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# Matrix metalloproteinase and heparin-stimulated serine proteinase activities in post-prostate massage urine of men with prostate cancer

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ABSTRACT

Proteinases secreted by the prostate gland have a reproductive function in cleaving proteins in the ejaculate and in the female reproductive tract, but some may have a fundamental role in disease and pathological processes including cancer. The purpose of this study was to determine if there were differences in proteinase activities in urine samples collected following prostate massage of men positive (CaP) or negative (no evidence of malignancy, NEM) for biopsy determined prostate cancer. Matrix metalloproteinase (MMP) and serine proteinase activities were detected using protein substrate zymography. There were no differences in activities of MMP-2, proMMP-9, and MMP-9/NGAL (neutrophil gelatinase associated lipocalin) complex (gelatin substrate) in men with detected prostate cancer, although the latter two were somewhat diminished. A caseinolytic activity of about 75 kDa inhibited by calcium did not differ between the NEM and CaP groups. Heparin stimulated calcium sensitive gelatinolytic activities of approximately 22, 42, and 60 kDa, but did not affect activities of MMP-2, MMP-9, or the 75 kDa caseinolytic activity. The 22, 42, and 60 kDa activities appear to be serine proteinases since they were inhibited by benzamidine. There was a significant decrease in the 22 kDa heparin-stimulated serine proteinase activity in urines of men with cancer. Proteinase expression and activities, perhaps in combination with other potential markers, may prove useful in urine for detection and evaluation of prostate cancer.

## 1. Introduction

The secretion of a variety of potent proteolytic enzymes by the prostate gland has long been appreciated (Wilson, 1995). Proteomic studies have now identified over 100 proteases and protease inhibitors in human seminal fluid, many of which are of prostatic origin (La Flamme and Wolfner, 2013). Such proteinases include plasminogen activators, pepsinogen II, prostate specific antigen (PSA, kallikrein-KLK3), matrix metalloproteinases (MMPs), gelatinoytic and caseinolytic activities, and trypsinogen (Tauber et al., 1980; Lilja, 1985; Lee et al., 1989; Wilson et al., 1993; Paju et al., 2000). Membrane associated proteinases such as prostasin (Yu et al., 1994), TMPRSS2 (Afar et al., 2001), and cathepsins B, L, and S (Inayat et al., 2012) are prostasome-localized or are present as enzymatically active proteolyzed cleavage products. Activities of secreted proteinases may thus be regulated in part by membrane localization, but their activity may also be controlled by interaction with endogenous inhibitors. For example, the proteinase matriptase is secreted as a complex with its endogenous Kunitz-type protease inhibitor hepatocyte growth factor activator inhibitor (HAI)-1 (Wang et al., 2009).

The roles of prostatic proteinases in semen include liquefaction of the postejaculatory seminal coagulum and degradation of seminal proteins. PSA has a predominant function in initiation and carrying out this process (Lilja, 1985; Lee et al., 1989) followed with pepsin (activated from pepsinogen II) degradation of residual proteins in the acidic environment of the vagina (Szecsi et al., 1989). Other human tissue kallikrein-related peptidases (15 member family) of prostatic origin appear to affect spermatozoa motility via proteolysis of seminal

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coagulum and female reproductive tract proteins (Viveris-Lowe et al., 2007; Karakosta et al., 2016). Proteinases may also function in activation or inactivation of growth factors in the seminal plasma (Wilson, 1995) and the modulation of spermatozoan motility by peptides generated by neutral endopeptidase and aminopeptidase N (Bosler et al., 2014).

However, proteinases can have a fundamental role in disease and pathologic processes, including tumor cell progression, invasion and metastasis. Because of this concept they have been studied as possible biomarkers of prostatic disease (Wilson, 1995). Most prominent has been the use of serum levels of PSA in the detection and evaluation of prostate cancer. Different forms of PSA are detected in the blood; including catalytically active PSA bound to a variety of proteinase inhibitors, the most prominent being α1-antichymotrypsin, and non-catalytic proprotein and proteolytically nicked subtypes (Thorek et al., 2013). The fusion of the TMPRSS2 gene and members of the ETS oncogene family produces a key oncogene predominant in prostate cancer and is detected in about half of prostatic tumors (Mosquera et al., 2009; Pettersson et al., 2012). Interestingly, the serine peptidase inhibitor, kazal type 1 (SPINK 1) is overexpressed in TMPRSS2-ERG negative prostate cancers (Tomlins et al., 2008). Urine stands out as a non-invasively, easily accessible media to sample for possible prostate cancer biomarkers, and several urinary components such as prostate cancer antigen 3 (PCA3) and TMPRSS2-ERC may have some utility (Dijkstra et al., 2014; Strephan et al., 2014). However, significant levels of AMACR and hepsin in urines were only found in samples collected after prostate massage (Srorka et al., 2015). We previously described a number of metalloproteinase activities in expressed prostate secretions (EPS), including activities of molecular size of MMP-2 and MMP-9, and serine proteinases (Wilson et al., 1993). We have now undertaken the present study to determine whether heparin-stimulated serine proteinase activities, recently described in in prostate and seminal vesicle tumors of the Lobund-Wistar rat (Wilson et al., 2015), or those of MMPs detected in post-prostate massage urine samples may be indicative of prostate cancer.

