Novel Role of Prostate-Specific Membrane Antigen in Suppressing Prostate Cancer Invasiveness

Arundhati Ghosh,1 Xinning Wang,1 Eric Klein,2 and Warren D.W. Heston1,2

1Department of Cancer Biology and 2Urological Institute, Cleveland Clinic Foundation, Cleveland, Ohio

Abstract
Prostate-specific membrane antigen (PSMA), a type II transmembrane glycoprotein, is overexpressed in prostate cancer. PSMA is a unique cell surface marker, negatively regulated by androgen and extensively used for imaging of hormone refractory carcinomas and metastatic foci. PSMA is a carboxypeptidase with two important enzymatic functions, namely, folate hydrolase and NAALADase. PSMA also exhibits an endocytic function, in which it spontaneously recycles through endocytic vesicles. PSMA is overexpressed at various stages of prostate cancer, including androgen-sensitive and -independent disease, increased in expression with early relapse after therapy. We have used in vitro invasion assays to explore the possible role of PSMA in the metastasis of prostate cancer cells. Androgen-dependent prostate cancer lines, which express PSMA endogenously (e.g., LNCaP, MDA PCa2b, and CWR22Rv1) are less invasive compared with androgen-independent PC3 or DU145 cells, neither of which expresses PSMA. Ectopic expression of PSMA in PC3 cells reduced the invasiveness of these cells, suggesting that this reduction in the invasion capability of PSMA-expressing cells is due to PSMA expression and not to intrinsic properties of different prostate cancer cell lines. Furthermore, knockdown of PSMA expression increased invasiveness of LNCaP cells by 5-fold. Finally, expression of PSMA mutants lacking carboxypeptidase activity reduced the impact of PSMA expression on invasiveness. Thus, it seems that the enzymatic activity is associated with the effect of PSMA on invasiveness. (Cancer Res 2005; 65(3): 727-31)

Introduction
Prostate cancer is the most commonly diagnosed form of cancer among men in the United States and is second only to lung cancer as a cause of cancer-related death. Death from prostate cancer occurs largely in patients with aggressive androgen-insensitive metastatic disease (1). Prostate-specific membrane antigen (PSMA) protein has been correlated with aggressive disease (2). PSMA is a transmembrane-carboxypeptidase (3, 4), which is up-regulated 10-fold or more in prostate cancer, from androgen-sensitive to androgen-independent prostate cancer, and in metastatic deposits. PSMA expression is negatively regulated by androgen (5). Overexpression of PSMA in primary prostate cancer correlates with other traditional prognostic factors and can independently predict the disease outcome such as early relapse after therapy (6). It is still not known whether PSMA has any effect on prostate cancer metastasis. Thus, PSMA overexpression is associated with a poor prognosis and we therefore hypothesized that PSMA expression is associated with biological processes of tumor invasion and metastasis. In our present report, we have tested our hypothesis that PSMA expression modulates the invasion capability of prostate cancer cells. We used prostate cancer cell lines that do not express PSMA and expressed PSMA or PSMA mutants within them or knocked down expression of the cell lines that normally express PSMA with small interfering RNA approach. We examined the invasiveness of these lines on a Matrigel invasion chamber. We report here the results of these studies.

Materials and Methods

Cell Lines and Reagents. PC3 cells (CRL-1435), CWR22Rv1 (CRL-2505), LNCaP (CRL-1740), and DU145 (HTB-81) were obtained from American Type Culture Collection (Manassas, VA). Retroviral transfected PC3PSMA and the transfection control PC3Vector cells were obtained from Dr. Michel Sadelain (Laboratory of Gene Transfer and Gene Expression, Gene Transfer and Somatic Cell Engineering Facility, Memorial-Sloan Kettering Cancer Center, New York, NY, ref. 7). The cells were maintained in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 2 mmol/L of l-glutamine and 10% fetal bovine serum (United States Biochemical, Cleveland, OH).] PSMA cDNA was originally cloned in our laboratory (5). Monoclonal antibodies used in this study (J591 and PMIT 485.5) were obtained from Dr. Neil Bander (Division of Hematology and Medical Oncology, Department of Medicine, Weill Medical College of Cornell University, New York, NY) and Hybritech (subdivision of Beckman Coulter, Inc., San Diego, CA), respectively. For making stable lines, different constructs were precipitated with pIRE5-puro at the ratio of 20:1 and transfected to the cells. The stable clones were selected with puromycin.

