfections were used to introduce the plasmid constructs. One day prior to transfection, 5 × 10^5 DU-145 cells were plated into 100-mm plates and allowed to adhere overnight. The calcium phosphate/DNA precipitates were formed by a published method (23) using 40 µg of plasmid DNA/plate. The precipitate was combined with complete MEM and left on the cells for 24 h for uptake of the plasmid DNA. The plates were then washed with complete MEM, and the cells were fed with complete MEM containing 500 µg/ml of Geneticin (Gibco). The medium was changed twice per week until colonies were visible (3 to 4 weeks); individual colonies were isolated using cloning rings and expanded for freezing and further analysis. Colonies derived from pH-ß-APr-neo-1-transfected cells were designated M-#, and the pH-ß-matrilysin-transfected cell lines were designated DUC-#.

Northern Analysis. All cell lines were grown to ~80% confluency, total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform method (24), and Northern blotting was performed. Tumors were frozen in liquid nitrogen and ground to a powder in liquid nitrogen. Total RNA was then isolated as above. Total RNA was then resolved on a 1% agarose, 3-[3N-morpholino]propane-sulfonic acid/formamide hydrogel; the gel was then capillary transferred to a GeneScreen (NEN Research Products, Boston, MA) nylon membrane and cross-linked by UV using a Stratalinker (Stratagene, La Jolla, CA). The blots were prehybridized for 18 h and hybridized for 24 h in a dextran sulfate/formamide-based hybridization solution according to the GeneScreen manufacturer. 32P-labeled cDNA probes were made by random priming (U.S. Biochemical, Cleveland, OH), washed out at high stringency (0.1 x standard saline citrate = 150 mM NaCl, 15 mM sodium citrate, pH 7.0; 55°C), and exposed to Kodak X-Omat film. The blots were stripped and reprobed according to the manufacturer’s instructions. Full-length cDNA inserts (with no polyadenylation) were used for probes. The CDNAs for human matrilysin and interstitial collagenase (14) were generously provided by R. Breathnach, and the cDNA for human TIMP-1 (25) was provided by G. P. Stricklin. The cDNA for rat gyceraldehyde-3-phosphate dehydrogenase was obtained from the American Type Culture Collection.

Antibody Production. BALB/c mice were immunized by standard procedures with full-length matrilysin protein isolated from Chinese hamster ovary cells transfected with the matrilysin cDNA. Spleen cells were harvested and fused with SP-2 myeloma cells using polyethylene glycol. Colonies were initially screened using an enzyme-linked immunosorbent assay with the whole matrilysin protein. Final cell lines were cloned three times by limiting dilution, and the final supernatants were characterized by immunoprecipitation and Western blotting against the whole matrilysin protein. One monoclonal antibody that specifically immunoprecipitated matrilysin, 10D2, was used for immunohistochemical staining of tissues for matrilysin. Frozen tissues were sectioned and fixed for 10 min in 0.3% glutaraldehyde in phosphate-buffered saline. The slides were washed with phosphate-buffered saline prior to addition of the antibody. The antigen-antibody complex was detected with a secondary antibody linked to horseradish peroxidase by previously described methods (26). Immunohistochemical detection of human cytokeratins 8 and 18 was performed on fixed tissue using the 10.11 antibody as described (26).

Western Analysis. For Western analysis, cell lines were fed with serum-free MEM for 24 h, and 50 µl of serum-free conditioned medium from each of the cell lines to be tested were combined with 2X Laemmli sample buffer, boiled, and separated on a 15% sodium dodecyl sulfate-polyacrylamide gel (27). The gel was transferred onto nitrocellulose paper (Schleicher and Schuell, Keene, NH). The blot was blocked with 2.5% nonfat dry milk in TBST for 1 h and then washed with TBST. The blot was incubated for 1 h with a 1:1000 dilution of a matrilysin polyclonal antibody generated against the COOH-terminal 100 amino acids of the matrilysin protein (18). The blot was washed three times for 5–10 min with TBST. An anti-rabbit IgG alkaline phosphatase conjugate was used as the secondary antibody and incubated for 30 min and washed again. Matrilysin protein localization was achieved by the ProtOBiot alkaline phosphatase detection system (Promega, Madison, WI).

