Presence and enzymatic activity of prostate-specific antigen in archival prostate cancer samples

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Abstract. Patients with advanced prostate cancer frequently
have a poor prognosis as a result of metastasis. The serum
prostate-specific antigen (PSA) test is widely used for the
diagnosis of prostate cancer. The enzymatic activity of PSA
may be involved in the invasion of prostate cancer. We set out
to determine the prevalent form of PSA in human prostate
adenocarcinoma samples by ELISA and Western blot analysis
and its enzymatic activity using a synthetic substrate S-2586
and fibronectin. Our results show that in serum from
prostate cancer patients and in tumour homogenates, the
prevalent form was PSA bound to α1-antichymotrypsin. All
homogenates showed enzymatic activity towards a synthetic
PSA substrate, whereas only five samples showed activity at
28 kDa towards fibronectin as determined by enzymography,
which is most likely due to active PSA. Human prostate cancer
LNCaP cells produced largely inactive PSA. In comparison,
22Rv1 cells produced 29-fold less PSA, but with high specific
activity. Similarly, our results from the human prostate cancer
tissue samples also show that free PSA appears to exist in
diverse forms of very different specific activity. Since PSA, as
a serine protease, may be involved in the invasion of prostate
cancer, our results suggest that prostate cancers have
potentially diverse invasive capacity due to differences in
specific enzymatic activity of PSA.

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Abbreviations: ACT, α1-antichymotrypsin; CM, conditioned
medium; PSA, prostate-specific antigen; TPCK, tosyl phenylalanyl
chloromethyl ketone

Introduction

Patients with advanced prostate cancer frequently have a poor
prognosis as a result of metastasis. The 5-year survival rate for
men diagnosed with advanced prostate cancer is only 33.5%,
compared to 100% when the cancer is initially diagnosed as
localised (1). There are several lines of evidence suggesting
that the serine protease, prostate-specific antigen (PSA), could
be involved in cancer cell invasion and metastasis. PSA is
generally known as a diagnostic marker for prostate cancer and
is also used for monitoring of the disease. Physiologically PSA
hydrolyzes semenogelins I and II to increase sperm motility
(2). Other substrates of PSA include fibronectin (3) and
insulin-like growth factor binding proteins (4). The cleavage of
these substrates by PSA may be related to its involvement in
cancer cell invasion (5,6).

Increased serum PSA levels correlate to tumour volume (7)
and most probably result from leakage of PSA from the
prostatic ductal system into the prostatic stroma and
subsequently into the blood stream. The serum PSA test
measures total PSA, which has been defined to consist of all
immunodetectable PSA and comprises mostly of free PSA and
PSA bound to the protein inhibitor α1-antichymotrypsin
(ACT) (8). One of the various forms of free PSA, pro-PSA, the
latent form of PSA, has to be activated by the action of specific
other proteases, likely kallikrein-2, -4 or -15 (9).

As opposed to serum, where PSA correlates to tumour
volume, PSA staining intensity in tumour tissue has been
shown to inversely correlate to the Gleason score. By
immunohistochemistry (7,10) and similarly by *in situ*
hybridization (11), prostate cancer tissue shows less staining
for PSA than benign prostatic hyperplasia. In tissue
homogenates, PSA correlates inversely with tumour stage
and cytological grade and is a much better indicator of
prostate cancer than serum PSA (12,13).

We set out to determine the prevalent form of PSA and its
activity in human prostate tumour samples towards several
substrates and compare the results to serum from prostate
cancer patients and human prostate cancer cells. The enzymatic
activity of PSA in prostate cancer tissue has not been studied,
with the exception of a study by Denmeade *et al* (14) in which

explant tissue from human prostate tumour samples was used to determine that >80% of PSA is active. The activity was measured using a fluorescent synthetic oligopeptide, which may not be biologically relevant, as PSA cleaves its physiological substrates, semenogelins I and II and fibronectin, at many different sites and amino acids (15).

