

Original Article

Potential Clinical Importance of the Activation Peptide of Prostate-specific Antigen

Laura M. Voegtly¹, Ida B. Thøgersen², Zuzana Valnickova², Kristian W. Sanggaard², Charleen T. Chu¹, Tim D. Oury¹ and Jan J. Enghild²

¹Department of Pathology, University of Pittsburgh Medical Center, Pittsburgh, PA 15261, USA and ²Center for Insoluble Protein Structures, Department of Molecular Biology, Science Park, University of Aarhus, Denmark

Received 29 April 2009; Accepted 23 May 2009; Available online 20 June 2009

Abstract: Prostate cancer is the second leading cause of cancer death in men. Prostate specific antigen (PSA) is currently the best marker available for screening and monitoring disease recurrence, but its use has limitations. This study investigates the biosynthesis, secretion and activation of PSA in a prostate adenocarcinoma cell line. PSA is secreted as a pro-enzyme containing a seven amino acid activation peptide (APLILSR). Because the activation peptide is removed extracellularly *in vivo*, we hypothesized that it may be detected in the blood or urine. Activated PSA is a serine protease and reacts rapidly with protease inhibitors in the blood. These protein complexes are removed from the circulatory system by hepatocyte-mediated endocytosis. This rapid clearance likely interferes with detection of PSA in the early stages of prostate cancer. Notably these clearance mechanisms are not considered when PSA levels are determined clinically. We used radio-labeled proteins to determine the clearance of PSA in complex with its inhibitors as well as *in vivo* clearance of APLILSR. Dot blotting was used to determine the presence of APLILSR in human urine samples. Our data indicates that PSA- α 1-antichymotrypsin only accumulates in the blood when large amounts of PSA are present and saturate clearance mechanisms. We found that APLILSR is filtered from the bloodstream by the kidney, and is detectable in the urine of patients with prostate cancer, but not controls. We propose that urine detection of the PSA activation peptide may represent a clinically sensitive measure of PSA production/secretion.

Key Words: PSA, prostate cancer, activation peptide

Introduction

Substantial amounts of prostate-specific antigen (PSA, EC 3.4.21.77) gain access to the blood compartment in a variety of prostatic disorders where it can be detected by immunological methods. This procedure has been utilized both as an adjunct for diagnosis of prostate cancer (CaP) and to monitor the effectiveness of therapy [1-5]. According to the American Cancer Society, CaP will account for 11% of male cancer-related deaths (surpassed only by lung cancer) in the United States in 2008. Currently, the digital rectal exam, serum PSA measurement, and Gleason grade determined from the biopsy cores are the most useful prognostic factors [6] whereas diagnostic screening for CaP relies on digital rectal exams, detection of PSA in the blood, and transrectal ultrasound [7, 8]. However, the cost and the invasiveness of rectal imaging

techniques preclude these from use in routine or large-scale screening of prostate cancer.

Despite the extensive use of serum PSA testing, 30% of men with CaP have locally advanced or metastatic disease at the time of diagnosis. These men are substantially less likely to be cured than men diagnosed with localized disease. Also, there is a very high false positive rate associated with serum PSA testing. Approximately 70% of men with "abnormal" PSA levels (above 4 ng/ml) do not have prostate cancer. In addition, PSA testing has a significant false negative rate. More than 20% of men with normal PSA values between 2.5 and 4 ng/ml have prostate cancer [3, 9, 10]. The complex biology of PSA makes assessments of stage and prognosis difficult for individual prostate cancer patients. Inaccuracies in predicting pathologic stage and the biology of prostate cancer often result

in over treatment of some men and under treatment of others. A better understanding of PSA biosynthesis, regulation, and clearance will enhance efforts to develop a more sensitive and specific test for prostate cancer.

PSA is a 33 kDa serine protease similar in structure to the trypsin-like tissue kallikreins but exhibits substrate specificity similar to chymotrypsin [11]. Analogous to other serine proteases, the activation involves a conformational change initiated by proteolysis of the Arg71Le8 peptide bond. PSA, like most serine proteases, is secreted as an inactive precursor [12].

PSA is detected in the plasma in three distinct forms (i) free-PSA; (ii) PSA- α 1-antichymotrypsin complexes (PSA- α 1ACT) and (iii) PSA- α 2-macroglobulin complexes (PSA- α 2M) [13-15]. However, PSA- α 2M is not detectable by most clinical immunoassays. The plasma half-life of α 1ACT- and α 2M-protease complexes is short because they are rapidly removed by hepatocyte receptors [16-19]. The plasma clearance of these complexes is independent of the proteases involved. The α 2M complexes are cleared from the circulation by the low density lipoprotein receptor [20, 21]. Serpin complexes are recognized by two serpin receptors: SR2, which recognizes and eliminates proteinase- α 2-antiplasmin complexes, and SR1 which recognizes complexes between proteinases and α 1-proteinase inhibitor, anti-thrombin III, heparin cofactor II, or α 1ACT [22, 23]. These receptors usually maintain undetectable levels of protease-inhibitor complexes in the blood. Since the level of PSA- α 1ACT in malignant disease may rise to several hundred ng/ml, we hypothesize that pathological PSA levels result from saturation of the clearance mechanisms. It follows that the PSA concentration depends both on how much PSA gains access to the blood stream and how efficiently it is removed. However, to date, the impact of clearance mechanisms has not been well studied.

Current use of PSA testing is directed toward detecting the major PSA forms in the blood (free-PSA, PSA- α 1ACT, and more difficult to detect PSA- α 2M) as well as complexes of PSA with other serine protease inhibitors including inter- α -inhibitor and α 1-protease inhibitor. However, the use of PSA as a screening or diagnostic test for the presence of prostate cancer has several limitations. PSA is known to

interact with other proteins in the blood. These interactions affect the half-life and interfere or prevent detection [24, 25]. Benign conditions in patients with altered hepatic function may cause an elevated serum PSA level, resulting in unnecessary biopsies or additional testing; it is also true that some prostate cancers are associated with normal serum PSA concentrations. This study demonstrates that PSA is activated and releases its activation peptide extracellularly and the activation peptide is subsequently filtered into the urine. We propose that detection of the PSA activation peptide in the urine may prove to be a viable alternative/addition to current PSA assays.