PSMA Mutants. Generation of cDNA encoding N336A, N459A, N476A, and N638A mutants of PSMA have been described earlier (8). The constructs L4AL5A and H380G were generated by using Stratagene (La Jolla, CA) QuickChange site-directed mutagenesis kit as described before (8). Various mutants are summarized below:

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Internalization</th>
<th>Enzymatic activity</th>
<th>Location/domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>N336A</td>
<td>No</td>
<td>Yes</td>
<td>Glycosylation site, E</td>
</tr>
<tr>
<td>N459A</td>
<td>No</td>
<td>No</td>
<td>Glycosylation site, E</td>
</tr>
<tr>
<td>N476A</td>
<td>No</td>
<td>No</td>
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<td>N638A</td>
<td>Yes</td>
<td>Yes</td>
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<td>H380G</td>
<td>Yes</td>
<td>No</td>
<td>Active site, E</td>
</tr>
<tr>
<td>L4AL5A</td>
<td>No</td>
<td>Yes</td>
<td>Internalization motif, A</td>
</tr>
</tbody>
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Cell Membrane Preparation and Folate Hydrolase Assay. Cell membrane preparation was done as described in Ghosh and Heston (8). Twenty micrograms of protein were used for Western blot and 2 μg of protein were used for folate hydrolase assay. Folate hydrolase
assay was done using high-performance liquid chromatography separation and spectrometric analysis of reaction products as described before (8). The released glutamate from MTXGlu2 was measured and expressed as nanomoles of glutamate released per milligram of protein per hour.

RNA Interference Using Short Hairpin RNA Construct. Short hairpin RNA generating hairpin primers were designed by using RNA interference oligo retriever (available from www.cshl.org/public/SCIENCE/hannon.html). A pGEM1 plasmid containing the human U6 promoter was used as a template for the PCR reaction. The vector contains ~500 bp of upstream U6 promoter sequence with an SP6 sequence that flanks the upstream portion of the U6 promoter sequence. Therefore, the 5’ primer was SP6 universal primer oligo sequence, and the 3’ primer was composed of the hairpin loop forming a sequence along with the 3’ region of the U6 promoter. Two different PCR products containing two different hairpin sequences (against two different regions of PSMA sequence) under the U6 promoter were cloned in two different regions of pCDNA3.1 vector sequences (against two different regions of PSMA sequence) under the U6 promoter. The clones expressing short hairpin RNA were selected against puromycin.

Invasion Assays Using Boyden Chamber. Matrigel invasion assays were done with cells expressing or nonexpressing PSMA as described by Albini et al. (9). A fixed number of cells (5 × 10^5 cells/mL) in serum-free medium was added to the upper chamber of the Boyden chamber coated with Matrigel (coated on a polyvinyl pyrrolidone-free polycarbonate filter with 8-μm pore size) inserts (BD Pharmingen, San Diego, CA) and the lower chamber contained 10% serum-containing medium. The cells were incubated for 48 hours. Invasion of cells to the underside of the Matrigel-coated membrane was detected by staining the cells with Mayer’s hematoxylin solution and visualizing the cells under a microscope. After staining, cells were counted under a microscope in four random fields (magnification ×100) and results were expressed in the form of a bar graph.

Results

PSMA Expression Reduces Invasiveness of Prostate Cancer Cell In vitro. LNCaP and CWR22Rv1 cells endogenously express PSMA, whereas PC3 and DU145 do not (Fig. 1A). LNCaP cells have a higher PSMA expression compared with CWR22Rv1 or PC3PSMA (ectopically expressing PSMA in PC3 cells), as shown in Fig. 1A. The enzymatic activity profile of these different cell lines corresponds with their protein profile (Fig. 1B). PC3 and DU145 cells are non-PSMA-expressing and highly aggressive, whereas PSMA-expressing cell lines show less invasion through a Matrigel membrane (Fig. 1C). Quantification of cells that invaded through the Matrigel matrix showed that PSMA expression in PC3 cells reduces the invasion by 5-fold (Fig. 1D). LNCaP and CWR22Rv1 cells are likewise minimally invasive. Inducible expression of PSMA using the tetracycline system in PC3 cells also supported this finding. These PC3 (Tet+) cells with tetracycline off-regulatory system did not express PSMA protein (Fig. 2A and B) and were as invasive as wild-type PC3 cells (10). The same cells when induced to synthesize PSMA (Tet−, i.e., upon removal of tetracycline) attenuated their invasion through the Matrigel-coated membrane and this reduction level was comparable to that of PC3PSMA cells (with comparable amounts of expression of enzymatically active PSMA protein) as described earlier (Fig. 2C).
Enzymatic Activity of PSMA Correlates with Reduced Cell Invasiveness. Several proteases have been found to play a role in cell invasion and metastasis. To explore whether PSMA enzymatic activity or intracellular alteration of cellular rigidity or internalization was involved, we used various mutant-expressing cell lines (lacking one or the other of these properties) for invasion assay. PC3 cells expressing H380G mutant of PSMA (where the mutation is located at the zinc binding site at the active center, renders it enzymatically inactive; ref. 11), invaded through the Matrigel membrane very efficiently. Other enzymatically inactive PSMA mutants express PSMA protein with mutation at the various sugar-attachment sites (8), that is, N336A, N459A, N476A, N638A, and pIRES-neo. D, top, Western analysis of 50 μg equivalent protein PC3-PSMA and PC3 cells expressing L4AL5A mutant of PSMA and parental PC3 cells. Bottom, left, enzyme assay of 2 μg equivalent protein of PC3-PSMA and PC3-L4AL5A and parental PC3 cells expressed in terms of rate of Glu released from MTXGlu2 (nmol/L/mg of protein/h); right, Matrigel invasion assay using 5 × 10⁵ cells of the different cell lines mentioned above.