## 2. Materials and methods

### 2.1. Patient description and post-prostate massage urine collection

Men at the Minneapolis VA medical center, who were about to undergo a biopsy of the prostate for clinical purposes (usually an elevation of serum prostate specific antigen, PSA), were asked to participate in this study by undergoing a digital prostate massage followed by collection of a urine sample. Patient exclusion criteria for the study were prior prostate surgery, treatment for prostate cancer before the biopsy, and active prostatitis in the last 4 months before the biopsy. In addition, the patient had to be cognitively able to understand the nature of his involvement in the study. All samples and patient information were given a study deidentifying code number for all sample labeling and all patient related information. Patient samples were divided into two groups based on the diagnosis from the biopsy: no evidence of malignancy (NEM) ($N = 12$) or prostate cancer (CaP) (N = 12). The mean age +/− S.D. and range were NEM 65.2 ± 3.9, 59–71 years and CaP 63.3 ± 6.5, 57–73 years. The mean PSA level +/− S.D. and range were NEM 6.0 ± 4.4, 0.3–15.8 ng/ml and CaP 7.0 ± 6.0, 2.6–23.7 ng/ml. The study was done with approval of the Institutional Review Board (IRB) committee at the Minneapolis VA Medical Center.

### 2.2. Processing of urine samples

Urine samples were centrifuged at 960 × *g* for 10 min at 4 °C to remove cells and particulate matter. The supernatant was then concentrated about 1:15 fold by centrifugation over an Amicon Ultra-15 3000 molecular weight cut off centrifugal filter (EMD Millipore Products, Billerica, MA) at 4 °C and then frozen and stored at −80 °C. The concentration of protein in the concentrated urine samples was estimated using bicinchoninic acid (Pierce Chem. Co., Rockford, IL) with bovine serum albumin as standard (Smith et al., 1985).

### 2.3. Zymography of proteinase activities

Aliquots of 20 μg protein were subjected to electrophoresis in gelatin (0.1%) or casein (0.1%) containing polyacrylamide (8.5% acrylamide) gels in the presence of sodium dodecyl sulfate under non-reducing conditions (Heussen and Dowdle, 1980; Wilson et al., 2004). The gels (0.75 mm thick) were electrophoresed for about 1.5 h at 130 v in a BioRad Mini-Protean II system. Following electrophoresis, the gels were rinsed with distilled water, washed with gentle shaking at room temperature with 2.5% Triton X-100 (2 changes) for 1 h, again rinsed with distilled water and then incubated in 50 mM Tris-HCl containing 5 mM $CaCl_2$ (pH 8.4), or buffer alone, overnight (18–20 h) at 37 °C (Wilson et al., 1992). Heparin effects on proteinase activities in zymograms were investigated by adding 3 μg heparin sulfate (Sigma Chemical Co, St. Louis, MO) to samples before loading onto the protein substrate gels as described above (Yu and Woessneer, 2001; Wilson et al., 2004, 2015). In some experiments the serine proteinase inhibitor benzamidine at concentrations given were added to the incubation buffer of gelatin containing zymograms, and effects of the inhibitor compared with duplicate zymograms in which no inhibitor was added. After electrophoresis the gels were washed and incubated as described above. Areas of proteolysis appear as clear zones against a blue background. Molecular mass determinations were made in reference to prestained protein standards (BioRad, Richmond, CA) co-electrophoresed in these gels. Areas of proteolysis were quantified using Image J.

### 2.4. Statistics

Statistical analysis was performed using the Prism 6 software and the multiple *t*-test with Holm-Sidak modifications and ANOVA with Fisher Least Significant Difference post hoc modifications.

## 3. Results

### 3.1. Characterization of gelatinolytic and caseinolytic proteinase activities

Concentrated urine samples were examined for proteinase activities that can cleave gelatin or casein. Prominent gelatinolytic activities were detected at about 90 and 125 kDa when incubated in the presence of calcium (Fig. 1A). The metalloproteinase characteristics of the 90 kDa activity observed in zymograms incubated without calcium were substantiated by total inhibition upon the addition of EGTA to the incubation (Fig. 2A). The 90 and 125 kDa activities correspond to proMMP-9 and MMP-9 complexed with human neutrophil gelatinase-associated lipocalin (NGAL) (Roy et al., 2008). A very low level of MMP-2 activity is observed at approximately 68 kDa. The activities of proMMP-9 and MMP-9/NGAL complex were diminished somewhat in samples from men with cancer, whereas there was no difference in MMP-2 activities in men with or without detected prostate cancer (Fig. 1C).

Caseinolytic activity in urine samples were detected at about 72, 75 and 80 kDa (Fig. 1B). Activities at 30 and 41 kDa were also detected when no Calcium was included in the gel incubation. The sensitivity to calcium of the caseinolytic proteinase activities was evidenced by enhanced activity of the 75 kDa form with EGTA added to the incubation (Fig. 2B). There was no difference in activities of the 75 kDa caseinolytic proteinase in the NEM and CaP groups.

### 3.2. Characterization of heparin stimulated proteinase activities

We recently described heparin-stimulated serine proteinase activities in sex accessory gland tumors induced in Lobund-Wistar rats