Materials and Methods

Reagents

ECL Western blotting detection reagents were from Amersham (Arlington Heights, IL). RPMI Medium 1640, RPMI 1640 select amine kit, Dulbecco's phosphate buffered saline, Earls' balanced salt solution, penicillin-streptomycin were from Gibco (Grand Island, NY). Epidermal growth factor, L-glutamine was from Sigma (St. Louis, MO). PSA antiserum was produced by DAKO Corporation (Carpinteria, CA). Human metastatic prostate adenocarcinoma (LNCaP) cells were obtained from American Type Culture Collection (Rockville, MD). Radiochemicals were from DuPont/NEN (Boston, MA). α 1-ACT was purified as previously described [27]. Urine samples were collected from the Duke University Medical Center Urology Clinic. Human prostatic tissues (normal, benign prostatic hypertrophy and malignant) were obtained from Duke University Medical Center for purification of PSA. Pathologists confirmed histology of each tissue independently.

Purification of PSA

All steps were performed at 4°C. 100 g of prostate tissue was homogenized (Vitris, Tempest) in 300 ml of 0.05 M Tris-Cl, 0.1 M NaCl, 0.01 M EDTA, pH 7.4. The homogenate was filtered through cheesecloth and cleared by centrifugation. The supernatant was digested with 0.1 mg/ml RNase, 0.2 mg/ml DNase and 0.005 M MgCl₂ for 4 h at 4°C. Following 4h incubation the supernatant was dialyzed overnight against 0.01 M HEPES, pH 8. The sample was clarified by centrifugation

and applied to a Q-Sepharose FF (Pharmacia, Piscataway, NJ) column (2.5 x 20 cm) equilibrated in 0.01 M HEPES, pH 8. The charged column was washed extensively in equilibration buffer and then developed with a linear gradient (total volume of 2 liter) from 0 M NaCl to 0.4M NaCl. Fractions of 4 ml were collected and tested for PSA by western blotting. The active fractions were pooled and concentrated by ultrafiltration (Amicon, Bedford, MA) and applied to a S-200 HR gelfiltration (Pharmacia, Piscataway, NJ) column (2.5 x 150 cm) equilibrated in 50 mM HEPES, 150 mM NaCl. Fractions of 4 ml were collected and assayed for PSA by western blotting. The PSA containing fractions were pooled and dialyzed into 10 mM HEPES, pH 8 and separated on a MONO-Q 5/5 HR (Pharmacia, Piscataway, NJ) connected to a Pharmacia FPLC system. The column was equilibrated in 10 mM HEPES and developed using a linear gradient from 0 M NaCl to 400 mM NaCl.

Preparation of Pro-PSA Antiserum

A peptide, Ala-Pro-Leu-Ile-Leu-Ser-Arg-Cys, corresponding to the N-terminal activation peptide of PSA was synthesized (Bio Synthesis, Lewisville, TX) and purified by RP-HPLC (RP-300, 4,6 mm column) and analyzed by mass spectrometry. The peptide was coupled to ovalbumin at a molar ratio of 10:1 using m-maleimidobenzoyl-N-hydroxysuccinimide ester (Pierce, Rockford, IL). Coupling was confirmed by SDS-PAGE. The purified peptide-ovalbumin complex was used for injection into rabbits (1 mg/injection). Antibodies from the final bleed were purified on a Protein G column.

Polyacrylamide Gel Electrophoresis

The supernatants from immunoprecipitates were recovered by centrifugation and separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) through 5-15% gradient gels [26]. The gels were stained, destained, dried, and subjected to imaging on a PhosphorImager (Molecular Dynamics 410A). Immunoprecipitates for radiosequence analysis were transferred to Immobilon membranes. Following electrophoresis, the Immobilon membranes were dried and exposed directly to X-ray film overnight at -70 °C.

Metabolic Labeling and Pulse-Chase Analysis

Human metastatic prostate adenocarcinoma (LNCaP) cells were maintained in RPMI Medium 1640 (ATCC, Rockville, MD) supplemented with 10% fetal bovine serum, epidermal growth factor (5 mg/500 ml), L-glutamine (150 mg/500 ml) and 1% penicillin-streptomycin in 5% CO₂. For standard biosynthetic radiolabeling, cells were grown in 50 mm tissue culture plates until 80% confluent. The cells were washed twice with Earls' balanced salt solution, and incubated for 30 min in RPMI without fetal bovine serum and lacking the amino acids that would be used for metabolic labeling. Cells were incubated for 5 min with either [³⁵S]Met or [³⁵S]Cys (pulse period). If the immunoprecipitated proteins were destined for radiosequence analysis [³⁵S]Cys was added. At the end of the labeling period, cells were rinsed twice with serum free RPMI and chased with "cold" complete medium for various periods of time.

Lysis and Immunoprecipitation

Conditioned medium was collected and frozen. Cell lysates were prepared by three rapid freeze-thaw cycles in high salt buffer containing 0.5% Triton X-100 and a proteinase inhibitor cocktail [11, 26]. Prior to immunoprecipitation, the samples of lysates and conditioned medium were cleared by the addition of a pre-immune serum followed by the addition of protein-G Sepharose 4 FF (Pharmacia, Piscataway, NJ). The supernatants were incubated overnight with the relevant specific antiserum. The next day protein-G Sepharose 4 FF was added and immunoprecipitates were collected by gentle centrifugation. The immunoprecipitates were then washed several times and bound proteins were released from the protein G Sepharose 4 FF by boiling in SDS sample buffer or by 100 mM glycine-HCl (pH 2.7) before SDS-PAGE.

