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PSA-alpha-2-macroglobulin complex is enzymatically active in the serum of patients with advanced prostate cancer and can degrade circulating peptide hormones

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Abstract

Background: Prostate cancer cells produce high levels of the serine protease Prostate-Specific Antigen (PSA). PSA is enzymatically active in the tumor microenvironment but is presumed to be enzymatically inactive in the blood due to complex formation with serum protease inhibitors α−1-antichymotrypsin and α−2-macroglobulin (A2M). PSA-A2M complexes cannot be measured by standard ELISA assays and are also rapidly cleared from the circulation. Thus the exact magnitude of PSA production by prostate cancer cells is not easily measured. The PSA complexed to A2M is unable to cleave proteins but maintains the ability to cleave small peptide substrates. Thus, in advanced prostate cancer, sufficient PSA-A2M may be in circulation to effect total A2M levels, levels of cytokines bound to A2M and hydrolyze small circulating peptide hormones.

Methods: Total A2M levels in men with advanced prostate cancer and PSA levels above 1000 ng/mL were measured by ELISA and compared to controls. Additional ELISA assays were used to measure levels of IL-6 and TGF-beta which can bind to A2M. The ability of PSA-A2M complexes to hydrolyze protein and peptide substrates was analyzed ± PSA inhibitor. Enzymatic activity of PSA-A2M in serum of men with high PSA levels was also assayed.

Results: Serum A2M levels are inversely correlated with PSA levels in men with advanced prostate cancer. Il-6 Levels are significantly elevated in men with PSA >1000 ng/mL compared to controls with PSA <0.1 ng/mL. PSA-A2M complex in serum of men with PSA levels >1000 ng/mL can hydrolyze small fluorescently labeled peptide substrates but not large proteins that are PSA substrates. PSA can hydrolyze small peptide hormones like PTHrP and osteocalcin. PSA complexed to A2M retains the ability to degrade PTHrP.

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CONFLICTS OF INTEREST

None of the authors have any relevant affiliations to disclose with any organization that has a direct interest, particularly a financial interest, in the subject matter discussed.

Conclusions: In advanced prostate cancer with PSA levels >1000 ng/mL, sufficient PSA-A2M is present in circulation to produce enzymatic activity against circulating small peptide hormones. Sufficient PSA is produced in advanced prostate cancer to alter total A2M levels, which can potentially alter levels of a variety of growth factors such as IL-6, TGF-beta, basic FGF, and PDGF. Alterations in levels of these cytokines and proteolytic degradation of small peptide hormones may have profound effect on host-cancer interaction.

Keywords

hormone; macroglobulin; prostate cancer; PSA; serum

1 | INTRODUCTION

Prostate cancer cells, like normal prostate epithelial cells, produce high levels of the differentiation marker Prostate-Specific Antigen (PSA).[1–3] PSA is used extensively as a biomarker to screen for prostate cancer, to detect recurrence following local therapies and to follow response to systemic therapies for metastatic disease. However, previous studies have suggested that PSA may play a role in prostate cancer pathobiology through a variety of mechanisms that include alterations in prostate cancer cell invasive capabilities, gene expression, and morphology.[4–7] Previously, we documented using that shRNA knock-down of PSA expression dramatically reduced growth rates of PSA-expressing LNCaP prostate cancer cells while expression of enzymatically active PSA in PSA-null DU145 cells enhanced growth rates in vitro and in vivo.[7] These results suggest that the enzymatic activity of PSA can affect cell growth. Functionally, PSA is chymotrypsin-like serine protease with unique substrate specificity that can directly cleave, or release from binding proteins, cytokines present in the stromal compartment that are involved in growth stimulation and inflammation. For example, PSA can cleave insulin-like growth factor binding proteins (IGFBP) resulting in local release of IGF-1,[8] and can specifically activate the small latent form of transforming growth factor beta-2 (TGFβ2).[9,10] PSA can also hydrolyze parathyroid hormone related protein (PTHrP), which may convert it from an osteoclastic to an osteoblastic growth factor, capable of playing a role in the osteoblastic bone metastasis, phenotype typically observed in men with advanced prostate cancer.[11,12]

PSA is present in the serum in a number of different forms, all of which are thought to be unable to cleave serum proteins.[13–15] These forms can be classified into two general categories: complexed PSA (ie, bound to serum protease inhibitors) and free PSA (ie, unbound, inactive PSA).[13–15] PSA forms a covalent complex by binding to the serum protease inhibitor alpha1-antichymptrypsin (ACT) to produce PSA-ACT, which is the predominant form of PSA (65–95%) in the serum in men with normal prostates, BPH or prostate cancer, including those patients with poorly differentiated (ie, Gleason score 8–10) cancers.[13–15] The remaining PSA consists of free PSA forms (5–35%) and PSA complexed with the major serum protease inhibitor alpha-2-macroglobulin (PSA-A2M).[15,16] Active proteases in the serum, including PSA, become engulfed upon binding to A2M making these proteins completely inaccessible to antibodies. Thus, the A2M-PSA complex cannot be measured using standard ELISA based methodologies.[15–17] Therefore, although the current clinical diagnostic test measures “total PSA,” consisting of free PSA + PSA-ACT in the

serum, the true amount of total PSA in the serum is the sum of all forms of PSA (ie, free PSA + PSA-ACT + PSA-A2M).[15]

PSA binds covalently to ACT and in vitro, this binding takes several hours at 37°C.[13,18,19] Once formed, the PSA-ACT complex is very stable and does not dissociate in vivo.[18,19] The half-life of PSA-ACT has been difficult to calculate accurately because of non-exponential elimination, but available data suggest that PSA-ACT is cleared slowly and linearly over hours to days.[19] In contrast, PSA binds very rapidly (in minutes) to A2M in human plasma.[16,19,20] Similar to other active serum proteases, PSA cleaves a peptide sequence in the bait region of the large macroglobulin protein causing a conformation change. The catalytic site of PSA is not covalently bound to A2M, but PSA does become cross-linked to A2M at regions outside the PSA catalytic site. A2M-bound PSA therefore can still hydrolyze small peptide substrates that can enter into the A2M cavity, but is unable to cleave larger protein substrates. Once the A2M undergoes conformational change after PSA cleavage of the bait region, it becomes a ligand for A2M-receptor/low density lipoprotein receptor-related protein present in the liver, and the PSA-A2M complex is subsequently rapidly cleared from the plasma with a half-life of a few minutes.[16]

While PSA-ACT is the predominant PSA complex found in human serum, the in vitro kinetics of complex formation are consistent with PSA should preferentially bind to A2M,[19,20] Figure 1A. When enzymatically active PSA is added to human serum almost all of the PSA rapidly forms the PSA-A2M complex, Figure 1B. The addition of methylamine, a small molecule that hydrolyzes the thiol ester within A2M inducing a conformational change that prevents PSA binding, restores the ability to measure the majority of PSA added to serum, Figure 1C. These results suggest that several orders of magnitude more enzymatically active PSA may be potentially entering the blood in prostate cancer patients and forming complexes with A2M, which are then rapidly cleared and not measurable.[15] The exact magnitude of this complex formation and clearance is unknown. High amounts of PSA-A2M in the serum could alter levels of A2M-bound growth factors and potentially inactivate small peptide hormones through PSA-A2M hydrolysis. In this study we document decreased serum A2M levels in patients with PSA levels >1000 ng/mL. Measurable PSA enzymatic activity, based on small peptide substrate hydrolysis, is observed in the serum from these patients. Finally, we demonstrate that A2M-bound PSA is able to hydrolyze the osteoclast growth factor PTHrP. These results point to a potential role for enzymatically active, A2M-bound PSA in modifying disease progression in advanced prostate cancer.

