Alternative Pathways to Prostate Carcinoma Activate Prostate Stem Cell Antigen Expression

Purnima Dubey, Hong Wu, Robert E. Reiter, and Owen N. Witte

Departments of Microbiology, Immunology and Molecular Genetics [P. D., O. N. W.], Molecular and Medical Pharmacology [H. W.]; and Urology [R. E. R.], Howard Hughes Medical Institute [O. N. W.], Los Angeles, California 90095-1662.

Abstract

Prostate Stem Cell Antigen (PSCA) is a glycosylphosphatidylinositol-anchored cell surface protein that is expressed in normal human prostate and overexpressed in human prostate cancers. To test whether different pathways that generate prostate cancer would affect PSCA expression, a murine model system was developed. Monoclonal antibodies were generated against murine PSCA (mPSCA). mPSCA is expressed on ~20% of cells in normal prostate epithelium, and this number decreases with increasing age. In the transgenic adenocarcinoma of the mouse prostate (TRAMP) model of prostate cancer, tumors develop between 19 and 25 weeks of age. Murine PSCA was strongly expressed on ~60% of the cells of TRAMP tumors, at an age where the number of PSCA+ cells and the level of expression of PSCA is very low in the normal prostate. Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) +/- mice develop a number of different cancers, including prostate cancer. The incidence of prostate cancer is low and occurs after a relatively long latency. Fluorescence-activated cell sorter analysis of prostate tissue from 11–18-month-old PTEN +/- mice showed elevated numbers of PSCA+ cells in the prostate, and immunohistochemical analysis showed high mPSCA expression in the tumors of these mice. Together, these results show that two distinct mechanisms of carcinogenesis lead to expression of a common target antigen.

Introduction

Cancers develop as a result of multiple genetic alterations, which have been clearly defined for some cancers, such as colorectal carcinoma (reviewed in Ref. 1). In contrast, the etiology of prostate cancer is unclear, although genetic alterations have been identified that may provide some hints as to how prostate carcinoma develops. Several different chromosomal rearrangements or deletions have been found, including loss of the short arm of chromosome 8 (2). The PTEN gene can also be mutated or deleted in prostate cancer samples, and cell lines and PTEN expression levels are reduced in prostate cancer xenografts derived from advanced cancers. Additional changes include β-catenin mutations, which occur in a relatively long arm of chromosome 8, and mutation of the androgen receptor. The HER2/neu protein has also been reported recently to be amplified in some prostate cancers (3).

A limited number of antigens have been identified on the surface of prostate cancers that may serve as targets for therapy. One such antigen is PSMA, a type II transmembrane glycoprotein overexpressed in ~75% of prostate cancers (4). PSMA is also expressed at low levels in some normal tissues and in the neovascularization of many human cancers. The first monoclonal antibody that was developed against PSMA, 7E11, is specific for an internal epitope on the molecule (5) and is used presently for the imaging of prostate cancer (4, 5). Other antibodies against PSMA recognize cell surface epitopes (6).

PSA is a GPI-anchored cell surface protein and is the prostate homologue of SCA-2 (7). PSA expression is largely restricted to the normal prostate, with lower levels of expression detected in normal bladder epithelium and placenta. Human PSA is expressed in ~80–90% of human prostate cancers and in 100% (nine of nine) of metastatic cancers to bone that were examined (8). In contrast to other molecules expressed on prostate cancers, PSA is expressed at all stages of the disease from benign hyperplasia to androgen-independent metastatic disease. Although homologues of PSMA and prostate-specific antigen are not found in the mouse, a murine homologue (mPSMA) that is 70% identical to hPSMA has been identified (7).