Materials and methods

Patient population and sample collection. The research project was approved by the Thunder Bay Regional Health Sciences Centre Research Ethics Team and the Board Ethics Committee of St. Joseph's Care Group (Thunder Bay, ON, Canada). Signed informed consents were obtained from patients before the processing of the samples. Serum of six prostate cancer patients with PSA >100 ng/ml was available. Patients had histologically documented prostate adenocarcinoma and ranged from 43 to 83 (median 73) years of age. Venous blood samples were collected in 10 ml tubes and allowed to clot. The serum was frozen at -80°C until performance of the analysis. Human prostate adenocarcinoma samples were obtained after prostatectomy between 1992 and 1995. The Gleason scores for each of the samples were obtained from records in the Pathology Department and were blindly assessed by S. V.-P. using midway sections from the frozen samples (16,17). Samples were snap-frozen in liquid nitrogen and kept at -80°C. Up to ten 8-μm thick sections of frozen tumour samples were homogenised in 1% SDS using a Potter-Elvehjem tissue grinder. The amount of total protein was determined using the CBQCA protein quantitation kit (Molecular Probes, Eugene, OR). Midway sections were mounted and stained with H&E after methanol fixation.

Cell culture. Mycoplasma-free human prostate adenocarcinoma LNCaP (18) and 22Rv1 cells (19) were obtained from ATCC (Manassas, VA) and maintained in RPMI-1640 supplemented with 10% FBS (Hyclone, Logan, UT), 1 mM sodium pyruvate, 10 mM Hepes and antibiotics (100 U/ml penicillin sodium, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B). Media and supplements were obtained from Invitrogen (Burlington, ON, Canada). Prostate cancer cells were grown till 70-80% confluency and washed three times with PBS after which serum-free medium was added. After 5 days, the medium was collected and the cells were lysed using 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1% NP-40. The conditioned media (CM) were concentrated 12-20 times using Centricon centrifugal devices (Fisher Scientific, Ottawa, ON, Canada) with a cut-off of 10 kDa.

Western blot analysis and PSA ELISA. Cell number-standardised CM, cell lysates and protein-standardised tumour homogenates were resolved in non-reducing 10% SDS-PAGE. Prestained protein molecular weight standards were obtained from Invitrogen. Proteins were transferred onto a Hybond-C nitrocellulose membrane. The membrane was incubated with the primary antibody [anti-PSA, 1/400, Santa Cruz Biotechnology Inc. (Santa Cruz, CA) or anti-PSA-ACT, 1/500, Fitzgerald Industries Int. Inc. (Concord, MA)]. Secondary anti-mouse antibody conjugated with HRP (1/2000, Santa Cruz) was used in conjunction with chemiluminescence

detection. The positive control, PSA isolated from seminal fluid, was obtained from Chemicon International (Temecula, CA).

The amount of PSA in serum, CM and tumour homogenates was quantified using a commercially available ELISA kit (Medicorp, Montreal PQ, Canada) against a standard curve (0.2-50 ng PSA/ml) provided with the kit. The amount of PSA is expressed as ng/μl in the case of serum and CM or as ng/μg of total protein for the homogenates.

Enzymatic activity assays. Enzymatic activity was determined using the synthetic chromogenic substrate S-2586 (DiaPharma, West Chester, OH) or using fibronectin as a substrate. Hydrolysis of S-2586 upon incubation with the samples in 0.1 M sodium phosphate buffer and 0.01% Tween-20 (pH 8.0) at 37°C was followed spectrophotometrically at 405 nm. The rate of degradation was determined against a standard curve of *p*-nitroaniline (1 μM - 0.4 mM) and expressed as nmol/h. The specific activity is calculated per ng of PSA per sample. Fibronectin degradation experiments were carried out by incubating tumour samples (5 μg) in the presence of 2.5 μg human fibronectin (Sigma-Aldrich, Oakville, ON, Canada or Chemicon) for 4 or 24 h at 37°C, followed by separation on 5-20% SDS-PAGE and staining with Coomassie Blue. Fibronectin enzymography was carried out by adding 0.4 mg/ml fibronectin to the separating gel of a 12% SDS-PAGE. After protein separation, the gel was incubated for 30 min in 2.5% Triton X-100 and subsequently in 30 mM Tris-Cl (pH 7.5), 100 mM NaCl at 37°C. After 72 h, the gel was stained with Coomassie Blue.