2 | MATERIALS AND METHODS

Enzymatically active PSA purified from human seminal plasma was acquired from AbD Serotec (Catalog #7820-0504, Oxford, UK), and stored as recommended by the manufacturer.[22] Information supplied with PSA from this vendor indicated that PSA is purified from human seminal fluid (≥95% pure by SDS-PAGE) supplied in a liquid form in sterile filtered PBS, pH 7.4–7.6 with <0.1% azide as a preservative. Enzymatic activity was confirmed by vendor using peptide substrates. A previously characterized PSA substrate Mu-SRKSQQY-AMC was purchased from California Peptide Research (Napa, CA).[21,23] PSA inhibitor Ahx-FSQn(boro)Bpg was synthesized in our laboratory as previously

described.[24] PRX302, a PSA-activated form of proaerolysin, a pore-forming bacterial protoxin, was generated in our laboratory as previously described.[25] Unless otherwise specified, all other materials were from Sigma.

2.1 | PSA measurements and enzymatic activity

Levels of free and total PSA in media and mouse plasma were determined by the Clinical Chemistry laboratory at Johns Hopkins using the Hybritech assays on the Beckman Access Immunoassay System (Beckman Coulter, Inc., Brea, CA).[20] PSA activity was determined using the Mu-SRKSQQY-AMC substrate as previously described.[21] The PSA concentration was 5 μg/mL with a PSA substrate concentration of 300 μM. The PSA inhibitor was used at 10 μM where appropriate. All assays were performed in PSA buffer (50 mM Tris, 100 mM sodium chloride, pH 7.5) at 37°C. Assays were performed in triplicate in a black half-area 96-well plate and read every 3 min for 42 min by a Beckman Coulter DTX-880 plate reader (excitation 370 nm, emission 465 nm).

2.1.1 | Assays of PSA activity in human plasma—

PSA containing serum and control serum was obtained from the clinical chemistry laboratory at Johns Hopkins from discarded samples. These samples were collected from women, men without prostate cancer and men with clinical diagnosis of prostate cancer. While disease state was known for each sample, no other patient identifiers were available on these samples. The activity of PSA in patients' plasma was determined by monitoring the change of fluorescence of AMC substrate (ex. 370 nm; em. 465 nm) due to hydrolysis by PSA[20] Product formation was followed over 2 h and readout was collected every 5 min. Reactions were performed on black 96-well plate where the final concentration of the substrate was 300 μM. The assay mixture contained 50% serum, 300 μM Mu-SRKSQQY-AMC and 5 μg/mL PSA. The plate was read immediately after the substrate was added. To inhibit the activity of PSA, 20 μL of 100 μM inhibitor Ahx-FSQn(boro) Bpg was added to the serum and the mixture was incubated for 5 min before addition of the substrate.[24] The velocity of each reaction (change in RFU/time) was determined and plotted versus concentration of the total PSA present in sera.

The A2M/PSA complex used for enzymatic assays and western blot standards was prepared by incubating 100 μL of 1 mg/mL A2M (Sigma), and 1.5 μL of 1 mg/mL PSA (Serotech) at room temperature overnight. Cation-exchange SP Sepharose (100 μL) was prewashed four times with 0.1M PBS, 50 mM NaCl, pH 6.6. The PSA-A2M mixture was added to the beads and incubated for 15 min at 4°C on a slow shaker. The supernatant containing the PSA/A2M complex was put through another round of SP Sepharose separation, to remove anytraces of free PSA. The purity of the complex was checked by Western blot, using primary rabbit PSA antibody (1:2500 dilution) supplied by Dako.

Interaction of PSA with its endogenous purified protease inhibitors or in plasma in absence and presence of PSA inhibitor was examined by Western blot using anti-PSA polyclonal rabbit antibody (Dako, A-0562).[24] All mixtures were incubated for overnight at room temperature. PSA-A2M standard was prepared as described.[24] PSA-ACT complex was prepared by incubating 100 μL of ACT (100 μg/mL, Sigma) with 1 μL of 1 mg/mL PSA at

room temperature overnight. Plasma samples (10 μL) and protein standards were diluted to 200 μL with sample buffer. All samples were run on 4–15% gradient gel from Bio-Rad. Anti-rabbit secondary antibody and bioluminescence assay used to visualize bands as previously described.[24]

2.1.2 | Protein digestion—PTHrP peptide 1–34 (1.0 μM) and osteocalcin (1.0 μM) were digested with 2 nM PSA in PSA assay buffer ± A2M (20 μg/mL) at 37°C. Aliquots of 20 μL of the mixture were desalted by using P10-C_{18} ZipTips (Millipore) then injected in the HPLC at indicated time points.

The HPLC used for the analysis was equipped with photodiode array detector and purchased from Waters (Milford, MA). The products of the hydrolysis were separated on an analytical HPLC column from Phenomenex: Luna C18, particle size 10 μm, 250 × 10 mm. Peptides were quantified by HPLC absorbance at 215 nM based on area under the curve calculations. The molecular weights of all hydrolyzed peptides were confirmed by mass spectrometry.

2.1.3 | ELISA assays—A2M and IL-6 cytokine circulating levels were evaluated in PSA containing human serum samples by a quantitative sandwich enzyme immunoassay technique (Human alpha two Macroglobulin AssayMax ELISA kit by Assaypro; Human IL-6 and Human TGF-1 Beta Quantikine Immunoassay ELISA by R&D Systems) according to the manufacturers' instructions.