Radiosequence Analysis

These analyses were performed as previously described [11, 27]. Briefly, following immunoprecipitation and SDS-PAGE, the [³⁵S] labeled proteins were electrotransferred to Immobilon membranes [27]. The proteins were identified by autoradiography and bands of interest were excised and analyzed by

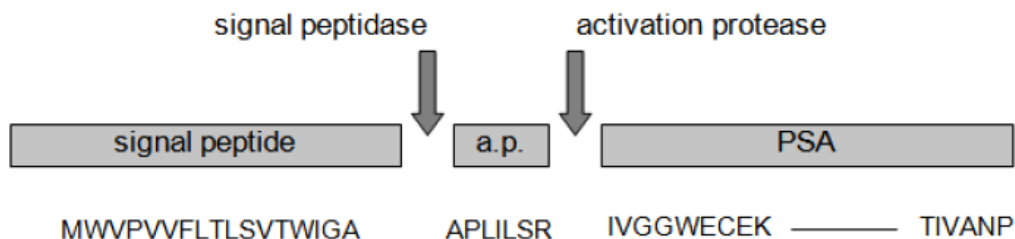


Figure 1 Schematic diagram of proteolytic processing of pro-PSA. Pro-PSA is composed of 244 amino acid residues. The signal peptide and the putative activation peptide (a.p.) are indicated. The arrows indicate the positions of the expected proteolytic cleavage sites. The solid bar labeled PSA represents mature, active PSA, with the majority of the sequence abbreviated.

automated Edman degradation in an Applied Biosystems 477A sequencer (Foster City, CA). The anilinothiazolinone (ATZ) amino acids released after each cycle were collected and counted for [³⁵S]. In the experiments destined for radiosequence analysis, the metabolic labeling were performed using [³⁵S]Cys which is positioned as residue 14 in the PSA zymogen. Subsequent radiosequence analysis of the bands and release of radioactive ATZ-amino acid in the anticipated cycle of Edman degradation provided identification of the protein band.

Radiolabelling

Approximately 100 µg of α1ACT and of the N-terminal activation peptide (synthesized by NEN™ Life Science Products, Boston, MA) were labeled with Iodine-125. α1ACT was labeled using Iodo-beads (Pierce, Rockford, IL) and the peptide (APLILSR) was labeled using the Bolton-Hunter method according to manufacturers recommendations. (Pierce, Rockford, IL).

Plasma Clearance Experiments

0.25 µg ¹²⁵I-APLILSR, 2.0 µg ¹²⁵I-α1ACT, or a 1:1 molar basis of PSA-¹²⁵I-α1ACT (2 µg and 0.97 µg respectively) was injected intravenously into the lateral tail vein of 50-60 day old CD-1 mice. Identical volumes of blood were collected by heparinized capillary tubes from the retro-orbital venous plexus. Radioactivity was measured in a γ-radiation counter and expressed as percentage radioactivity present compared to the first sample, drawn approximately 5 seconds after injection.

Dot Blotting

Membranes were developed using the ECL Western blotting kit from Amersham™ (Piscataway, NJ). Briefly, following transfer, the PVDF membranes were blocked for 1 h in 20 mM Tris-Cl, 137 mM NaCl, pH 7.6 containing 0.1% Tween (TBS-T buffer) and 5% of the supplied blocking reagents. The membrane was washed in TBS-T buffer before the primary antibody was added (1/2000 dilution). Following 1 h incubation the membrane was washed in TBS-T buffer and horseradish peroxidase labeled second antibody was added (1/20,000 dilution). The membranes were incubated for 1 h and washed with TBS-T buffer and developed using the supplied reagent.

Results

Biosynthesis of PSA

The cDNA sequence encoding PSA predicts an N-terminal 7 amino acid activation peptide [29] (**Figure 1**), although this putative activation peptide has not been detected in purified PSA [30-34]. To confirm that the activation peptide is removed extracellularly after secretion we characterized both intracellular and secreted PSA.

This analysis was performed using a polyclonal PSA antibody (**Figure 2A**) and antisera specific to the activation peptide (**Figure 2B**). PSA was immunoprecipitated from the cell lysates and medium, demonstrating that PSA was produced and that it was not degraded following secretion (**Figure 2A**). To investigate the kinetics of the activation, a pulse chase

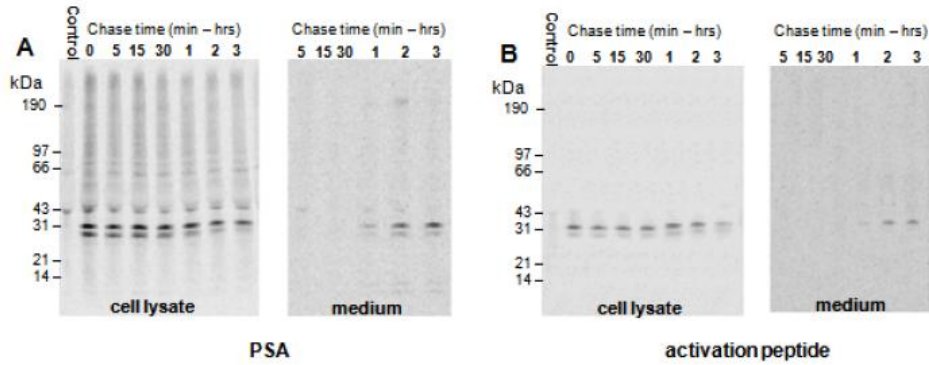


Figure 2 Posttranslational processing of PSA. LNCaP cells were radiolabeled using a pulse-chase protocol. The cells were chased for the indicated times and the lysates and medium were probed with specific antisera to the whole PSA (**A**) and to the activation peptide (**B**). The samples were analyzed by reduced SDS-PAGE. Pro-PSA is secreted (~36 kDa). The doublet sometimes observed reflects carbohydrate heterogeneity.

experiment was performed using antibodies directed specifically against the activation peptide (**Figure 2B**). The analysis of the gels suggested that the pro-PSA was detected intracellularly and, significantly, also in the cell culture medium. The results indicate that PSA does not undergo any N-terminal processing events in this culture system. This was confirmed by radiosequence analysis of both

intracellular and secreted PSA (**Figure 3**). These results show that PSA is secreted as an inactive pro-enzyme and contains the Ala-Pro-Leu-Ile-Leu-Ser-Arg- N-terminal activation peptide. The activation of pro-PSA as an extracellular event allows for the detection of the dissociated activation peptide in the extracellular space.