Results

PSA expression in human prostate cancer cell lines. Human prostate adenocarcinoma LNCaP and 22Rv1 cell lines express PSA, albeit to differing degrees (20-22). We confirmed the expression of PSA in these cell lines by quantitative RT-PCR, Western blot analysis and ELISA. By RT-PCR, LNCaP cells expressed 96.4 times more PSA than 22Rv1 cells (data not shown). PSA secreted into the medium by the LNCaP and 22Rv1 cells amounted to 846.9±154.1 and 21.6±0.56 ng/day/10^5 cells, respectively, as determined using ELISA. By Western blotting, however, PSA could not be detected in the 22Rv1-CM. In LNCaP-CM the majority of the PSA was present in the 29-kDa form (Fig. 1B). Western blots probed with an antibody specific for PSA bound to α1-antichymotrypsin (PSA-ACT) demonstrated this form of PSA was absent in the LNCaP-CM (Fig. 1A).

PSA expression in human samples. Serum of six patients with histologically documented prostate adenocarcinoma and a level of serum PSA >100 ng/ml was collected. As determined by Western blot analysis only PSA-ACT (at 79 kDa), but not free PSA, was detectable in serum (Fig. 1A and B). Tumour specimens were also collected from nine prostate cancer patients with Gleason scores between 6 and 9. The Gleason score for each sample was obtained from the original patient records and independently assessed using one H&E-stained midway section from each of the frozen samples. One of the nine samples, R238, was recorded as Gleason score 5, but this
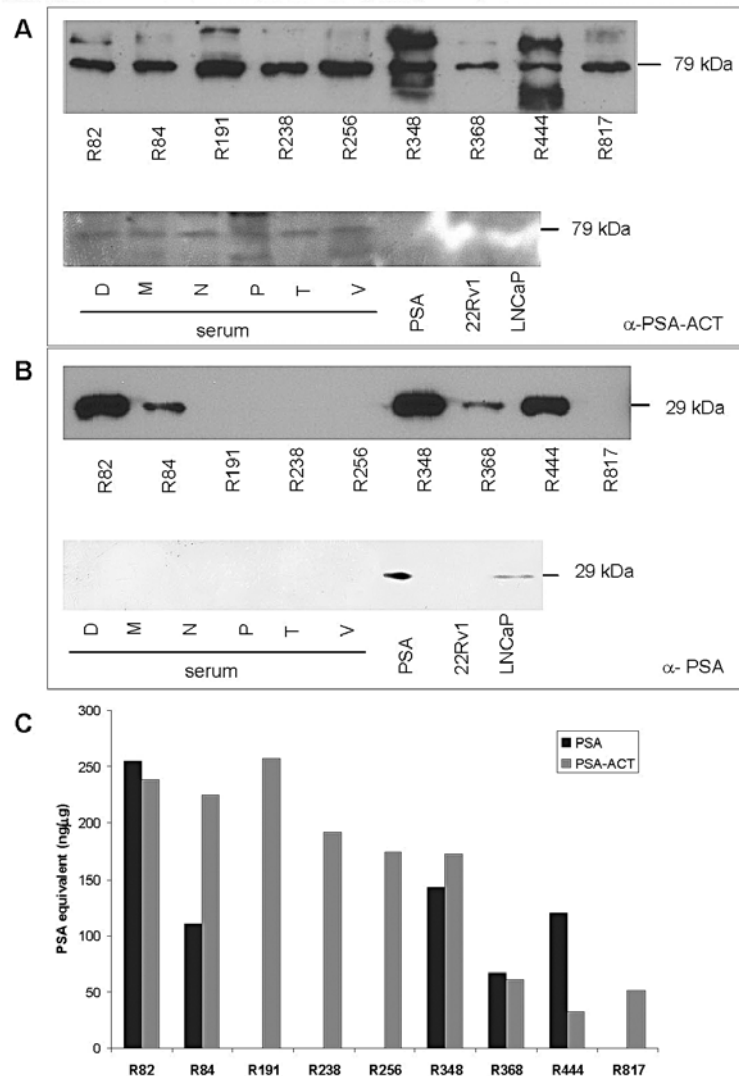
Figure 1. PSA expression determined by Western blot analysis. (A) Homogenised prostate tumour samples (5 µg per sample) were run under non-reducing conditions, transferred to nitrocellulose membrane and probed for (A) PSA bound to α1-antichymotrypsin (PSA-ACT), or for (B) PSA. Samples also included serum samples (D, M, N, P, T, and V), concentrated CM obtained from human prostate adenocarcinoma LNCaP or 22Rv1 cells cells (20 µl) and 25 ng PSA. For the sera an equivalent of 6 ng PSA, as calculated from the results of the PSA-ELISA, was loaded per lane. (C) Densitometric analyses of the bands corresponding to PSA and PSA-ACT as observed in each of the nine tumour homogenates by Western blot analyses shown in (A) and (B).