3 | RESULTS

To determine if a correlation between PSA levels and A2M exists, serum samples were obtained from women, men without clinical prostate cancer and PSA levels <2 ng/mL, men with known prostate cancer with PSA levels <20 ng/mL and men with advanced prostate cancer and PSA levels >100 ng/mL. A2M levels were measured by ELISA and plotted against PSA levels. Overall there was a decline in serum levels of A2M with increasing PSA levels, Figure 2A. The median of A2M in normal men with PSA <2 ng/mL was 8.33 μg/mL, Table 1, which is significantly higher than in men with prostate cancer and PSA <20 (median A2M 5.84 μg/mL) or >100 ng/mL (median A2M 4.44 μg/mL) ($P = 0.002$), Table 1, Figure 2B. In addition, within each group there was no correlation (Spearman) found between A2M and PSA in either normal men with PSA < 2 ng/mL ($r = 0.04$; $P = 0.9$) or men with prostate cancer with PSA < 20 ng/mL ($r = 0.01$; $P = 0.96$); however, the correlation between A2M and PSA in men with advanced prostate cancer was statistically significant ($r = -0.5$; $P < 0.01$), Figure 2C.

A2M has been demonstrated to bind to TGF-Beta and IL-6 in the serum.[6] Therefore, effects of decline in serum A2M level on serum levels of TGF-Beta and IL-6 were measured using ELISA assays. The median of TGF-Beta 1 in normal men with PSA <2 ng/mL was 17609 ng/mL, Table 1, which is higher than in men with prostate cancer and PSA <20 or >100 ng/mL, but this difference was not statistically significant ($P = 0.55$), Table 1, Figure 2A. Within each group there was no significant correlation found between A2M and TGF-β1 in either normal men with PSA <2 ng/mL ($r = 0.10$; $P = 0.85$) or men with prostate cancer with PSA <20 ng/mL ($r = 0.3$; $P = 0.21$); however, the correlation between A2M and TGF-Beta1

in men with advanced prostate cancer was statistically significant ($r = -0.5$; $P = 0.01$), Figure 3B.

IL-6 levels were subsequently determined in a group of men with no prostate cancer and no measurable PSA levels (ie, <0.1 ng/mL) and compared to levels in men with marked elevation in PSA to >1000 ng/mL. In this analysis, the median IL-6 level was ~14-fold higher in the men with prostate cancer and high PSA levels compared to those with PSA <0.1 ng/mL, Figure 3C. Median A2M levels in the men with no prostate cancer was 2510 μg/mL compared to 1816 μg/mL for those men with advanced prostate cancer and PSA > 1000 ng/mL. Among those patients with prostate cancer and high PSA levels (removing one outlier whose A2M is >3300), A2M and IL-6 showed a significant positive association after adjusting for PSA levels ($P = 0.028$), Figure 3D.

3.1 | Enzymatic activity of plasma PSA-A2M can be inhibited by peptidomimetic boronic acid inhibitor

In previous studies, we described a series of peptide-based inhibitors of PSA including the peptidomimetic boronic acid inhibitor Ahx-FSQn(boro)Bpg,[24] Figure 4A. This inhibitor has a PSA K_i of 72 nM with a K_i for chymotrypsin of 580 nM. This PSA inhibitor is able to block PSA complex formation with A2M and ACT, Figure 4B. To assess activity, the PSA substrate Mu-SRKSQQY-AMC was incubated with commercially purified PSA in 1% BSA containing buffer versus PSA-A2M complex that was purified to remove any uncomplexed free PSA. In this assay, A2M-bound PSA had a similar reaction velocity for substrate hydrolysis compared to uncomplexed enzyme, Figures 4C and 4D. The activity was completely abolished following addition of the PSA inhibitor, Figures 4C and 4D.

Previously, antibodies to the catalytic site have been described that inhibit the enzymatic activity of purified PSA.[21] However, A2M-bound PSA is not accessible to these anti-PSA antibodies and therefore this approach cannot be used to assess binding specificity of PSA in human serum. Therefore, given the ability of the Ahx-FSQn(boro)Bpg to inhibit A2M-bound PSA, this inhibitor was evaluated for its ability to inhibit PSA-A2M activity in plasma. Following pre-incubation with buffered plasma, PSA maintained the ability to hydrolyze the Mu-SRKSQQY-AMC substrate, although the reaction velocity was ~3.3-fold lower compared to the purified PSA-A2M, Figures 4C and 4E. The PSA inhibitor maintained the ability to inhibit PSA following addition to human plasma, although some non-specific substrate hydrolysis was observed as evidenced by a rate of hydrolysis that was ~1.8-fold higher compared to PSA + inhibitor in buffer only, Figure 4C.

While PSA added to plasma to a final concentration of 10 μg/mL was able to readily hydrolyze the low molecular weight fluorescent PSA substrate, this concentration of PSA was completely inhibited in its ability to activate PRX302, a 52 kDa pore-forming bacterial toxin that has had the PSA substrate HSSKLQ substituted for the wild type furin activation site in the wild type toxin,[21,25] Figure 4F. In contrast, PSA in buffer readily activates the PRX302 toxin resulting in rapid lysis of red blood cells. These results suggest that in human serum, A2M bound PSA should be unable to hydrolyze large serum proteins, but could maintain the ability to cleave low molecular weight peptide containing putative PSA-recognized cleavage sites.

3.2 | Enzymatic activity of PSA-A2M in serum of patients with prostate cancer

PSA-A2M has a short serum half-life due to rapid clearance by the liver. PSA-A2M complexes cannot be measured by standard clinical chemistry testing. Therefore, to evaluate PSA-A2M activity we selected men with advanced cancer and high total serum PSA levels >1000 ng/mL (range 1174–6887 ng/mL) based on our hypothesis that the total PSA (PSA-ACT + free inactive PSA) would correlate with levels of PSA-A2M. Incubation of the Mu-SRKSQQY-AMC with these samples resulted in measurable hydrolysis of the substrate and the rate of substrate hydrolysis (ie, reaction velocity) correlated with total PSA level, Figure 5A. Western blot analysis of serum samples from patients with high total PSA demonstrated detectable levels of PSA-A2M complex in some samples, Figure 5B. To establish that the PSA activity in the serum was due to PSA-A2M, samples with high total PSA were subject to filtration through a 100 kDa cut-off filter to separate the 33 kDa free PSA fraction from the high MW (>720 kDa) PSA-A2M fraction in the samples. After filtration, all of the enzymatic activity is present in the filter with no activity in the <100 kDa filtrate consistent with activity being due to PSA-A2M, Figure 5C. The PSA-A2M activity in the serum samples was abolished upon addition of the PSA-specific Ahx-FSQn(boro)Bpg inhibitor to the high PSA serum samples, Figure 5D.