SCID Mouse Invasion Assay. The SCID mouse colony at the University of Arizona Health Sciences Center are BALB/c C.B-17 mice that were originally provided by L. Schultz at Jackson Laboratories. They were maintained in a specific pathogen-free environment and in compliance with USPHS guidelines governing the care and maintenance of animals. All animals in this study were tested for plasma levels of IgM, and any animal with an IgM level higher than 5 µg/ml was not used in this study. The cells were tested to be harvested during exponential growth and resuspended in sterile phosphate-buffered saline. The cells were counted, and the numbers were corrected for cell viability. Four-week-old SCID mice were given i.p. injections of 5 × 10^6 cells of the lines to be tested. Three weeks later the mice were sacrificed by cervical dislocation, and all abdominal and thoracic internal organs, including the diaphragm, were fixed and sectioned. Organs that were involved sporadically included liver, spleen, prostate, kidneys, and adrenal glands. Tumors growing on or invading organs were visualized by either hematoxylin and eosin staining or immunohistochemical staining for human cytokeratins 8 and 18, which specifically detects the human DU-145 cells against a negative murine tissue background (26). To quantitate the invasive ability of these cells the diaphragm sections were scored as invasive or noninvasive. One random section from each diaphragm was scored, and if there was evidence of BL invasion by any of the tumors in that section then it was scored as invasive. If there was no evidence of BL invasion, then the section was scored as noninvasive. The scoring was performed in a blinded fashion as to which cell line formed the tumor. The Pearson χ2 analysis was used to test for statistical significance between vector-only transfected cells and matrilysin-transfected cells. The results from individual cell lines were pooled to eliminate clonal differences not related to matrilysin expression. Subcutaneous tumors were formed by s.c. injection of 5 × 10^6 DU-145 or DUC-26 cells into SCID mice. The tumors were allowed to grow for 6 weeks before the mice were sacrificed, and the tumors were snap frozen for RNA isolation and Northern analysis.

RESULTS

Transfection of DU-145 Cells with Expression Constructs. The transfections yielded 6 vector-only and 49 matrilysin-transfected Geneticin-resistant cell lines. All of the cell lines were initially screened by Northern analysis, and 13 of the matrilysin-transfected cell lines were positive for matrilysin mRNA, while the parental and all of the vector-only transfected cell lines remained negative. The four matrilysin-transfected DUC cell lines and two vector-only transfected M cell lines that were selected for further analysis are shown (Fig. 1). Steady-state levels of matrilysin mRNA were similar in the four DUC cell lines. Longer exposures of DU-145, M-1, and M-38 failed to show any expression of matrilysin (data not shown). These cell lines were also screened by Northern analysis for expression of TIMP-1, a known endogenous inhibitor of matrilysin (14) (Fig. 1). Steady-state levels of TIMP-1 mRNA appeared to be unaffected by the transfection and selection process that produced the cell lines, regardless of which construct was used. Therefore, four prostate cell lines were derived that demonstrated the overexpression of the matrilysin gene observed.
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Matrilysin

TIMP-1

GAPDH

Fig. 1. Northern (RNA) analysis of DU-145 and transfected cell lines. Northern blots of the indicated cell lines were probed simultaneously with random primed cDNA probes for matrilysin (1.2 kilobases) and TIMP-1 (0.9 kilobases). The blots were stripped and reprobed for glyceraldehyde-3-phosphate dehydrogenase (1.2 kilobases) as a RNA loading and transfer control. All cell lines were grown to ~80% confluency, total RNA was isolated, and Northern blotting was performed as described in “Materials and Methods.”

Fig. 2. Western blot analysis of DU-145 and transfected cell lines. Serum-free conditioned medium (50-μl aliquots) from DU-145 and the transfected cell lines were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotted as described in “Materials and Methods” with a polyclonal antibody raised against the carboxy-terminal 100 residues of the matrilysin protein (18). This antibody recognizes both the M, 29,000 promatrilysin and the M, 18,000 activated matrilysin.