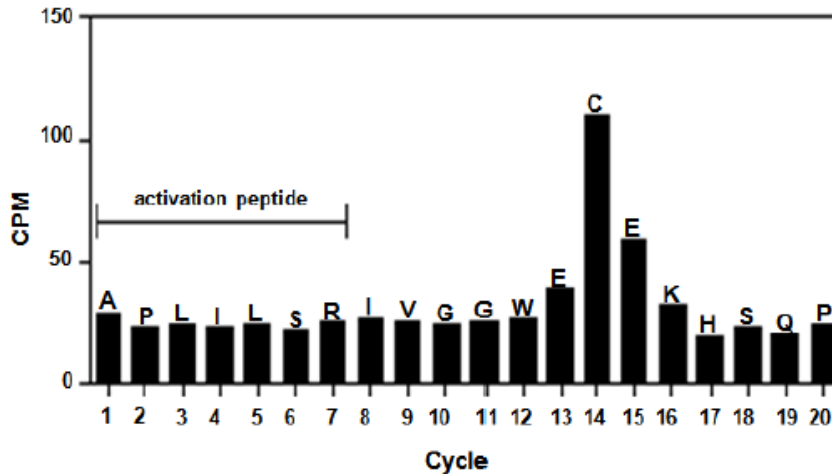


Figure 3 N-terminal radiosequence analysis. Radiosequence analysis of radiolabeled pro-PSA immunoprecipitated from the medium of LNCaP cells. The cells were pulse labeled with [³⁵S]Cys and subjected to immunoprecipitation, electrophoresis, autoradiography and radiosequencing. The vertical bars represent the level of [³⁵S] radioactivity released during each cycle of Edman degradation.

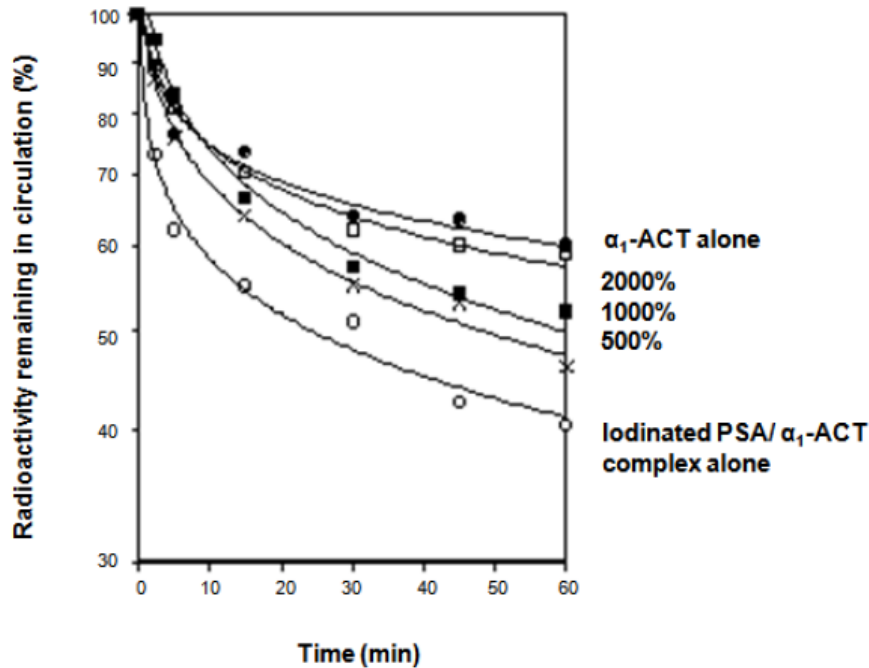


Figure 4 Plasma elimination of PSA- α_1 ACT complexes. α_1 ACT was injected alone (●) and in complex with active PSA (○). The half-life of the PSA- α_1 ACT complex was significantly reduced compared to native α_1 ACT. PSA- 125 I- α_1 ACT complexes were injected with a 500 (x), 1000 (■), and 2000 (□) fold molar excess of “cold” PSA- α_1 ACT complexes. These experiments demonstrate that PSA • α_1 ACT complexes are removed from the blood and do not begin to accumulate until the clearance mechanism is saturated.