could not be independently confirmed due to the insufficient quality of the frozen section. Frozen tumour specimens were homogenised and subjected to Western blot analysis and ELISA to detect PSA. All tumour homogenate samples contained PSA bound to ACT, to differing amounts (Fig. 1A). Free PSA was detected in five out of the nine prostate tumour samples (Fig. 1B). The same five PSA-positive homogenates as determined by Western blot analysis were also clearly positive for PSA by ELISA (Table I). The amount of PSA detected by ELISA ranged from 3.14±0.67 to 12.21±3.62 ng/µg protein. Densitometry of the bands corresponding to 29 kDa PSA-form as determined by Western blotting (Fig. 1C) correlated very well with the results obtained by PSA ELISA results (r^2=0.962, p=0.000).

Activity of PSA as measured by the cleavage of the synthetic substrate S-2586. To detect enzymatic PSA activity in the different samples, the synthetic chymotrypsin substrate S-2586

was used. Concentrated CM obtained from human prostate cancer LNCaP and 22 Rv1 cells showed little, but detectable PSA activity towards S-2586 (Table II). The activity amounted to 0.011 pmol/h/ng PSA in the LNCaP cells and 0.29 pmol/h/ng PSA in the 22Rv1 cells. PSA activity was unaffected by the chymotrpysin inhibitor, Tosyl Phenylalanyl Chloromethyl Ketone (TPCK) (data not shown). Of the nine tumour homogenates, R256 showed very high activity against S-2586 (Table I), followed by R817. The activity of the tumour samples against S-2586 was inhibitable to a certain extent by TPCK. The highest inhibition of activity by 100 µg/ml TPCK amounted to 55% in R256. In samples with low activity, such as R368 and R444, the inhibition was 34 and 19%, respectively. In R191, the inhibition only amounted to 1.8%. Serum samples showed comparable activity to five of the tumour samples with low activity. The most activity was found in serum V, which contained the lowest amount of PSA (0.20±0.03 ng/µl).