Enzyme activity of PSMA is important for invasion assay. A, Western analysis of 50 μg equivalent protein PC3-PSMA and different PSMA mutants or vector-transfected cell lines (N380G, N336A, N459A, N476A, N638A, and pIRES-neo). B, folate hydrolase assay of 2 μg equivalent protein from different cell lines expressing different mutant proteins. C, invasion assay using 5 × 10⁵ cells/mL from different cell lines PC3-PSMA, H380G, N336A, N459A, N476A, N638A, and pIRES-neo. D, top, Western analysis of 50 μg equivalent protein PC3-PSMA and PC3 cells expressing L4AL5A mutant of PSMA and parental PC3 cells. Bottom, left, enzyme assay of 2 μg equivalent protein of PC3-PSMA and PC3-L4AL5A and parental PC3 cells expressed in terms of rate of Glu released from MTXGlu2 (nmol/L/mg of protein/h); right, Matrigel invasion assay using 5 × 10⁵ cells of the different cell lines mentioned above.

Discussion
Association of PSMA with progression of disease at various stages of prostate cancer suggests a positive role in prostate cancer tumor cell growth and metastasis. Contrary to our expectation, in our studies with prostate cancer cell lines we

Figure 2. Enzyme activity of PSMA is important for invasion assay. A, Western analysis of 50 μg equivalent protein PC3-PSMA and different PSMA mutants or vector-transfected cell lines (N380G, N336A, N459A, N476A, N638A, and pIRES-neo). B, folate hydrolase assay of 2 μg equivalent protein from different cell lines expressing different mutant proteins. C, invasion assay using 5 × 10⁵ cells/mL from different cell lines PC3-PSMA, H380G, N336A, N459A, N476A, N638A, and pIRES-neo. D, top, Western analysis of 50 μg equivalent protein PC3-PSMA and PC3 cells expressing L4AL5A mutant of PSMA and parental PC3 cells. Bottom, left, enzyme assay of 2 μg equivalent protein of PC3-PSMA and PC3-L4AL5A and parental PC3 cells expressed in terms of rate of Glu released from MTXGlu2 (nmol/L/mg of protein/h); right, Matrigel invasion assay using 5 × 10⁵ cells of the different cell lines mentioned above.
found PSMA expression to have a strong role in decreasing the invading capacity of cells across the Matrigel membrane. Expression of cell surface peptidases are known to play a key role in the control of growth and differentiation by modulating the activity of peptide factors and by regulating their access to adjacent cells by two basic mechanisms; either by inactivation of stimulatory peptide or by activation of inhibitory factors (14). The enzymatic function of PSMA seems to decrease the ability of prostate cancer cells to invade the extracellular matrix. The pathway through which PSMA functions in controlling the invasiveness is still unidentified.

To detect whether the enzymatic function of PSMA has any role in the invasiveness of the PC3 cells, we have used enzymatically inactive mutants of PSMA with mutations located in different regions of PSMA protein and used them for invasion assays. All such mutants ectopically expressed in PC3 cells show invasiveness comparable to parental PC3 cells, indicating that enzyme activity of PSMA expressed on the surface of PC3 cells plays an important role in determining its ability to invade the extracellular matrix. The pathway through which PSMA functions in controlling the invasiveness is still unidentified.

We also used an inducible line of PSMA in our invasion assay and we have found that such a line has an attenuated invading capacity when induced to express PSMA protein. Knockdown of PSMA expression from LNCaP cells showed a similar result in which removal of PSMA expression could increase the ability of LNCaP cells to invade the extracellular matrix, indicating that the enzymatic activity of PSMA is altering the invasiveness of LNCaP cells as well. Knockdown of PSMA from LNCaP cells will similarly allow these cells to invade more efficiently because the peptidase function of PSMA is not available anymore to modulate putative factor(s). Similarly, expressing wild-type PSMA into PC3 cell surface made these cells less invasive.

