**Advances in Brief**

**Prostate-specific Antigen, a Serine Protease, Facilitates Human Prostate Cancer Cell Invasion**

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**Abstract**

Human prostatic epithelial cells constitutively secrete prostate-specific antigen (PSA), a kallikrein-like serine protease, which is a normal component of the seminal plasma. PSA is currently used as a specific diagnostic marker for the early detection of prostate cancer. We demonstrate that PSA degrades extracellular matrix glycoproteins fibronectin and laminin and, thus, may facilitate invasion by prostate cancer cells. Blocking PSA proteolytic activity with PSA-specific mAb results in a dose-dependent decrease in vitro in the invasion of the reconstituted basement membrane Matrigel by LNCaP human prostate carcinoma cells which secrete high levels of PSA. A novel PSA-SDS-PAGE zymography method for the detection of matrix degrading ability of PSA is also described. We propose that: (a) because of the dysplastic cellular disorganization in early neoplastic lesions called prostatic intraepithelial neoplasia (PIN), PSA may be secreted not only at the luminal end but also, abnormally, at the cell-basement membrane interface, causing matrix degradation and facilitating invasion; and (b) PSA, along with urokinase, another serine protease secreted by prostatic epithelium, may be involved in the proteolytic cascade during prostate cancer invasion and metastasis. The discovery of the extracellular matrix degrading ability of PSA not only makes it a marker for early detection but also a target for prevention and intervention in prostate cancer.

**Introduction**

Prostate cancer is the most common cancer in adult men in the United States. An estimated 244,000 new cases and 40,400 deaths from prostate cancer will occur in the United States in 1995 (1). The incidence increases with age, and about 80% of prostate cancers are diagnosed after the age of 65 years (2). Because of the increasing life span and an aging American male population, prostate cancer is a major health concern. The most threatening and primary cause of death from prostate cancer is locally advanced disease (3). Serum PSA levels in patients decline. A subsequent increase in serum PSA level indicates cancer recurrence (12). Although elevated levels of PSA in the serum of prostate cancer patients were observed over 25 years ago, PSA measurement has come into wide use only recently for early detection of prostate cancer, for monitoring patients following radical prostatectomy, and for identifying metastatic tumors of unknown origin (13). Serum PSA measurement in combination with digital rectal examination and transrectal ultrasonography has greatly increased the ability to detect prostate-confined cancer (12, 13).

PSA is an important component and one of the most abundant serine proteases in the seminal plasma, where it is found at an average concentration of about 1.0 mg/ml (9). Immediately after ejaculation, the seminal plasma coagulates into a viscous gel which liquefies within about 20 min. PSA mediates this liquefaction (14). An understanding of the liquefaction process provides clues to the mechanism of matrix degradation by PSA in prostate cancer invasion. The seminal coagulum is formed by the two most abundant, large molecular...
weight proteins in semen, semenogelin and fibronectin, both of which are contributed by the seminal vesicles. During liquefaction, PSA-mediated degradation of semenogelin and fibronectin runs parallel with the dissolution of the seminal gel (14). Laminin, type IV collagen, and fibronectin are major components of the BM and ECM, respectively, and their degradation is the initial step in the metastatic cascade. Our objective was to determine whether PSA was involved in ECM degradation and invasion.

**Materials and Methods**

**Materials.** PSA, Calbiochem 539832, mAb to PSA, DAKO M750; polyclonal, anticatalytic antibody to u-PA, American Diagnostica 389; ε-aminoacaproic acid, A7824, monoclonal mouse antihuman antibody against fibronectin, F-7387, monoclonal mouse antihuman antibody against laminin, L-8271, human plasminogen 5661, and BSA, A-2153 (Sigma); gelatin, Bio-Rad 170-6537; fetal bovine serum, Intergen 1020-L-827!, human plasminogen 5661, and BSA, A-2153 (Sigma); monoclonal mouse antihuman antibody against laminin, DAKO M750; polyclonal, anticatalytic antibody to u-PA, Vector ABC kit, AK-5001 for Western blots; Vector peroxidase Elite ABC kit PK-6102, and 3,3'-diaminobenzidine (DAB) nickel kit SK-4100 for immunostaining (Vector Laboratories); RPMI 1640 medium, GIBCO 320-1875AJ; Nucleopore filters, 8-μm pore size, Costar 150446, and HEMA-3, Curtin Matheson 122-911 were used.