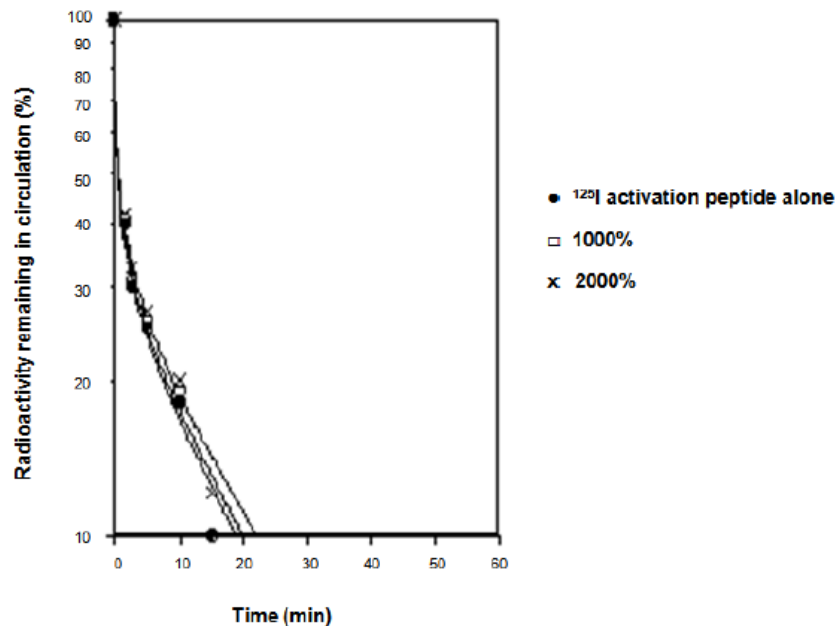


Figure 5 Plasma elimination of activation peptide. 125 I labeled activation peptide was injected into the lateral tail vein and the plasma elimination was monitored. The half life of the activation peptide injected alone was less than 2 min (●). 125 I labeled peptide was co-injected with a 1000 (□) and 2000 (x) fold excess of cold activation peptide. The half-life of the peptide was not affected significantly by the amount of peptide injected demonstrating that unlike plasma PSA, urine levels of activation peptide may correlate directly with of amount of pro-PSA produced.

Elimination of PSA- α 1ACT Complexes

The clearance rate of ^{125}I - α 1ACT was compared to the clearance rate of PSA- ^{125}I - α 1ACT complexes in mice (**Figure 4**). The half-life of α 1ACT alone was estimated to be several days. When α 1ACT was in complex with PSA, the half-life was reduced to 20 minutes. To mimic a situation in which increasing amounts of PSA are secreted, PSA ^{125}I - α 1ACT complex was injected with a 500-fold, 1000 fold and 2000 fold excess of unlabeled "cold" PSA- α 1ACT complexes (**Figure 4**). These experiments suggest that initially the PSA- α 1ACT complex is cleared from the blood, but as its concentration increases its clearance mechanism becomes saturated and only then do the PSA- α 1ACT complexes accumulate in the blood.

Plasma elimination of the activation peptide was studied by injecting ^{125}I labeled activation peptide (APLILSR) into the lateral tail vein of a mouse (**Figure 5**). Plasma elimination of ^{125}I -labeled activation peptide was followed for 1 hour, revealing that the half-life of the peptide was less than 2 minutes. The ^{125}I -labeled activation peptide was also injected with a 1000-fold and 2000-fold excess of unlabelled activation peptide. The clearance rate was not significantly affected by the level of activation peptide in the blood stream, suggesting a non-receptor mediated clearance mechanism. Following plasma elimination experiments, the organs of the test mice were examined for radioactivity using a γ -counter. The predominance of radioactivity in bladder and kidney indicates that the peptide is removed from the blood stream by renal filtration (**Figure 6**).

Plasma Elimination of Activation Peptide

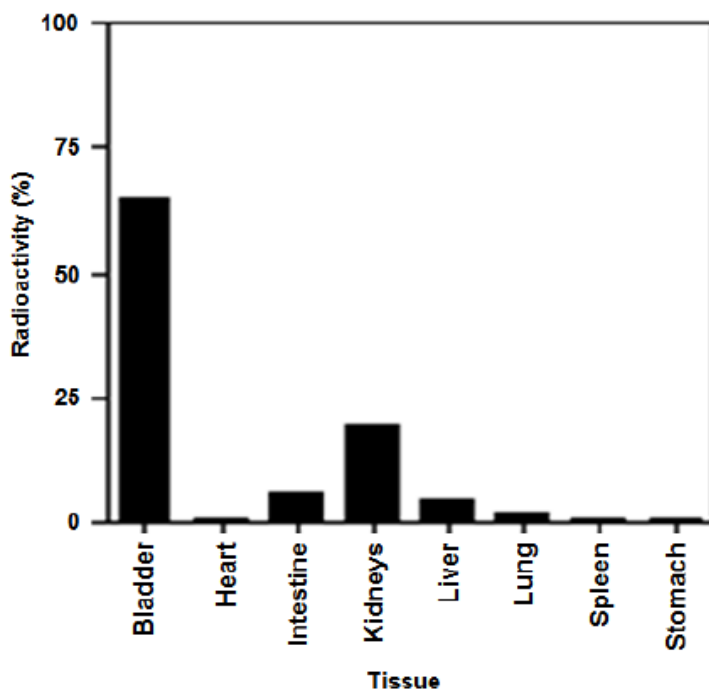


Figure 6 Tissue distribution of the ^{125}I -activation peptide. Following the plasma elimination experiments the mice were perfused and the organs were removed and counted in a γ -counter. It is apparent that the activation peptide is removed by renal filtration.

Detection of PSA Activation Peptide in Biological Samples

To test whether PSA activation peptide is measurable in the urine of patients with

prostate cancer, the activation peptide was synthesized and a polyclonal peptide antiserum was prepared in rabbits. The specificity of the antisera was verified by dot blot against the synthetic activation peptide,