3.3 | A2M-bound PSA maintains ability to cleave low molecular weight peptide hormones

Previous studies have documented the ability of PSA to hydrolyze a variety of high MW proteins including the putative physiologic substrates semenogelin I and II, the latent form of TGF-Beta, IGFBP-3, complement factors 3 and 5, and fibronectin.[2,7,26] PSA can also cleave low molecular weight peptide hormones such as PTHrP.[11,12,27] As documented, PSA-A2M was unable to cleave the 52 kDa PRX302 toxin containing a PSA activation site. However, PSA-A2M may maintain the ability to cleave small peptide hormones that may be able to access PSA within the A2M cavity. To evaluate this possibility, PSA was incubated with osteocalcin and PTHrP, two low molecular weight peptide hormones that regulate bone metabolism. Osteocalcin has a molecular weight of approximately 5.8 kDa and consists of 49 amino acids. Incubation of osteocalcin with purified PSA results in production of multiple small degradation fragments that can be detected by HPLC separation, Figure 6A.

Previously we demonstrated that purified PSA degradation could also degrade a 34 amino acid PTHrP peptide to produce two fragments of MW 2781.40 and 2193.06.[27] To study if A2M-PSA could cleave a low molecular weight peptide hormone, the PTHrP 1–34 peptide was used to assay cleavage by PSA versus PSA-A2M. In this analysis, purified PSA reduced the area under the curve of the PTHrP peak by 45% after 26 h incubation and generated two product fragments (PR1, PR2) that were separable on HPLC, Figure 6B. Incubation of the PTHrP 1–34 peptide with PSA-A2M led to 65% reduction in PTHrP at 24 h post-incubation. PSA-A2M incubation also produced two product fragments, one of which, Pr3, exhibited a longer retention time than the parent peak, Figure 6C. These results demonstrate that PSA-A2M retains the ability to hydrolyze low molecular weight peptide hormones.

4 | DISCUSSION

Both normal and malignant prostate epithelial cells can produce large amounts of PSA. PSA is present in the seminal fluid at mg/mL concentration with the major form being enzymatically active free PSA.[6] High amounts of total PSA (>1000 ng/mL) can also be found in the serum of men with advanced prostate cancer. However, as previously described, the absolute total amount of PSA released (ie, PSA-ACT + free PSA + PSA-A2M) into the blood by prostate cancer cells is difficult to measure because encapsulation of PSA epitopes by A2M makes it impossible to measure this form using standard ELISA assays.[6] However, the PSA in PSA-A2M can be rendered immunoreactive by denaturation of A2M with high pH and detected in serum with high PSA levels by immunoblotting.[17,24] Using this approach Zhang et al[17] measured low levels of PSA-A2M in patients with total serum PSA levels in the range of 10–20 ng/mL. In addition, these PSA-A2M complexes have a serum half-life of only a few minutes as they are rapidly cleared by the liver. However, the kinetics of complex formation suggest that the majority of PSA released into the blood should complex with A2M and not ACT.[19,20] Thus, these findings would lead to the conclusion that several orders of magnitude higher amounts of PSA are being produced by prostate cancers beyond what is measured in the serum.

One possible explanation for the decline in A2M levels in patients with very high PSA levels >1000 ng/mL could be that the cumulative sustained production of large amounts of PSA by prostate cancer sites within a patient with advanced disease can overwhelm production of A2M by the liver. Prostate cancer cells also produce other proteases such as KLK2 that also bind to A2M and could contribute to A2M decline. However, A2M is one of the most abundant serum proteins present at concentrations of 1.8–4.5 mg/mL.[17] Additionally, patients with prostate cancer typically have intact liver synthetic function even in the advanced disease state. Therefore, either PSA production occurs at a rate that is much higher than A2M production by the liver or PSA or other factors released by prostate cancer can affect A2M production to explain the observed decline in A2M levels.

A2M plays an underappreciated, but critical role in cancer biology based on its ability to act as a signaling molecule, a carrier for growth factors and a modulator of protease activity. Conformationally altered A2M can activate or inhibit signal transduction pathways through binding to membrane receptors.[28–31] A2M binding to A2M receptor/LRP activates G-protein activated pathways.[29,30] For instance binding of methylamine activated A2M to A2M/LRP receptors stimulates macrophage to increase cellular and nuclear COX-2.[31] This COX-2 stimulation could be blocked with inhibitors of Protein kinase C, phospholipase A_2, MAP kinase, and phosphoinositide-3-kinase (PI 3-kinase) consistent with A2M receptor mediated activation of these downstream signal transduction pathways.[31,32] Methylamine activated A2M was also demonstrated to bind to plasma membrane bound Grp78 in Lin-1 cells, a subclone of PC-3 human prostate cancer cell line.[33] Binding to Grp78 generated elevations in intracellular calcium and also led to activation of 21 kDa activated kinases (PAKs), an important mechanism for increasing cell motility.[33]

In addition to its role as a general protease inhibitor, A2M regulates a variety of cytokines in serum and tissue. Several, including interleukin-4 and −10, transforming growth factor-β

(TGF-β) and nerve growth factor-β (NGF-β), bind covalently with A2M via disulfide bonds.[6,34,35] A2M can also form non-covalent complexes with interferon, tumor necrosis factor-α (TNF-α), vascular endothelial growth factor (VEGF) and Interleukin-6.[36–38] Specific interactions are dependent on the conformation of A2M with most interactions only occurring when A2M is in its conformationally altered protease-bound state.[39] The significance of A2M binding to growth factors is unclear but evidence indicates a role for increased sequestration and degradation of growth factor/A2M complexes.

In this study, we observe marked ~14-fold elevation of IL-6 levels in patients with prostate cancer and high PSAlevels >1000 ng/mL compared to prostate cancer patients with PSA levels <0.1 ng/mL. In the patients with high PSA, levels of IL-6 directly correlated with A2M after adjusting for PSA levels. A statistically significant correlation was also observed between TGF-Beta levels and A2M levels in patients with PCa and PSA levels >100 ng/mL but not in normal patients or patients with lower tumor burden and PSA levels <20 ng/mL. These results would be consistent with prior studies showing A2M functions as the primary carrier of IL-6 and TGF-Beta in the serum.[38,39] In other studies, IL-6 levels have been found to increase as overall disease burden increases.[40] Previously, Kanoh et al[41] measured levels of inflammatory biomarkers in patients with advanced prostate cancer with a condition they termed “macroglobulin deficiency,” defined as an A2M level <0.20 mg/mL. These patients had median PSA levels above 5000 ng/mL. Thus, in this context, macroglobulin deficiency was not due to a genetic condition or macroglobulin mutation, but the result of high levels of PSA production in these patients. In this study, levels ofIL-6 and two other A2M-dependentacuteinflammatoryproteins, C-Reactive Protein (CRP) and serum amyloid A, were not increased in PCa patients with low A2M levels but were increased in prostate cancer patients with normal serum A2M levels.[42] Thus, similar to our findings, in patients with advanced prostate cancer, lower A2M levels are associated with lower levels of inflammatory cytokines like IL-6 and growth factors like TGF-Beta.