**SDS-PAGE Zymography for Detection of Urokinase Activity.** PSA samples were analyzed using SDS-PAGE zymography (4) to confirm the absence of u-PA and plasmin activity. Substrates for u-PA and plasmin were incorporated at a concentration of 12 μg of a solid plasminogen preparation (activity, 0.44 units/mg solid) and 0.9 mg gelatin, respectively, per ml of acrylamide in a 10% separating gel. Six μg PSA/lane were run in minigels (200 V, 4°C, 45 min). The gels were further processed to renature the enzymes and stained with Coomassie blue. The presence of enzyme activity is indicated by bands of lysis against a dark background (4). This u-PA activity could be blocked by antibody to u-PA. Six μg PSA were mixed with 20 μg u-PA antibody and 50 μg ε-aminoacaproic acid and incubated for 2 h at 37°C before SDS-PAGE zymography.

**PSA-SDS-PAGE Zymography for Detection of PSA Proteolytic Activity and the Anticatalytic Ability of Antibody to PSA.** To determine whether PSA alone has protease activity and can degrade fibronectin, PSA samples were analyzed in our new and novel SDS-PAGE zymography method where fibronectin was incorporated into a 12% acrylamide gel to serve as a substrate for PSA. Gels were run as described above, but stained using a silver stain (15). To determine whether the antibody to PSA (PSA-Ab) has anticatalytic activity, 10-μg PSA samples were preincubated with 0.75 μg PSA-Ab (IgG) for 17 h at 37°C prior to SDS-PAGE zymography. It should be noted that not only does the anticatalytic ability of various PSA antibodies but also the protease activity of different PSA samples needs to be established because PSA can lose this activity during the purification process.

**SDS-PAGE and Western Blot Analysis.** Affinity-purified PSA derived from human seminal plasma was used for Western blots and for experiments to further examine fibronectin and laminin degradation by PSA. To determine whether PSA can degrade fibronectin and laminin, it was necessary to first establish whether the PSA sample had any contaminating u-PA activity. Therefore, PSA samples were subjected to Western blot analysis as well as zymography. Twelve μg PSA/lane were analyzed using SDS-PAGE on 4–15% gradient minigels (200 V, 4°C, 45 min) and transferred to Immunoblot membrane. Blots for PSA were stained with mAb to PSA (1:100), and for urokinase, with polyclonal antibody to urokinase (1:500), followed by avidin-biotin alkaline phosphatase using a Vector ABC kit.

**Fibronectin and Laminin Degradation.** Degradation of fibronectin and laminin by PSA is demonstrated by immunoblot analysis. Twelve μg PSA were incubated with 20 μg antibody to u-PA and 40 μg ε-aminoacaproic acid for 2 h at 37°C to block any trace of u-PA and plasmin activity. A 2.5-μg sample of fibronectin only was run as a control. For fibronectin degradation, 2.5 μg fibronectin were incubated (17 h, 37°C) with 12 μg PSA prepared as above, run on a 4–15% gradient gel, transferred to Immunoblot, and stained with mouse antihuman primary antibody against fibronectin (1:500), biotinylated goat antimouse secondary antibody (1:10,000), and avidin-biotin alkaline phosphatase. For a laminin control, 2.5 μg of reduced laminin only, boiled with β-mercaptoethanol for 5 min, were run. For laminin degradation, 2.5 μg laminin were incubated with 12 μg PSA prepared as described above, resolved by SDS-PAGE, and stained with mouse antihuman primary antibody against laminin (1:500). Laminin sample was reduced before loading into the gel. These experiments were repeated four times.