PSMA is known to internalize spontaneously like a number of well-established cell surface receptors (15). The internalization of PSMA requires an MXXXL motif. The cytoplasmic tail of PSMA interacts with actin-binding protein filamin A. Filamin binding to PSMA reduces its internalization and enzyme activity (16). We used a PC3PSMA cell line as contrasted with the PC3-L4AL5A (PC3 cells expressing PSMAL4AL5A mutant) cell line for invasion assays. The PSMAL4AL5A mutant does not internalize (13), and it is enzymatically as active as wild-type PSMA. We found that both PC3PSMA and PC3PSMAL4AL5A show similar patterns of decreased invasiveness as opposed to parental PC3 cells, which are highly invasive. It is known that PSMAL4AL5A interacts with filamin A (data not shown) but this does not resolve whether filamin A provides any role in invasion. This finding is in contrast with a similar function shown by matrix metalloprotease MT1-MMP, in which dynamic turnover by internalization of the protein regulates cell surface levels of enzyme during cell migration and invasion (17). Future planned experiments will examine the effect of PSMA expression on cell invasiveness.

**Figure 3.** Invasion assay of inducible cell line of PSMA expressed in PC3 cells with or without tetracycline (Tet)-containing medium. A, Western blot analysis of 50 μg equivalent proteins from PC3-PSMA cells (with or without tetracycline) analyzed by SDS-PAGE. B, enzymatic analysis of PC3-PSMA (+Tet) and (−Tet) in the presence or absence of tetracycline, respectively. The tetracycline-off situation expressed the protein (−Tet). C, Matrigel invasion assay showing the invading capability of PC3-PSMA cell line with and without tetracycline.

**Figure 4.** PSMA knockdown from LNCaP cells by RNA interference method increases their invasion activity. A, Western blot analysis of LNCaP cells and PSMA knockdown line from LNCaP knockdown cells (LNCaP kd) detected with J591 antibody. B, enzymatic activity of PSMA protein expressed on the membrane of LNCaP or LNCaP knockdown cells. C, invasion assay of a fixed number (5 × 10⁵ cells/mL) of LNCaP knockdown cells and parental LNCaP cells were analyzed and the number of hematoxylin-stained cells that have invaded through the Matrigel membrane were photographed. D, graphical representation of cell invasion assay shown in C.
of knockdown of filamin A expression to further delineate the role of PSMA-filamin interaction.

Therefore, the carboxypeptidase function of PSMA contributes to the low level of invasiveness of PSMA-expressing cells. One conclusion is that PSMA is proteolytically modulating invasion-promoting peptides or amino acids. Following is a list of PSMA-specific substrates:

- Unknown peptide \textsubscript{a} → peptide \textsubscript{a} + 1 + Glu
- N-acetyl-aspartyl-glutamate → N-acetyl-aspartate + Glu (4)
- Poly-γ-glutamated folates → (n - 1)-γ-glutamated folates + Glu (3)
- Ac-X-Glu (X is Ala, Asp, or Glu) → Ac-X + Glu (18)
- Ac-X-Met (X is Ala, Asp, or Glu) → Ac-X + Met (18)
- Ac-Asp-X (X is Glu or Met) → Ac-Asp + Glu or Met (18)

One of the constant products of PSMA enzymatic reaction is glutamate, which might also be a potential modulator and functions through glutamate receptors. Glutamate receptors have been reported to be present in prostate and LNCaP cells as well (19, 20). Alternatively, folates, which are produced as a by-product of this enzymatic reaction, could be a potential modulator for PSMA enzymatic activity. The role of folates and glutamates on the invasiveness of PSMA-expressing cells across the Matrigel membrane needs to be investigated further. However, discovery of the last three groups of substrates of PSMA open the possibility of the existence of many potentially important yet unidentified substrates, which could contribute to this effect on the invasiveness of PSMA-expressing prostate cancer cells.

Because of this interplay between proteases and peptidases and the receptors, it will be important to understand what moderators are being generated that affect this process of cell invasion. We are beginning to examine whether we can extend our finding in vitro to an in vivo model system. If it is shown to have the same effect as observed with prostate cancer cell lines in vitro, then one would want to identify factors that enhance PSMA enzymatic activity and not those that inhibit its enzymatic activity. We also plan to study the detailed mechanism behind this anti-invasive role of PSMA. Indeed, if there are nutritional or other environmental modifiers, it may be possible that they would contribute to the aggressiveness or lack of aggressiveness of prostate cancer by modifying PSMA enzymatic activity and as such may help to explain the extreme range of biological aggressiveness of prostate cancer.

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