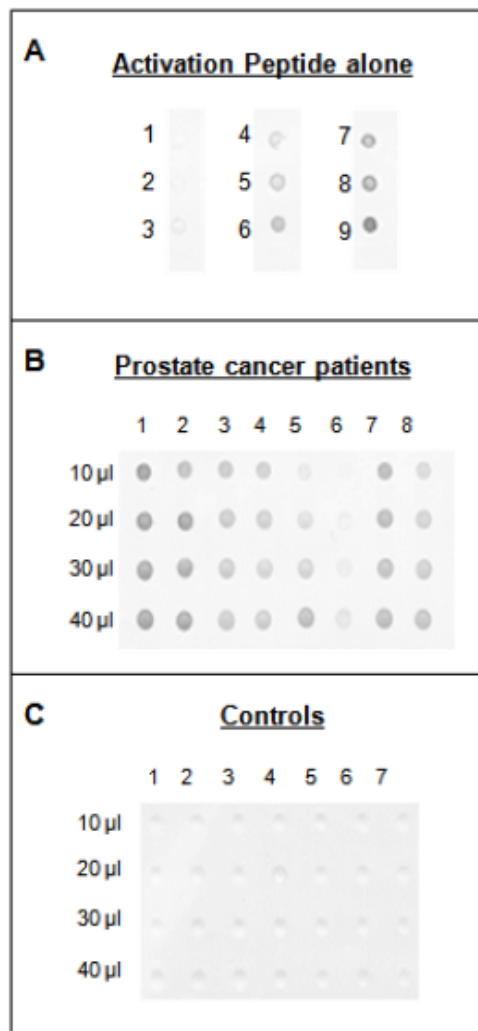


Figure 7 Detection of PSA activation peptide by dot blotting. The reactivity of the activation peptide antisera against synthetic activation peptide added to control urine samples was tested at concentrations ranging from 0.01 - 5 ng of peptide (A). Following these tests, urine from prostate cancer (B) and control patients (without prostate disease) (C) were analyzed. This shows that urine detection of the PSA activation peptide is feasible.

which was tested in concentrations ranging from 0.01 - 5.0 ng of peptide (Figure 7A; increasing concentration of activation peptide from dot 1 to dot 9). The antisera produced a dose-dependent reaction. Urine samples from seven control subjects with no evidence of prostate disease and eight patients with prostate cancer were tested for the presence of the activation peptide as discussed above. The activation peptide was detected in the urine (Figure 7B) of the cancer patients, but not in the urine (Figure 7C) of controls. These

results indicate that detection of the activation peptide in urine of subjects is feasible and correlates with the presence of prostate cancer.

Discussion

PSA in the blood is cleared very rapidly by one of two mechanisms: 1) combination with the serpin α 1ACT and then immediate clearance by the hepatocyte SR1 receptors or 2) combination with the α 2M and then immediate clearance by hepatocyte α 2M receptors (LRP/ α 2MR) [20, 21]. The present study suggests that PSA concentration in the blood remains low until the PSA- α 1ACT clearance is saturated. In contrast to intact PSA, we show that the activation peptide from PSA can be detected in the urine of prostate cancer patients, and our mouse clearance studies indicate a linear rate of clearance unaffected by saturation kinetics. We conclude that urine detection of PSA activation peptide may form a useful screening tool, particularly for early stage diseases, and serve as a sensitive measure for monitoring recurrence of disease.

We found that PSA- α 1ACT complexes are rapidly removed from the circulatory system. Moreover, we have found that serum PSA concentration is dependent on how much PSA gains access to the bloodstream and how efficiently it is removed. The capacity of the clearance mechanism is unknown but it is evident that it eventually becomes overwhelmed. Indeed PSA complexes only begin to accumulate after very high levels are introduced. These results imply that rapid removal of PSA from the circulation in the early stages of prostate cancer, when there is a low tumor cell burden and lower levels of PSA production may form a heretofore-unrecognized problem with conventional PSA testing. It is possible that, in cases of prostate cancer associated with a normal serum PSA concentration, the plasma elimination mechanism has not yet been saturated, so that PSA is quickly removed from the bloodstream.

While studies performed in male patients undergoing radical prostatectomy have shown that PSA in complex with inhibitors has a long kinetic half life [35], such that PSA- α 1ACT levels remain constant in the bloodstream following prostatectomy for up to six hours

[36], the present study demonstrates that the PSA- α 1ACT complex in a mouse model is rapidly cleared from the plasma. This rapid clearance suggests that the complex is only detectable in the serum after the clearance mechanisms become saturated. It is therefore likely that the patients in the previous study had such a high serum level of PSA- α 1ACT that their clearance mechanisms would be saturated. This would be consistent with PSA- α 1ACT levels remaining elevated and detectable for six hours. Sudden decreases of the complex at around six hours post prostatectomy would suggest that the clearance mechanisms were no longer saturated and were able to adequately clear the complex. The clearance rate is likely to depend upon the overall health of the patient, including physical condition, body weight, and alcohol and tobacco consumption. These factors may account for some of the variability of serum PSA levels between individuals. This rapid clearance of PSA complexes limits the utility of serum PSA as an early diagnostic tool. Although serum PSA testing plays a useful role in monitoring treatment response and relapses in a given individual, the complex clearance mechanisms of serum PSA may also interfere in recurrence monitoring as well. On the other hand, these factors are not expected to significantly affect the half-life of the activation peptide. The mechanism responsible for the removal of the activation peptide is mainly a passive filtration event in contrast to the active receptor mediated endocytosis required for the removal of the PSA- α 1ACT complex. Moreover, we have shown in mice that plasma clearance of the activation peptide is not influenced by saturation kinetics like serum PSA complex levels.

Our results suggest that the presence of the PSA activation peptide in urine may be a reliable indicator of secreted PSA with fewer confounding factors than serum PSA analysis. This activation peptide is cleaved from PSA during activation of pro-PSA in the extracellular space. The rapid and simple kinetics of clearance suggest that it does not interact with other proteins, but is cleared from the blood by simple renal filtration. The activation peptide is easy to detect within the urine. Additionally, it represents a noninvasive sampling procedure, amenable to large-scale use.

It is important to note that the ratio of free PSA to total PSA in the serum has been shown to

be helpful in discriminating CaP from benign prostatic hypertrophy (BPH) [37, 38] where an increase in free PSA is suggestive of benign disease. Because a majority of free PSA is either enzymatically inactive pro-PSA ((-7)pro-PSA) or a significantly nicked variant of pro-PSA ((-4,-5)pro-PSA) [39], detection of the full 7 amino acid activation peptide in the urine would represent active PSA. This would include active PSA that has subsequently bound to α 1ACT or otherwise undetectably bound to α 2M. This is of great interest as there is currently no simple assay that can detect PSA bound to both α 1ACT and α 2M.