Experimentally lowering A2M levels by exogenous administration of proteases was previously shown to lower TGF-Beta levels and alter tumor growth kinetics.[43] These results suggest that high amounts of PSA-A2M complex in the blood could alter concentrations of key growth and survival factors that could affect prostate cancer progression. Changes in levels of these proteins could also affect some of the symptoms associated with advanced/end stage prostate cancer such as fatigue, cachexia and increased pain. Decreased levels of total A2M and increased levels of PSA bound to A2M also has the potential to disrupt protease inactivation and clearance in the blood which could lead to dysregulation of serum protease cascades such as those of the clotting and complement systems.

Our studies provide additional evidence that proteases like PSA complexed with A2M can maintain enzymatic activity toward small peptide substrates. Thus, A2M-protease complexes within the blood may play a role in clearance or modification of peptide hormones from the circulation.[44] In our study, PSA-A2M maintained the ability to cleave the 34 amino acid form of PTHrP, a peptide hormone involved in bone homeostasis. PTHrP is also an important mediator of osteolytic bone metastases, and inactivates PTHrP-stimulated cAMP accumulation in mouse osteoblasts.[12,45] Prior studies have suggested that PSA degradation of PTHrP could alter the bone metastatic phenotype from osteoclastic to osteoblastic.[46] Our

analysis also demonstrated that circulating PSA-A2M in advanced prostate could also play a role in altering this phenotype. Additionally, the peptide cleavage pattern of PTHrP was different for PSA versus PSA-A2M and this finding raises the possibility that, in addition to degrading peptide hormones, PSA-A2M could produce new forms of peptide hormones with altered function.

5 | CONCLUSIONS

These studies document that sufficient PSA-A2M activity is present in the serum of men with advanced prostate cancer to produce measurable enzymatic activity against peptide substrates and peptide hormone. The activity of PSA-A2M in the serum could be inhibited by a peptide-based boronic acid inhibitor of PSA.[24] In prior studies we documented that in vivo administration of this inhibitor produced a slight effect on growth of PSA-producing human prostate cancer subcutaneous xenografts. These peptide inhibitors are not ideal drug candidate given the short circulating half-life. However, the results presented here suggest that further efforts to develop PSA inhibitors with better pharmaceutical properties are warranted. These inhibitors could be used to disrupt pro-tumor growth effects induced by the large amounts of circulating PSA-A2M in patients with advanced cancer. These PSA inhibitors could also have the potential for a substantial palliative effect in patients with end stage, widely metastatic prostate cancer.

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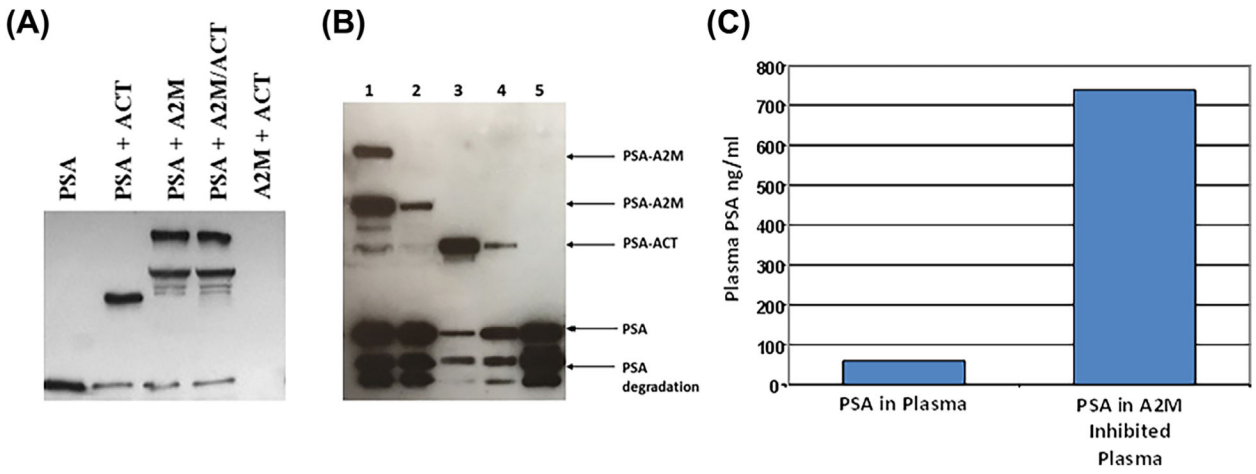

FIGURE 1.

A, Western blot of PSA complexed with ACT and A2M or combination of ACT and A2M. B, PSA added to human plasma preferentially forms complexes with A2M. Lane 1 PSA added to 50% buffered female plasma, Lane 2 PSA + purified A2M standard, Lane 3, 4 PSA + purified ACT standards, Lane 5 purified PSA (Serotec) standard (C) Measurable PSA in plasma in absence or presence of 0.2 M A2M inhibitor methylamine. Purified PSA (Calbiochem) added to final concentration of 1000 ng/mL. Plasma preincubated with 0.2 M methylamine for 5 min prior to adding PSA. Total PSA measured using ELISA assay (Beckman Coulter)