Table I. Enzymatic activity of PSA in human prostate tumour homogenates.[a]

Sample	Gleason Score	Amount of PSA (ng/μg protein)	PSA activity (nmol/h/μg protein)	Specific activity (nmol/h/ng PSA)
R82	6	12.21±3.62	0.25±0.05	0.02±0.01
R84	6	3.43±0.67	0.29±0.05	0.09±0.02
R191	8	0.04±0.02	0.09±0.05	2.31±1.35
R238	5[b]	<0.04±0.02	0.40±0.07	>9.98±5.25
R256	9	<0.07±0.06	3.10±0.35	>44.27±38.27
R348	6	9.73±2.46	0.76±0.13	0.08±0.02
R368	7	3.14±0.67	0.34±0.03	0.11±0.03
R444	6	4.90±1.41	0.48±0.08	0.10±0.03
R817	9	<0.03±0.02	0.78±0.13	>26.09±17.84

[a]The enzymatic activity of PSA (± SEM) was determined in prostate tumour homogenates obtained from nine prostate cancer patients by the rate of cleavage of S-2586. The amount of PSA (± SEM) was determined by ELISA. The activity is expressed per ng PSA or μg total protein. [b]Gleason score could not be independently confirmed.

Table II. Enzymatic activity of PSA in serum samples and LNCaP- and 22Rv1-conditioned medium.[a]

Sample	Amount of PSA (ng/μl)	Specific PSA activity (nmol/h/ng PSA)
D	2.10±0.18	0.10±0.05
M	1.06±0.09	0.09±0.02
N	0.92±0.07	0.12±0.05
P	0.23±0.05	0.16±0.08
T	3.77±0.17	0.05±0.01
V	0.20±0.03	0.70±0.12
		(pmol/h/ng PSA)
LNCaP-CM	3843.2±699.2	0.011±0.001
22Rv1-CM	72.63±1.87	0.29±0.09

[a]The enzymatic activity of PSA (± SEM) was determined in serum samples obtained from six prostate cancer patients and in concentrated medium conditioned for 5 days by human prostate adenocarcinoma LNCaP or 22Rv1 cells by the rate of cleavage of S-2586. The amount of PSA (± SEM) was determined by ELISA. The enzymatic activity is expressed per ng PSA.

Activity of PSA as measured by the cleavage of fibronectin. Fibronectin is one of the physiological substrates of PSA (23). Enzymatic activity of PSA was measured against fibronectin in two different ways. Enzymography was carried out by adding 0.4 mg/ml fibronectin to the separating gel and subsequent incubation to allow degradation followed by Coomassie Blue staining. Activity towards fibronectin was observed at 29 kDa in LNCaP CM (Fig. 2B). Fibronectin degrading activity was also observed in sera at molecular weights corresponding to 28 and 79 kDa. Fig. 2A shows the activity observed in the tumour homogenates. In the tumour samples R191, R238, and R444, activity was observed at molecular weights corresponding to 28 and 29 kDa, whereas R256 showed fibronectinolytic activity at a slightly higher molecular weight and at >90 kDa. Tumour sample, R82, clearly showed three bands ~30 kDa.

Fibronectin was also directly incubated with tumour homogenates at 37˚C for 4 and 24 h and subsequently run on a gradient SDS-PAGE of 5-20% (Fig. 3). Tumour sample, R256, clearly contained degradative capacity towards fibronectin, as all three higher molecular weight protein bands ~170, 140 and 105 kDa disappeared upon 4 h incubation. The same also happened in the presence of R82, but to a lesser extent. The degradation could be inhibited by the addition of 200 μM Zn^{2+}. PSA (200 ng), however, was unable to degrade fibronectin over 4 h, whereas 2.8 μg PSA was sufficient, especially when the incubation time was increased to 24 h. The degradation of fibronectin by R256 could be inhibited by TPCK, but again only partially.