**Immunocytochemical Staining for PSA in LNCaP Cells.** LNCaP cells were plated on glass coverslips in RPMI 1640 medium and 15% fetal bovine serum at a density of 20,000/400 μl medium/well in 24-well culture plates. The following steps were performed at room temperature. Cells were rinsed with PBS (10 min) between all successive steps after primary antibody application. Cells were fixed in 50:50 methanol:acetone, blocked with horse serum (Vector kit) for 1 h, and incubated for 24 h with mAb to PSA diluted 1:20 in horse serum. Coverslips lacking primary antibody served as controls. Prior to staining, cells were treated with 3% hydrogen peroxide for 3 min to quench endogenous peroxidase activity, then stained using a Vectorstain Elite ABC avidin-biotin peroxidase complex for 30 min and developed with diaminobenzidine-nickel chloride substrate for 5 min.

**Invasion Assay.** Nucleopore filters were coated with 500 μg/ml Matrigel (16). LNCaP cells were released from mother flasks using 1 mM EDTA and suspended in RPMI 1640 medium containing 0.1% BSA. Two hundred thousand or 400,000 cells/200 μl medium were plated on the coated filter in Boyden chambers containing 650 μl medium. The lower chamber contained 220 μl of the chemoattractant conditioned medium from NIH/3T3 fibroblasts grown for 24 h in serum-free medium containing 50 μg/ml ascorbic acid. Cells were allowed to migrate for 6 to 48 h, fixed on the filter, and stained with HEMA-3. Nuclear stain was extracted for 15 min with 0.1 N HCl, and absorbance was measured at 620 nm using a Titertek microplate reader (17). Three replicate cultures were prepared per treatment, and the mean values were plotted as percentage of control, taking untreated invaded cells as 100%. In experiments to determine the role of PSA in invasion, PSA activity was blocked...
with PSA antibody (IgG) at 6.25, 12.5, 25, 50, and 100 ng/ml in the final cell suspension. A suspension of one or two million LNCaP cells/ml of serum-free RPMI 1640 medium containing 0.1% BSA was incubated with the antibody for 2 h before performing the assay. A nonspecific immunoglobulin (IgG) was used as a control. Nine such separate experiments were conducted.

Results and Discussion

To attribute degradation of fibronectin and laminin to PSA, it was necessary to first ascertain whether any u-PA activity was present in the PSA sample to be used to assess its potential for matrix degradation. Both PSA and urokinase are normally secreted by the prostate and constitute the prostate’s contribution to the seminal plasma, from which PSA was purified. As stated earlier, urokinase is also involved in matrix degradation (4, 5).

In the immunoblot in Fig. 1A, both lanes received a PSA sample. Lane 1 was stained with human PSA-specific and Lane 2 with u-PA-specific antibody. In Lane 1, a series of bands at Mr ~33,000 represent PSA. Variations in prostatic PSA molecular weight (20,000–35,000) due to glycosylation and alternative splicing have been reported (6, 18). Absence of staining in Lane 2 indicates that u-PA was not detected in the PSA sample using immunoblot analysis. A more sensitive zymographic assay, which can detect as little as 10^-14 mol u-PA (19), was then used to detect trace u-PA activity in the PSA sample by SDS-PAGE in which the gel also contained plasminogen, a substrate for urokinase (4). The faint zone of lysis at Mr ~54,000 (Fig. 1B, Lane 1) represents the high molecular weight u-PA, indicating the presence of trace u-PA activity in the PSA sample. This activity could be completely blocked by urokinase-specific antibody (Fig. 1B, Lane 2). Even traces of urokinase are sufficient to generate plasmin in the presence of plasminogen. Plasmin, also a serine protease, is formed by urokinase-mediated catalysis of plasminogen to plasmin, and it can degrade both fibronectin and laminin. The zone of lysis in the PSA zymogram in Fig. 1C (6 µg PSA), which used fibronectin as a substrate, shows that PSA has protease activity and it can degrade fibronectin. In Fig. 1D (Lane 1), when a larger amount (10 µg) of PSA was run in a similar gel, two zones of lysis, one at Mr ~31,000 and the other at a lower molecular weight (~25,000) were observed. This lysis could be blocked by preincubation of PSA with PSA-Ab (Fig. 1D, Lane 2), indicating that the antibody to PSA used has anticatalytic ability.