The PSA activation peptide is derived solely from secreted PSA, and its detection is not affected by plasma protein interactions and saturation of clearance methods. Therefore screening for the presence of the activation peptide in urine may give a more accurate representation of PSA production.

Because pro-PSA has been shown to have different forms, we must consider that an antibody against APLILSR may also detect slightly truncated forms of the activation peptide in the urine. Because it would be difficult to discern between 5 and 7 amino acid peptides in an immunoassay, it is possible that the APLIL peptide that is cleaved from the (-2)pro-PSA form will also be detected in the urine with an antibody against APLILSR. While this would seemingly implicate a lower specificity for detection, it has been shown that the presence of (-2)pro-PSA in the serum is a sensitive indicator of cancer [40]. This detection might therefore increase the sensitivity of the assay to detect CaP.

We propose that an assay to detect APLILSR will constitute a clinically sensitive method of screening for cancers associated with increased levels of secreted PSA. As protease activation is a common occurrence in many other diseases, this activation peptide detection technique may also prove useful for the diagnosis or monitoring of other protease specific conditions.

In summary, while serum PSA screening is useful, particularly in following treatment response in individuals with known prostate cancer, it is clear that a better understanding of mechanisms involved in PSA biology and regulation will lead to improved prostate cancer screening. Our biosynthesis and

clearance studies indicate that prior to detectable elevations of serum PSA in patients, there is likely significant pathology developing in the prostate, and elevated serum PSA levels develop only after saturation of hepatic clearance mechanisms. A saturation-independent method of detecting increased PSA secretion would enhance early detection of prostate cancer and would improve treatment outcomes. We propose that urine detection of the PSA activation peptide may represent a sensitive and reliable early detection method that would also be useful in following treatment response in patients with known prostate cancer.

Please address all correspondences to Laura M. Voegtly, Department of Pathology, University of Pittsburgh, 200 Lothrop St, BSTWR W904 Pittsburgh, Pennsylvania 15261. Tel: 412-624-8888; Fax: 412-648-9172; Email: lmn20@pitt.edu

References

- [1] Babaian RJ, Mettlin C, Kane R, Murphy GP, Lee F, Drago JR, Chesley A., Partin AW, Oesterling JE, Frydenberg M, Stricker PD, Kaye KW, Gleason DF and Mellinger GT. The relationship of prostate-specific antigen to digital rectal examination and transrectal ultrasonography. Findings of the American Cancer Society National Prostate Cancer Detection Project. *Cancer* 1992;69:1195-200.
- [2] Brawer MK, Chetner MP, Beatie J, Buchner DM, Vessella RL and Lange PH. Screening for prostatic carcinoma with prostate specific antigen. *J Urol* 1992;147:841-845.
- [3] Catalona WJ. Clinical utility of measurements of free and total prostate-specific antigen (PSA): a review. *Prostate Suppl* 1996;7:64-69.
- [4] Mettlin C. The status of prostate cancer early detection. *Cancer* 1993;72:1050-1055.
- [5] Partin AW, Oesterling JE. The clinical usefulness of prostate specific antigen: update 1994. *J Urol* 1994; 152:1358-68.
- [6] Gleason DF. Histologic grading of prostate cancer: a perspective. *Hum Pathol* 1992;23: 273-279.
- [7] Friedman GD, Hiatt RA, Quesenberry CP Jr and Selby JV. Screening for prostatic cancer. *Lancet* 1991;338:1271-1272.
- [8] Littrup PJ. The American Cancer Society's National Prostate Cancer Detection Project. *Can J Oncol* 1994;4(Suppl 1): 65-69.
- [9] Brawer MK, Crawford ED, Fowler J, Lucia MS and Schroeder FH. Prostate cancer: epidemiology and screening. *Rev Urol* 2000;2 (Suppl 4): S5-9.
- [10] Canto EI and Slawin KM. Early management of prostate cancer: how to respond to an elevated PSA. *Annu Rev Med* 2002;53:355-368.
- [11] Ban Y, Wang MC, Watt KW, Loo R and Chu TM. The proteolytic activity of human prostate-specific antigen. *Biochem Biophys Res Commun* 1984;123:482-488.
- [12] Corey E, Brown LG, Corey M, Buhler K and Vessella R. LNCaP produces both putative zymogen and inactive, free form of prostate-specific antigen. *Prostate* 1998;35: 135-143.
- [13] Christensson A, Laurell CB and Lilja H. Enzymatic activity of prostate-specific antigen and its reactions with extracellular serine proteinase inhibitors. *Eur J Biochem* 1990; 194:755-763.
- [14] Lilja H, Cockett AT and Abrahamsson PA. Prostate specific antigen predominantly forms a complex with alpha 1-antichymotrypsin in blood. Implications for procedures to measure prostate specific antigen in serum. *Cancer* 1992;70:230-234.
- [15] Stenman UH, Leinonen J, Alfthan H, Rannikko S, Tuhkanen K and Alfthan O. A complex between prostate-specific antigen and alpha 1-antichymotrypsin is the major form of prostate-specific antigen in serum of patients with prostatic cancer: assay of the complex improves clinical sensitivity for cancer. *Cancer Res* 1991;51:222-226.
- [16] Fuchs HE, Shifman MA and Pizzo SV. In vivo catabolism of alpha 1-proteinase inhibitor-trypsin, antithrombin III-thrombin and alpha 2-macroglobulin-methylamine. *Biochim Biophys Acta* 1982;716:151-157.
- [17] Imber MJ and Pizzo SV. Clearance and binding of two electrophoretic "fast" forms of human alpha 2-macroglobulin. *J Biol Chem* 1981;256: 8134-8139.
- [18] Mast AE, Enghild JJ, Pizzo SV and Salvesen G. Analysis of the plasma elimination kinetics and conformational stabilities of native, proteinase-complexed, and reactive site cleaved serpins: comparison of alpha 1-proteinase inhibitor, alpha 1-antichymotrypsin, antithrombin III, alpha 2-antiplasmin, angiotensinogen, and ovalbumin. *Biochemistry* 1991;30:1723-1730.
- [19] Pizzo SV, Mast AE, Feldman SR and Salvesen G. In vivo catabolism of alpha 1-antichymotrypsin is mediated by the Serpin receptor which binds alpha 1-proteinase inhibitor, antithrombin III and heparin cofactor II. *Biochim Biophys Acta* 1988;967:158-162.
- [20] Chu CT and Pizzo SV. alpha 2-Macroglobulin, complement, and biologic defense: antigens, growth factors, microbial proteases, and receptor ligation. *Lab Invest* 1994;71: 792-812.
- [21] Strickland DK, Ashcom JD, Williams S, Burgess WH, Migliorini M and Argraves WS. Sequence identity between the alpha 2-macroglobulin receptor and low density lipoprotein receptor-related protein suggests that this