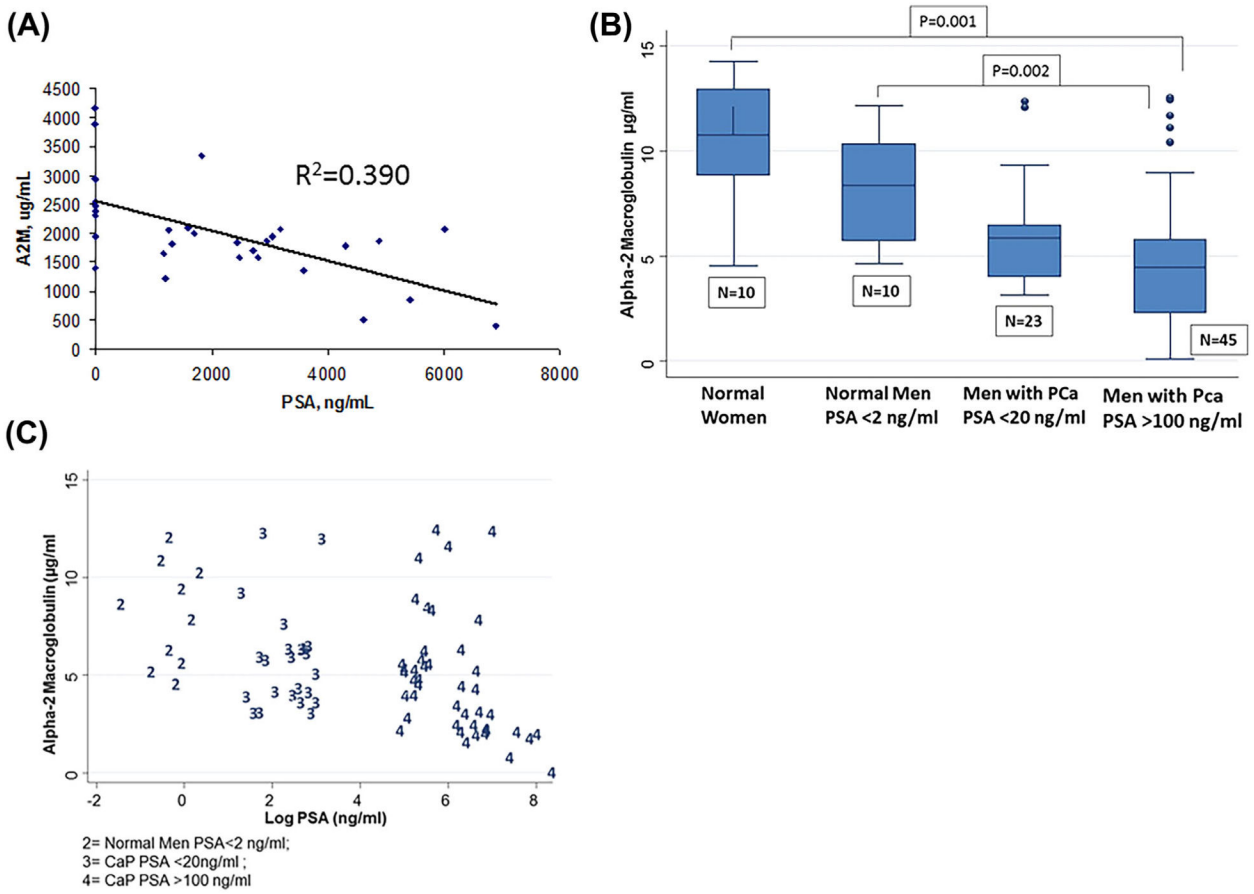

FIGURE 2.

A, Inverse correlation observed between serum A2M and PSA levels; B, Whisker plot of A2M levels in normal women and men with indicated disease state and level of PSA; C, Correlation A2M versus PSA according to PSA level

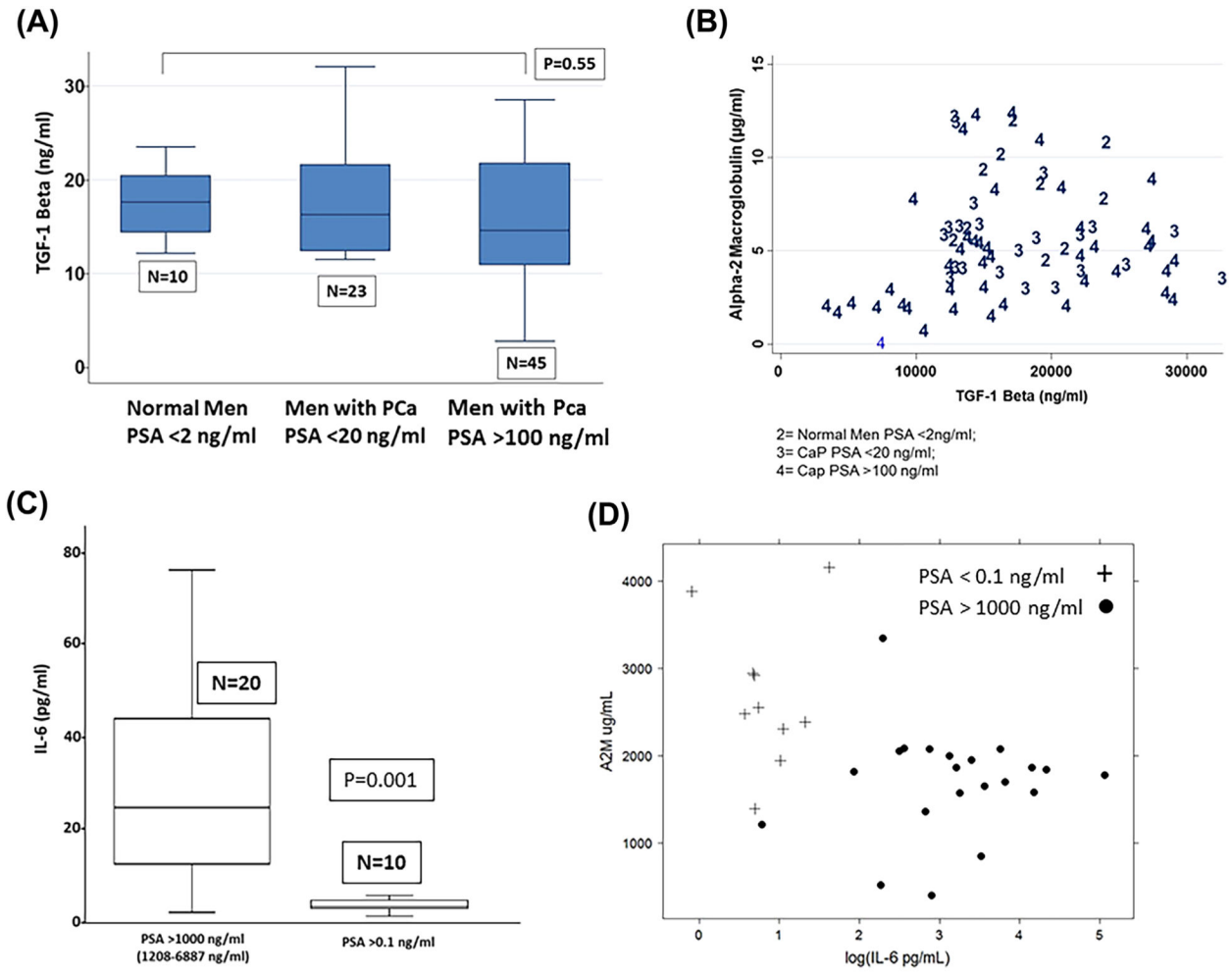

FIGURE 3.