Discussion

Western blot analysis shows that the majority of PSA was present in the 29-kDa form in LNCaP-CM, as previously reported (24). In the 22Rv1 cells, PSA expression was undetectable by Western blotting, but was confirmed by
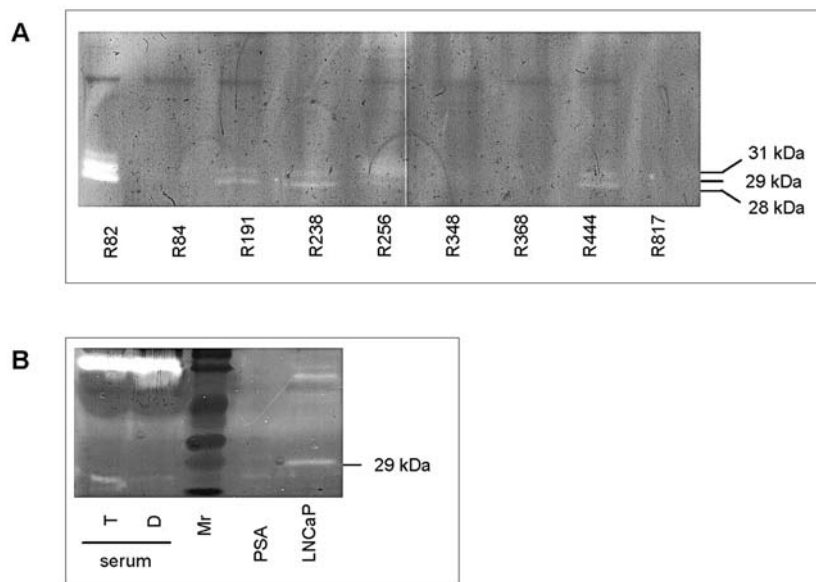
Figure 2. Detection of enzymatic activity as determined by fibronectin enzymography. (A) Fibronectin enzymography of tumour homogenates obtained from prostate adenocarcinoma (2.5 µg each). The activity of different forms of PSA towards fibronectin at M_r 28, 29, and 31 kDa is indicated. (B) Fibronectin enzymography using concentrated CM of human prostate adenocarcinoma LNCaP cells and sera from two prostate cancer patients. For the sera an equivalent of 6 ng PSA, as calculated from the results of the PSA-ELISA, was loaded per lane.
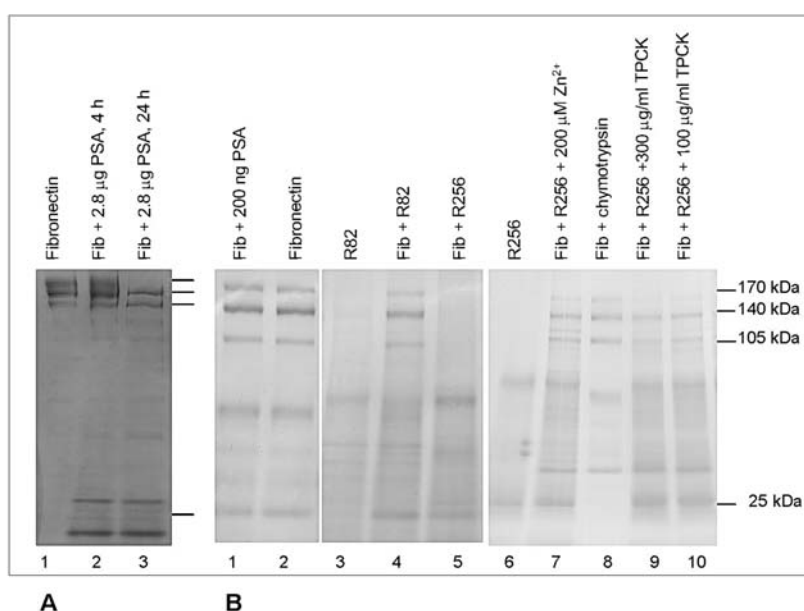