Western Analysis. The parental DU-145 cell line M-1 and the four DUC cell lines were then analyzed by Western blotting for expression and secretion of the matrilysin protein with a polyclonal antibody raised against the carboxy-terminal domain of the matrilysin protein (18) (Fig. 2). The four matrilysin-transfected cell lines all secreted the matrilysin protein into conditioned medium, while DU-145 and M-1 did not show any detectable protein in the conditioned medium. The amount of protein secreted into the medium by the DUC cell lines appeared to be equivalent, which is in agreement with the results from the Northern analysis. The molecular weight of matrilysin seen in this Western indicated that the protein was being secreted in the pro form in vitro.

SCID Mouse Assay. To determine if the matrilysin protein plays a functional role in the invasion of prostate tumors we have developed an experimental animal model to assay for early events in tumor cell invasion. All of the mice that were given injections developed i.p. tumors by 3 weeks postinjection. The tumor cells formed the most colonies on the mesenteric fat, the diaphragm, and the walls of the peritoneum. No evidence was found for distant metastasis by any of the cell lines tested during this 3-week assay. Each cell line was injected into five mice. M-1 and M-38 control transfected cells did not form grossly visible tumors by autopsy but did contain microscopic tumors on the diaphragm. In contrast, the DUC cell lines formed grossly visible tumors on the diaphragm and other organs, and three mice contained bloody ascites at the time of autopsy. This apparent growth difference was not due to increased in vitro growth rates of transfected cells as measured by cell growth kinetics (data not shown). Representative examples of the histology of diaphragmatic tumors derived from vector-only transfected and matrilysin-transfected cell lines are shown (Fig. 3). Diaphragmatic tumors arising from vector-only transfected cell lines appeared to be inhibited from crossing the BL of the diaphragm (Fig. 3A); matrilysin-transfected cells lines, however, invaded past the BL in a much higher percentage of the diaphragms examined (Fig. 3B). This qualitative difference was tested for quantitative significance by scoring the diaphragmatic tumors as invasive or noninvasive and comparing the vector-only transfected

Fig. 3. Immunohistochemical staining of SCID mouse diaphragmatic tumors. DU-145- (A) and DUC-26- (B) derived tumors were immunohistochemically stained for cytokeratin 8 and 18 as previously described (26). The sections are positioned such that the cytokeratin positive tumors are above the nonstaining BL and skeletal muscle of the diaphragm. At autopsy the thoracic side of the diaphragm was supported by a piece of absorbent paper to maintain the diaphragm as a sheet. This splinted diaphragm was divided for both formalin fixation and snap freezing; in both cases the diaphragm was oriented on edge in the mounting medium so that cross-sections of the tumors could be obtained for histological analysis. × 800.

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The antibody. The diaphragms were scored as invasive if the tumor cells penetrated the BL or injected with transfected cell lines were stained with the anti-cytokeratin 8 and 18 transfected cells and "natrilysin transfected cells.

noninvasive if the BL remained intact. The scoring was performed in a blinded fashion.

the parental cells.

highly significant with a \( P < 0.006 \), indicating that by expressing and matrilysin-transfected cell line-derived tumors (Fig. 4). The change in invasive phenotype that apparently resulted from the overexpression of a single MMP gene in the DU-145 cells was unexpected. There are at least two explanations for this change in biological behavior. The first potential explanation is that matrilysin is either directly or indirectly activating other MMPs being secreted from the DUC cells. Matrilysin can superactivate collagenase with a 5-fold increase in activity (14). We have shown here that subcutaneous tumors derived from DU-145 and DUC-26 cells express the mRNA for type I procollagenase; if these tumors are expressing the type I procollagenase protein then matrilysin may be acting in concert with collagenase to produce a more invasive phenotype. It has recently been shown that stromelysin (MMP-3) can proteolytically activate progelatinase B (28). Since matrilysin is a member of the stromelysin subclass of MMPs (8) it is possible that matrilysin may directly activate progelatinase B. We have shown that DU-145 cells express both gelatinase A and gelatinase B by immunohistochemistry (in vivo) and zymography (in vitro) (data not shown). It is also possible that matrilysin indirectly activates other MMPs through proteolytic cleavage of as yet undiscovered endogenous activators of MMPs. Therefore, by transfecting the gene for matrilysin into DU-145 cells that in vivo already express three members of the MMP family we may have enhanced the activation of the MMPs present, thus increasing the invasiveness of the transfected cells.