A, Whisker plot of TGF-1 Beta levels in men without prostate cancer and men with prostate cancer and indicated PSA levels; B, Correlation of A2M levels versus TGF-1 Beta levels in indicated groups; C, Whisker plot of IL-6 levels in men with prostate cancer and PSA > 1000 ng/mL versus PSA < 0.1 ng/mL ($P = 0.001$ via Kruskal-Wallis test). Correlation of A2M versus IL-6 Levels in indicated patients. D, After adjusting for PSA levels A2M and IL-6 showed a significant positive association $P = 0.028$

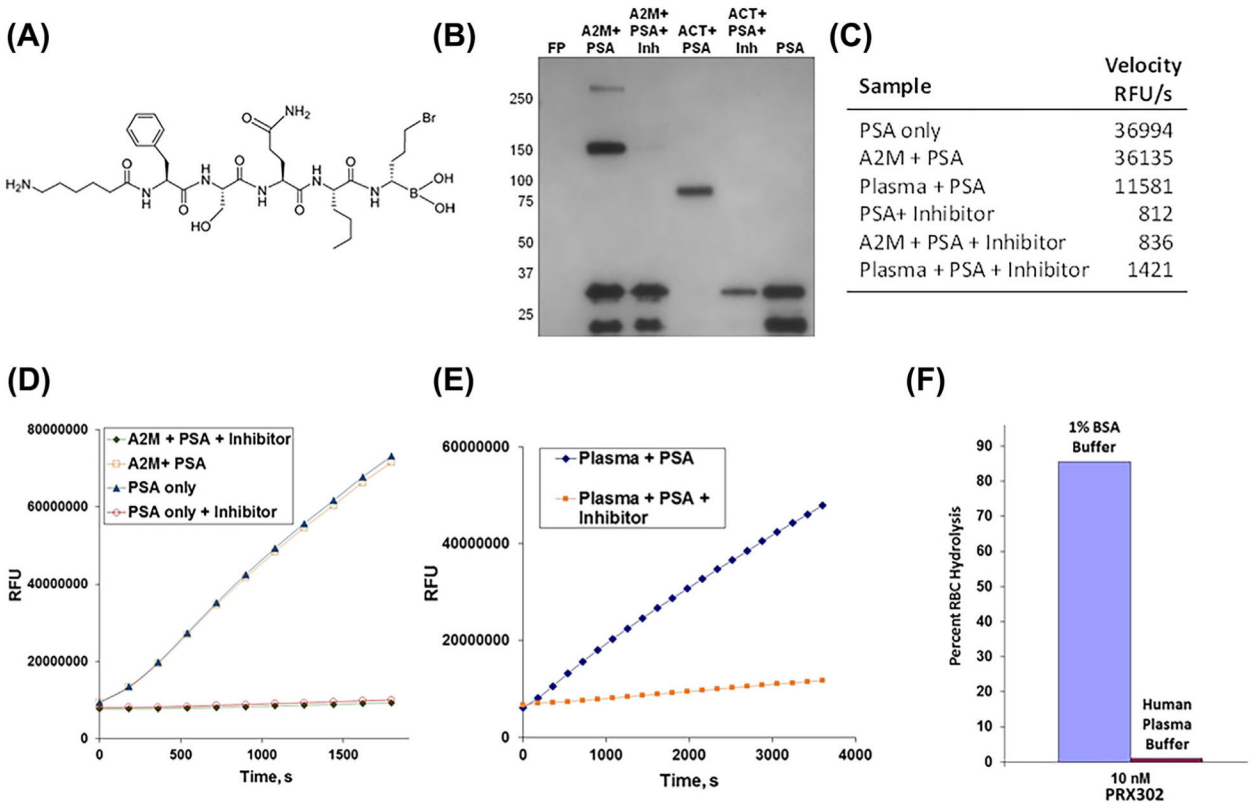

FIGURE 4.

A, Chemical structure of the peptidomimetic boronic acid PSA inhibitor Ahx-FSQn(boro)Bpg; B, Western blot demonstrating ability of PSA inhibitor (10 μM) to block complex formation between PSA (10 μg/mL) and A2M (20 μg/mL) or ACT (20 μg/mL); C, Velocity of hydrolysis of the PSA substrate Mu-SRKSQQY-AMC (300 μM [K_m = 140 μM]) by PSA (10 μg/mL) ± A2M (20 μg/mL in buffer with 1% BSA) or 50% buffered human plasma in the absence or presence of the PSA inhibitor (10 μM); D, Substrate hydrolysis in buffer with 1% BSA under indicated conditions; E, Substrate hydrolysis in buffered human plasma under indicated conditions; F, Activation of PRX302 (PSA-activated proaerolysin) by PSA (10 μg/mL) in buffered human plasma or buffer containing 1% PSA

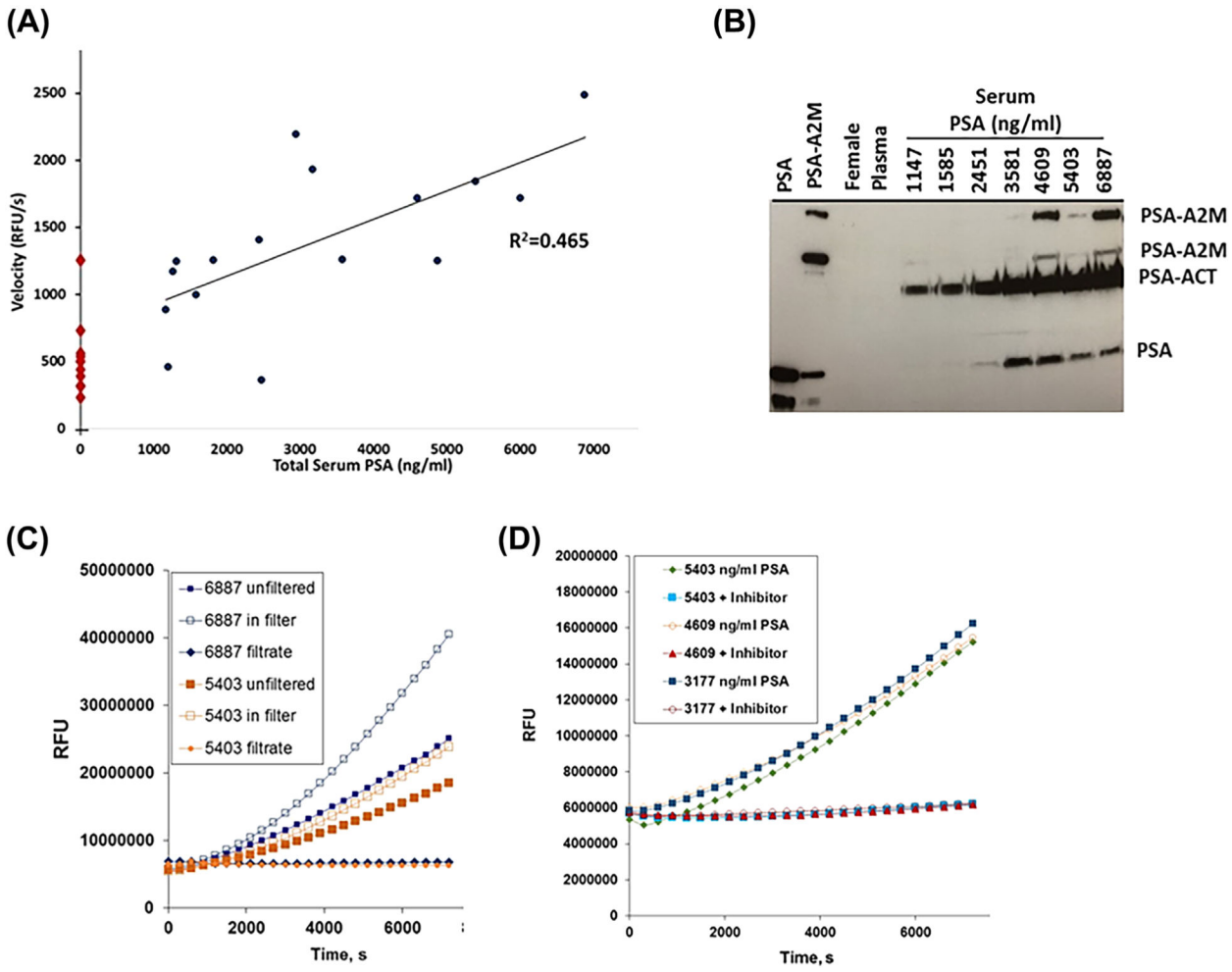

FIGURE 5.