Fig. 2  Degradation of fibronectin and laminin by PSA is demonstrated by Western blot analysis. Samples were resolved on 4–15% gradient gels using SDS-PAGE and transferred, and blots were stained with the respective antibodies. A, immunoblot to show fibronectin degradation by PSA. Lane 1, 2.5-µg sample of fibronectin only (control); Lane 2, 2.5 µg fibronectin were incubated with PSA prepared as described in ‘Materials and Methods.’ Fibronectin degradation products are shown. Blot was stained with mouse antihuman primary antibody against fibronectin (1:500). B, immunoblot for laminin degradation by PSA. Lane 1, 2.5 µg of reduced laminin only; Lane 2, 2.5 µg laminin were incubated with PSA prepared as described above. Laminin sample was reduced before loading into the gel. Blot was stained with mouse antihuman primary antibody against laminin (1:500).
manner were then used to examine the ability of PSA to degrade the matrix proteins. Samples of fibronectin or laminin were incubated with PSA, and the degradation products were separated by SDS-PAGE and detected using immunoblot analysis. Fig. 2A (Lane 1) shows the undegraded, control fibronectin bands at $M_r \sim 440,000$ dimer and at $M_r 220,000$ and 210,000 monomeric forms. After incubation of fibronectin with PSA, several low molecular weight fibronectin degradation bands were observed, indicating that PSA can degrade fibronectin (Fig. 2A, Lane 2). Similarly, in Fig. 2B, Lane 1 shows the undegraded, control laminin bands at $M_r \sim 400,000$ and 220,000, and in Fig. 2B, Lane 2 shows loss of the two high molecular weight laminin bands and the appearance of several low molecular weight degradation products, indicating that PSA degrades laminin.

To demonstrate that PSA may play a role in invasion by prostate cancer cells, the relationship between free, secreted PSA and the invasive ability of LNCaP cells was examined. LNCaP cells are a useful model for this study because they produce only a small amount of u-PA as compared to a more invasive DU145 human prostate carcinoma cell line, and they also secrete very low levels of gelatinases. Nevertheless, they are invasive, although much less than DU145 cells in an in vitro invasion assay. LNCaP cells were derived by Horoszewicz et al. (10) from a lymph node metastasis of prostate carcinoma, and they formed invasive tumors at the site of injection in nude mice 8 weeks after injection, but distal metastases were not apparent at that time. LNCaP cells express (Fig. 3) and secrete high levels of PSA. The possible role of PSA in invasion is further suggested by results which show (Fig. 4) that in the presence of PSA-specific antibody, the ability of LNCaP cells to invade a reconstituted BM Matrigel is markedly reduced in a dose-dependent manner. However, a nonspecific immunoglobulin did not inhibit invasion. PSA zymography results (Fig. 1D) already showed that the PSA-Ab used has anticatalytic ability. Therefore, a dose-dependent inhibition of invasion by this antibody in the invasion assay suggests that PSA may be associated with invasion by LNCaP cells.

On the basis of these results, we propose that PSA, along with u-PA, may be involved in the proteolytic cascade in prostate cancer. Evidence suggests that a proteolytic cascade independent of metalloproteases may exist and that degradation of type IV collagen, an important component of the BM, can occur via a plasmin-dependent but metalloprotease-independent pathway (20). Furthermore, kallikreins such as PSA can activate prourokinase to its active form and subsequently, both u-PA and plasmin can activate procollagenases (5). Our results show that ECM components can serve as substrates for PSA, and, thus, PSA may be involved in localized proteolysis in tumor cell invasion in prostate cancer.