An alternative hypothesis is that matrilysin substrate degradation creates proteolytic fragments of the extracellular matrix, which in turn bind through their respective integrin receptors and signal for an increase in the expression of genes involved in invasion (29). This is supported by evidence that shows proteolytic fragments of fibronectin and Arg-Gly-Asp-containing synthetic peptide fragments can transduce a signal through the fibronectin receptor and increase the expression of type I procollagenase and prostromelysin 1 in rabbit synovial fibroblasts (30). In contrast, native fibronectin does not have the same effect, indicating that it is not just a cell attachment phenomenon but a more specific response to a unique peptide. It has also been shown that a synthetic peptide derived from the laminin A chain can

and matrilysin-transfected cell line-derived tumors (Fig. 4). The difference between the control and matrilysin-transfected cell lines was highly significant with a \( P < 0.006 \), indicating that by expressing matrilysin in DU-145 we were able to alter the invasive phenotype of the parental cells.

To further analyze the in vivo characteristics of these tumors, Northern analysis of RNA from DU-145 and DUC-26 subcutaneous SCID mouse tumors was performed (Fig. 5). DU-145 cells in vitro did not express the matrilysin gene; however, subcutaneous tumors derived from DU-145 cells appeared to express low levels of the matrilysin message. This may be due to interactions between the DU-145 cells and the host tissue, or this finding may represent the expression of matrilysin in immune cells invading the tumor. MMPs have been shown to be expressed in both macrophages and neutrophils (9). The tumors derived from DU-26 cells were all found to express the larger matrilysin transcript encoded by the expression vector. The tumors, but not the cell lines, also expressed the type I collagenase message, which may be due to host-tumor interactions (Fig. 5). TIMP-1 expression appeared to be unaltered in the DU-145 and DUC-26 cell lines and tumors, suggesting that in vivo we have altered the ratio of matrilysin to TIMP-1 expression. To demonstrate that the matrilysin mRNA present in the tumors resulted in protein, we generated a monoclonal antibody to the full-length matrilysin protein for use in immunohistochemical analysis. This antibody, 10D2, was used to stain DU-145 and DUC-26 tumors on the diaphragms of SCID mice (Fig. 6). Fig. 6A shows a DU-26 tumor invading the diaphragm stained with the 10D2 antibody. There was homogeneous reactivity throughout the DUC-26 tumor cells, indicating increased expression of matrilysin in the tumors from matrilysin-transfected cells. The level of matrilysin protein in the DUC-26 tumor appears higher than that seen in the DU-145 tumor. Fig. 6B shows a DU-145 tumor stained with the same antibody. The reactivity that was slightly above background in this tumor indicates that the matrilysin mRNA expression seen in the DU-145 subcutaneous tumors was from the DU-145 tumor cells.

DISCUSSION

The change in invasive phenotype that apparently resulted from the overexpression of a single MMP gene in the DU-145 cells was unusual.
increase the amount of collagenase IV activity (31). We are currently investigating whether matrilysin-digested proteolytic fragments of fibronectin and laminin are able to alter MMP gene expression in DU-145 cells growing in culture. It is also possible that the effect seen is due to a combination of both theories in conjunction with matrilysin’s matrix-degrading activity.

In conclusion, our data show that the overexpression of the MMP matrilysin in a human prostate tumor cell line can alter its in vivo phenotype. This alteration manifests itself in the early events of BL invasion, thus allowing prostate tumor cells to colonize the underlying stroma. The molecular basis for biological variability seen in human prostate cancer is not well understood. We have observed in primary prostate tumors the increased loss of BL with increasing Gleason grade of tumor (5). We have also found that invasive prostate cancers overexpress matrilysin relative to normal prostate glands (12). In the study presented here we have obtained evidence in an experimental animal model that overexpression of the matrilysin MMP plays a functional role during early events in prostate tumor cell invasion. Therefore, the biological variability seen in human prostate cancer could be due, in part, to different levels of matrilysin expression in the primary tumors.

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