A, Correlation of total serum PSA levels and reaction velocity (blue dots). Red dots indicate reaction velocity of serum samples with PSA < 0.1 ng/mL. B, Western blot of human serum samples with indicated concentrations of total PSA. Bands for PSA-A2M and PSA-ACT complexes and PSA are indicated. C, Hydrolysis of PSA substrate by human serum containing 6887 or 5403 ng/mL total PSA following filtration through 100 kDa cut-off filter (Amicon). D, PSA inhibitor (10 μM) inhibits PSA-A2M activity in human serum containing indicated amounts of total PSA

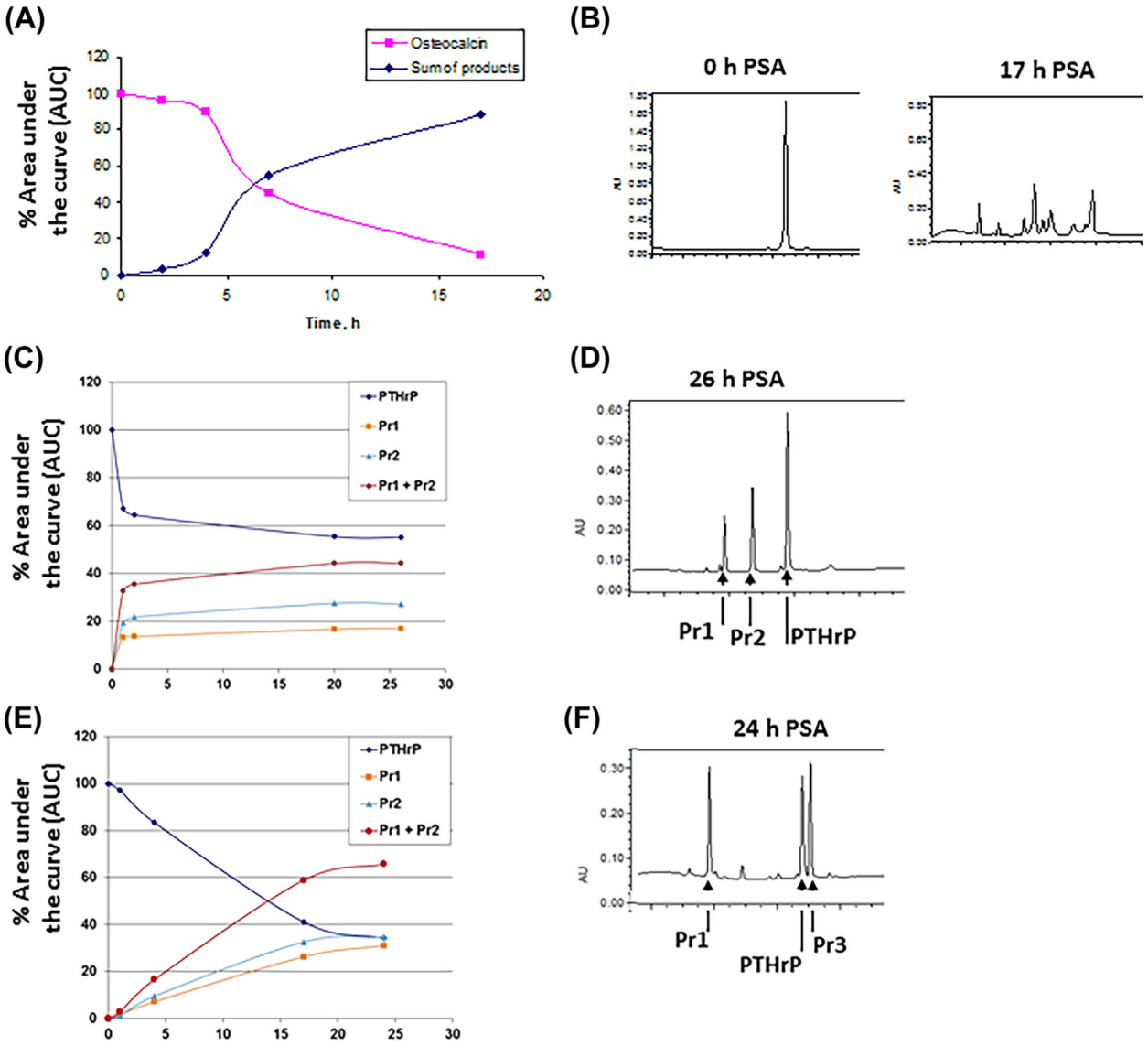

FIGURE 6.

PSA hydrolysis of peptide hormones. A, PSA (10 μg/mL) hydrolysis of osteocalcin (80 μM). Sum of products indicates sum of area under the curve for each peak detected by HPLC. B, HPLC trace of osteocalcin + PSA at 0 and 17 h. C, Hydrolysis of PTHrP-1–34 by PSA over 26 h demonstrating appearance of two products (Pr1, Pr2) over time. D, HPLC trace of PSA + PTHrP at 26 h. E, Hydrolysis of PTHrP-1–34 by purified PSA-A2M complex over 24 h demonstrating appearance of Pr1 and new product (Pr3) over time. F, HPLC trace of PSA-A2M + PTHrP at 24 h

TABLE 1

Characteristics of alpha-2 macroglobulin and TGF-beta 1 (Median [25th, 75th centile]) in study participants

	Normal women	**Normal men PSA <2 ng/mL**	**PCa PSA <20 ng/mL**	**PCa PSA >100 ng/mL**	***P*-value*,*a***	***P*-value*,*b***
Alpha-2 macroglobulin	10.74 (8.83, 13.00)	8.33 (5.71, 10.33)	5.84 (4.00, 6.47)	4.44 (2.30, 5.76)	0.0001	0.002
TGF-beta 1	NA	17 608 (14 387, 20 415)	16 316 (12 392, 21 560)	14 560 (10 941, 21 729)	NA	0.55

*Kruskal-Wallis test.

[a]Including normal women.

[b]Excluding normal women.

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