The distinct advantages of a syngeneic animal model system is the ability to evaluate the expression pattern of a target protein on a constant genetic background where the cancer is generated by a defined mutation. We evaluated the change in expression of mPSMA as a function of age and cancer progression. We generated monoclonal antibodies specific for mPSA and studied mPSA levels in two different experimental models of prostate cancer. In the TRAMP model, prostate cancer is generated by the specific oncogenic signal of SV40 T antigen (9). PTEN +/- mice develop prostate cancer because of inactivation of one allele of the tumor suppressor gene PTEN (10, 11). Murine PSA is overexpressed on the prostate cancers that develop in both of these experimental models, suggesting that distinct oncogenic signals that lead to prostate carcinogenesis can up-regulate expression of the same target antigen.

Materials and Methods

Plasmids. Nucleotides 61–297 were amplified from the mPSMA expressed sequence tag clone (Mouse expressed sequence tag clone W42223) by PCR and cloned into pGEX-2T (AP Biotech, Piscataway, NJ), generating a bacterial expression vector. The eukaryotic expression vector pcDNA3-N-FLG-mPSMA was generated by a two-step PCR and contains the entire murine PSA coding sequence. The FLAG tag sequence was inserted between the signal sequence and the S+ end of the mature protein.

Overproduction of GST-mPSMA Fusion Protein. The pGEX-2T-GST-mPSMA construct was transformed into TOPO Escherichia coli bacteria and overproduced by induction with isopropyl-1-thio-β-D-galactopyranoside, according to the manufacturer’s protocol (AP Biotech, Piscataway, NJ). The protein was isolated from the cell pellet with 8 M urea and then purified by binding to glutathione-Sepharose.

Animals and Cell Lines. Armenian hamsters (CytoGen R&D, West Roxbury, MA) were maintained in the specific pathogen-free facility at the California Institute of Technology. BALB/c and C57BL/6 mice were bred in the specific pathogen-free facility at UCLA and maintained according to the guidelines of the Department of Laboratory Animal Medicine at UCLA.

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2 To whom requests for reprints should be addressed, at University of California at Los Angeles, 5-748 MRL, 675 Charles E. Young Drive South, Los Angeles, CA 90095-1662.

3 The abbreviations used are: PTEN, phosphatase and tensin homologue deleted on chromosome 10; PSA, prostate-specific membrane antigen; PSCA, prostate stem cell antigen; mPSCA, murine PSCA; iPSCA, human PSA; G1P, glycosylphosphatidylinositol; TRAMP, transgenic adenocarcinoma of the mouse prostate; UCLA, University of California at Los Angeles; GST, glutathione S-transferase; FACS, fluorescence-activated cell sorter.

3256
TRAMP transgenic mice (FVB) were obtained from Dr. N. Greenberg (9), and Dr. Charles Sawyer at UCLA. Mice were castrated by surgical removal of both testicles. Surgery was performed under approved aseptic conditions using anesthetics and analgesics as per the guidelines of the Department of Laboratory Animal Medicine. Two weeks after castration, a 90-day, timerelease testosterone pellet (12.5 mg; Innovative Research of America, Sarasota, FL) was placed s.c. under the skin of the back. Animals were killed, and tissues were harvested 7 days after the addition of the testosterone pellet. 293T/17 cells were maintained in DMEM with 5% FCS, transiently transfected with pcDNA3-N-FLAG-mPSCA using the calcium phosphate precipitation method (12), and analyzed 48 h after transfection.

Production and Purification of α-mPSCA Monoclonal Antibodies. Female hamsters were immunized s.c. with 10 μg of purified GST-mPSCA emulsified in RIBI adjuvant. Hamster immune serum was checked by ELISA analysis for specific reactivity against GST-mPSCA. The immune spleen cells were fused to the mouse myeloma cell line HL-1 (NS-1 cells adapted to growth in HL-1 medium; BioWhittaker, Walkersville, MD). The immunization and fusion was performed by Dr. Susan Ou of the Monoclonal Antibody facility at the California Institute of Technology, Pasadena, CA. Hybridoma supernatants were tested using ELISA for specific reactivity against GST-mPSCA. Those hybridomas with at least 100-fold higher reactivity on GST-mPSCA as compared with GST alone were tested for recognition of mPSCA expressed in 293T/17 cells by FACS analysis of live cells. Two hybridomas that specifically recognized 293T-mPSCA cells were subcloned. The subclones were screened first by ELISA and then by FACS, and the ones with highest activity in both assays were selected. The fusion yielded two antibodies α-mPSCA-1 (3D10-A4) and α-mPSCA-2 (3C11–1E7) that were used for all experiments. These hybridomas were also screened for the ability to bind to protein A. Hybridomas were grown in the Cell Pharm 100 (Unisyn Technologies, Hopkinton, MA), and supernatants were concentrated by saturated ammonium sulfate precipitation and purified by binding to and elution from protein A-Sepharose (13).