Figure 3. Fibronectin degradation. (A) Degradative activity of 2.8 µg PSA incubated for 4 and 24 h (lanes 2 and 3, respectively) together with 5 µg fibronectin (Chemicon) as a substrate. Samples were incubated at 37˚C and run on a 5-20% gradient SDS-PAGE. Lane 1 represents 5 µg fibronectin alone incubated for 24 h. (B) Degradative activity determined in tumour homogenates R82 and R256 in the presence or absence of TPCK or Zn^{2+} using fibronectin (Sigma-Aldrich) as a substrate. R82 and R256 (2.5 µg) alone are shown in lanes 3 and 6, respectively. Fibronectin alone is shown in lane 2. Fibronectin incubated with 200 ng PSA, R82 or R256 in lanes 1, 4 and 5, respectively. TPCK at 100 µg/ml (lane 10) or 300 µg/ml (lane 9) or 200 µM Zn^{2+} (lane 7) were added to the incubation of R256 and fibronectin as inhibitors of chymotrypsin and PSA, respectively. Fibronectin incubated with chymotrypsin (3 ng) is shown in lane 8. Samples were incubated at 37˚C for 4 h and run on a 5-20% gradient SDS-PAGE. The fibronectin bands with calculated molecular weights of 170, 140, 105 and 25 kDa are indicated.

ELISA. The two minor additional bands that were observed in LNCaP-CM when an increased amount of sample was loaded (not shown), most likely represent slightly different forms of PSA, such as N-terminally truncated forms of PSA (21,25). In the LNCaP-conditioned media PSA-ACT was absent, even though these cells express ACT as by determined by RT-PCR (24). This suggests that PSA secreted by LNCaP cells is not in the active form, as ACT can only complex with activated PSA (26). In serum, the opposite was true; the predominant form in serum was PSA-ACT, free PSA was not detectable. This confirmed that PSA in serum is primarily bound to its inhibitors (26). PSA-ACT was also detectable in all nine tumour samples analysed, albeit to differing amounts. Free PSA was only detected in five out of the nine

prostate tumour samples. The same five PSA-positive samples were also clearly positive for PSA by ELISA. Regression analysis showed that the densitometry of the bands corresponding to 29 kDa PSA-form as determined by Western blotting (Fig. 1C) correlated very well to the results obtained by ELISA (r^2=0.962, p=0.000). Interestingly, when densitometric data for free PSA and PSA-ACT were combined, the correlation was less statistically significant (r^2=0.724, p=0.028), suggesting that the antibodies utilised by the ELISA do not have equal affinity for free PSA and PSA-ACT.

To measure the enzymatic activity of PSA in the various samples, several approaches were taken. Both S-2586, a synthetic chymotrypsin substrate and fibronectin were employed as substrates. S-2586 has been used extensively to measure PSA activity (21,22,27). However, PSA cleaves its physiological substrates at many different sites and amino acids (15), therefore the use of fibronectin as a substrate may be more biologically relevant. The LNCaP-CM showed little, but detectable PSA activity towards fibronectin by enzymography and S-2586 (Figs. 2B and 3), respectively. Using enzymography, the LNCaP-CM showed activity towards fibronectin at 29 and 79 kDa, indicative of PSA and PSA-ACT. From these results, combined with our ELISA data, we conclude that the LNCaP cells produced more, but less active PSA when compared to the 22Rv1 cells. This confirms earlier reports that PSA produced by LNCaP cells is N-terminally truncated or nicked (21,22,24,25). Surprisingly, when compared to the prostate cancer cells, the serum samples showed much higher activity towards S-2586. The most active serum was serum V, which contained the lowest amount of PSA. Fibronectinolytic activity was observed at 28 kDa in the sera, but the major activity was found at a molecular weight of >79 kDa consistent with the large amounts of PSA-ACT found by Western blot analysis.

Of the nine tumour homogenates, R256 showed very high activity using all three methods. R256 exhibited an activity of 44.3±38.3 nmol/h/ng PSA towards S-2586. Activity of three of the nine tumour samples (R238, R256 and R817) against S-2586 was at least 10-fold higher than the other six. These higher activities were in part due to the low presence of PSA, but were still high when the activity was expressed per μg protein (Table I). Since PSA activity is unaffected by TPCK, a chymotrpysin inhibitor, we used it to evaluate the contribution of PSA in the tumour homogenates. The highest inhibition by 100 μg/ml TPCK amounted to 55% in R256. The activity of most other tumour samples was inhibited only to a certain extent by TPCK (1.8-34%). By enzymography, activity towards fibronectin was observed in five tumour homogenates (R82, R238, R191, R256 and R444). In tumour samples R191, R238 and R444, activity was observed at molecular weights of 28 and 29 kDa, likely corresponding to PSA and pro-PSA. Tumour sample R82 clearly showed three bands, ~30 kDa, with molecular weights corresponding to preproPSA, pro-PSA and PSA. By Western blot analysis, we also observed these three bands when increasing amount of sample was loaded (data not shown).