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Unpublished data.
If PSA plays a role in the proteolytic cascade in prostate cancer, as suggested here, why are LNCaP cells, which secrete PSA, less invasive than DU145 human prostatic carcinoma cells, which do not secrete PSA? The answer may lie in the fact that DU145 cells secrete high levels of urokinase (4). In the presence of plasminogen, which is abundantly available in vivo in blood and extracellular fluids, and in vitro, in serum and pituitary extract, plasmin is generated by the activation of plasminogen by u-PA. Plasmin, a potent protease, has a wide substrate specificity and is able to degrade not only fibrin, fibronectin, and laminin but also type IV collagen (20). Therefore, plasmin-mediated proteolysis is much more efficient than that mediated by PSA. As a result, DU145 cells, which secrete high levels of u-PA, are more invasive than LNCaP cells. If prostate cancer cells are secreting urokinase, PSA would not be required for invasion. However, PSA may contribute to the invasive ability of prostate cancer cells. Our results suggest that in the absence of high urokinase secretion, PSA could mediate invasion, as in LNCaP cells. These proposed mechanisms do not, however, exclude the involvement of other proteases in the proteolytic cascade.

The presence of low grade PINs has been observed in men in their 20’s and 30’s, the frequency of preinvasive high-grade PIN increases with age and PINs may precede cancer by 10 or more years (21, 22). Focal proliferation, cellular disorganization and heterogeneity, and disruption of the basal epithelial cell layer, followed by disruption of the BM in high-grade PIN have been observed (22, 23). In the normal prostate gland in vivo, polarized secretion of prostatic proteases, such as PSA and u-PA, takes place at the apical, luminal end of glandular epithelium. Disorganization of prostatic epithelial cell monolayers in vitro can lead to a loss of polarized secretion so that cells begin to secrete proteases at their apical as well as at their basal end (24). Accepting the cellular disorganization as seen in dysplasia in vivo, changes in the polarity of secretion by epithelial cells could take place, leading to similar loss of polarized secretion. This would result in the secretion of PSA and u-PA not only into the gland lumen but also at the cell-BM interface, causing localized proteolysis of the BM and ECM which culminates in invasion and metastasis. Such protease secretion would lead to their leakage and entry into capillaries (Fig. 5), resulting in increased serum PSA levels in prostate cancer patients. This would also explain why increased serum PSA levels have been observed as early as 6 years before prostate cancer is discovered by rectal examination (25). For example, the mean serum PSA level in normal, high-grade PIN, and carcinoma patients is 4, 7, and 17.9 ng/ml, respectively (23). The increased serum PSA levels need not necessarily result from an increased production by carcinoma cells but may be due to its leakage into the blood vessels. Detection of PSA mRNA by PCR in cells from peripheral blood of prostate cancer patients also suggests that dissemination of prostate cancer may be a relatively early event (26).

We postulate that since prostatic epithelium secretes both urokinase and PSA, cells in preinvasive prostatic lesions, such as high-grade PIN, may have an inherent ability and predilection to invade and metastasize. This may explain why a majority of prostate cancer patients already have disseminated disease beyond the prostate at initial diagnosis by rectal examination. In invasion, the net extracellular proteolysis at the cell-BM interface will depend on the ratio between the levels of extracellular proteases and their natural inhibitors. The balance between the protease and its inhibitors must be in favor of the active protease in order for proteolysis to occur. Serine protease inhibitors (serpins; Ref. 27) and their modulators may serve as useful agents for blocking the progression of preinvasive to invasive prostatic carcinoma. Work on the association between the secretion of different proteases and the invasive ability of several different immortalized and malignant human prostate cell lines (28) is in progress. The discovery of the ECM-degrading ability of PSA not only makes it a marker for early detection but also a target for prevention and intervention in prostate cancer.

Acknowledgments

We thank Dr. Nikolay Dimitrov for helpful comments, Dr. Hynda K. Kleinman for providing the Matrigel, Aaron I. Steele for preparing the manuscript, and Kay Steele and Chris Fetters for graphics.

References


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