- molecule is a multifunctional receptor. *J Biol Chem* 1990;265:17401-17422.
- [22] Lilja H and Lilja DR. Early diagnosis and staging. *Prostate Cancer Prostatic Dis* 1999;2:15-18.
- [23] Malm J and Lilja H. Biochemistry of prostate specific antigen, PSA. *Scand J Clin Lab Invest* 1995;221 (suppl):15-22.
- [24] Salvesen GS, Catanese JJ, Kress LF and Travis J. Primary structure of the reactive site of human C1-inhibitor. *J Biol Chem* 1985;260: 2432-2436.
- [25] Kitagawa T and Aikawa T. Enzyme coupled immunoassay of insulin using a novel coupling reagent. *J Biochem (Tokyo)* 1976;79:233-236.
- [26] Liu FT, Zinnecker M, Hamaoka T and Katz DH. New procedures for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterization of such conjugates. *Biochemistry* 1979;18:690-693.
- [27] Thogersen IB and Enghild JJ. Biosynthesis of bikunin proteins in the human carcinoma cell line HepG2 and in primary human hepatocytes. Polypeptide assembly by glycosaminoglycan. *J Biol Chem* 1995;270:18700-18709.
- [28] Matsudaira P. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* 1987;262:10035-10038.
- [29] Bury AF and Barrett AJ. Plasma arginine esterase in cystic fibrosis: kinetics of activation, identification as plasma kallikrein, reaction with alpha 2-macroglobulin and comparison with levels in normal plasma. *Pediatr Res* 1982; 16:613-620.
- [30] Lundwall A and Lilja H. Molecular cloning of human prostate specific antigen cDNA. *FEBS Lett* 1987;214:317-322.
- [31] Schaller J, Akiyama K, Tsuda R, Hara M, Marti T and Rickli EE. Isolation, characterization and amino-acid sequence of gamma-seminoprotein, a glycoprotein from human seminal plasma. *Eur J Biochem* 1987;170:111-120.
- [32] Sensabaugh GF and Blake ET. Seminal plasma protein p30: simplified purification and evidence for identity with prostate specific antigen. *J Urol* 1990;144:1523-1526.
- [33] Watt KW, Lee PJ, M'Timkulu T, Chan WP and Loor R. Human prostate-specific antigen: structural and functional similarity with serine proteases. *Proc Natl Acad Sci USA* 1986;83: 3166-3170.
- [34] Zhang WM, Leinonen J, Kalkkinen N, Dowell B and Stenman UH. Purification and characterization of different molecular forms of prostate-specific antigen in human seminal fluid. *Clin Chem* 1995;41:1567-1573.
- [35] Martin BJ, Cheli C, Davis R, Ward M, Kokatnur M, Mercante D, Lifsey D and Rayford W. cPSA and fPSA elimination in African-American men. *Prostate Cancer Prostatic Dis* 2003;6: 163-168.
- [36] Stephan C, Jung K, Brux B, Lein M, Sinha P, Schnorr D and Loening SA. Act-PSA and complexed PSA elimination kinetics in serum after radical retropubic prostatectomy: Proof of new complex forming of PSA after release into circulation. *Urology* 2000;55:560-563.
- [37] Catalona WJ, Partin AW, Slawin KM, Brawer MK, Flanigan RC, Patel A, Richie JP, deKernion JB, Walsh PC, Scardino PT, Lange PH, Subong EN, Parson RE, Gasior GH, Loveland KG and Southwick PC. Use of the percentage of free prostate-specific antigen to enhance differentiation of prostate cancer from benign prostatic disease: a prospective multicenter clinical trial. *JAMA* 1998;279:1542-1547.
- [38] Woodrum DL, Brawer MK, Partin AW, Catalona WJ and Southwick PC. Use of the percentage of free prostate-specific antigen to enhance differentiation of prostate cancer from benign prostatic disease: a prospective multicenter clinical trial. *J Urol* 1998;159:5-12.
- [39] Mikolajczyk SD, Marker KM, Millar LS, Kumar A, Saedi MS, Payne JK, Evans CL, Gasior CL, Linton HJ, Carpenter P and Rittenhouse HG. A truncated precursor form of prostate-specific antigen is a more specific serum marker of prostate cancer. *Cancer Res* 2001;61: 6958-6963.
- [40] Mikolajczyk SD, Catalona WJ, Evans CL, Linton HJ, Millar LS, Marker KM, Katir D, Amirkhan A and Rittenhouse HG. Proenzyme forms of Prostate-Specific Antigen in serum improve the detection of prostate cancer. *Clin Chem* 2004; 50:1017-1025.