Tumour homogenates were also directly incubated with fibronectin at 37°C for 4 and 24 h and the degradation was determined. Again, tumour sample R256 clearly was able to degrade fibronectin, as all three higher molecular weight protein bands ~170, 140 and 105 kDa disappeared upon 4 h incubation. The degradation of fibronectin by R256 could only be partially inhibited by TPCK. Fibronectin incubated with chymotrpysin, however, remained largely intact. This suggests that a chymotrpysin-like TPCK-inhibitable enzyme may have been partly responsible for the degradative activity found in R256.

The different assays to determine PSA activity demonstrated somewhat inconsistent results. When fibronectin was incubated with tumour homogenates, it is clear that both R82 and R256 contained degradative capacity towards fibronectin. The degradation could be inhibited by 200 μM Zn^{2+}, indicating that PSA, or another related kallikrein, may be responsible for the degradation, at least in part (6,15). However, R256 only contained PSA-ACT and no detectable PSA, neither by ELISA nor Western blot analysis. Using the same method, we also showed that 200 ng PSA was not sufficient to degrade fibronectin over 4 h (Fig. 3B, lane 1). Other proteases can also degrade fibronectin, most importantly the matrix metallo-proteinases MMP-2, -7 and -9 (28). Matrilysin (MMP-7) would be a likely candidate with a molecular weight of 29.7 kDa in its latent form. However, Zn^{2+} does not inhibit MMP-7. The gelatinases MMP-2 and -9 have a higher molecular weight, of 72 and 92 kDa, respectively. Additional likely candidates are other prostatically expressed kallikrein-related peptidases, such as KLK2 and KLK4, that have many similarities with PSA, including overlapping substrate specificities and similar apparent molecular weights of 34 (29) and 40 kDa (30), respectively. Moreover, fibronectin is hydrolysed more efficiently by KLK2 than by PSA (31). On the other hand, S-2586 is not a substrate for KLK2 and R256 showed high activity towards S-2586. Tumour sample R82, however, did not show appreciable activity using S-2586 as a substrate, but exhibited clear PSA expression by Western blot analysis, ELISA and by fibronectin enzymography. The same holds true for R444, but to a lesser extent. Taken together, these data provide evidence for the fact that free PSA appears to exist in diverse forms of very different specific activity. The nature of the proteolytic band at ~30 kDa in R256 remains unclear and will be determined in future investigations.

The prevalent form of PSA in prostate tumour samples, according to molecular weight, appeared to be the 79 kDa PSA-ACT form; other forms of PSA, most likely corresponding to free PSA, were also detectable in five of the nine samples. The tumour homogenates from the patients with the higher Gleason scores of 8 and 9 had no detectable PSA, consistent with reports showing that PSA correlates inversely with tumour stage and cytological grade (12,13). The enzymatic activity of PSA in prostate cancer tissue has not been previously studied, with the exception of a report by Denmeade *et al* (14) using explant tissue from human prostate tumour samples to conclude that >80% of PSA is active. The activity was measured using a fluorescent synthetic oligopeptide, which may not be biologically relevant, and did not take into account the fact that PSA may exist in different forms of different specific activity. In conclusion, our results from the human prostate cancer tissue samples and two human prostate cancer cell lines showed that free PSA appears to exist in diverse forms of very different specific activity. This may

...ications for the invasive capacity of human prostate ...s evidence suggests that the enzymatic activity of PSA may be involved in these processes.

Acknowledgements

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