Isolation and Processing of Mouse Tissues. Tissues for immunohistochemistry were fixed in 10% buffered formalin and then embedded in paraffin and sectioned by the Human Tissue Research Center at UCLA. Tissues for Western blot analysis were chopped into fine fragments and boiled in SDS lysis buffer [0.1 M Tris (pH 6.8), 20% glycerol, 2% SDS, 5% β-ME, and 0.1% bromophenyl blue] at 1 ml/100 mg of tissue. Tissues for FACS analysis were chopped into fine fragments, resuspended in DMEM with 5% FCS and 170 units/ml collagenase I (Worthington, Inc.), and incubated on a nutator at 37°C for 4 h. The single cell suspension was filtered through a 100 μm nylon mesh, washed thoroughly, and stained with antibodies.

FACS Analysis. 293T/17 (2 × 10^5) or dissociated prostate cells were preincubated for 5 min on ice in FACS buffer (PBS, 1% BSA, and 0.1% sodium azide) with 0.5 μg of F, blocking antibody 2.4G2 (PharMingen). Then, α-mPSCA-1 or control 145–2C11 antibody was added (2 μg/ml) and incubated on ice for 15–30 min. After two washes with FACS buffer, cells were incubated with goat antihamster FITC (1:200; Jackson Immunochemicals) for 15–30 min on ice, washed twice, and then analyzed on a FACSscan (Becton Dickinson) using CellQuest software. For α-FLAG staining, cells were incubated with 3 μg/ml of α-FLAG antibody (Sigma Chemical Co. Immunochemicals, St. Louis, MO), followed by donkey α-mouse PE (1:200; Jackson Immunochemicals).

Western Blot Analysis. Whole cell lysates were separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose. The membrane was blocked with 5% nonfat milk in high-salt TBST (0.02 M Tris, 0.13 M NaCl, and 0.2% Tween 20). Primary antibody (1 μg/ml in TBST, 60 min at room temperature), was followed by three washes with TBST and then horseradish peroxidase-conjugated secondary antibody (1:5000 in TBST; Jackson Immunochemicals, 60 min at room temperature). After three TBST washes, the membrane was developed with the Pierce Luminol kit.

Results

Antibodies Generated against a GST-mPSCA Fusion Protein Recognize mPSCA in Normal Mouse Tissues. Armenian hamsters were immunized with a GST-mPSCA bacterial fusion protein to develop antibodies that were specific for the extracellular portion of mPSCA. The fusion protein contained amino acids 21–99 of the mature protein minus the signal sequence and GPI anchor. Hybridomas were screened as described in “Materials and Methods,” yielding two antibodies α-mPSCA-1 and α-mPSCA-2. Human 293T/17 cells were transiently transfected with a CMV-driven FLAG-tagged mPSCA construct. Staining of live cells with α-mPSCA-1 and α-FLAG antibody showed that mPSCA was expressed on ~95% of the transfected cells and was recognized by both reagents (Fig. 1A).

To study cell surface expression of mPSCA in normal mouse prostates, a single cell suspension of cells was prepared by collagenase digestion. Although GPI-anchored proteins can be cleaved by some proteases, PSCA expression on the cell surface was unaffected by collagenase preparation. Staining of cells from the dorsolateral prostate of an 8-week-old C57BL/6 male mouse with α-mPSCA-1 showed that 17% of the cells expressed mPSCA on the cell surface, and the level of expression was ~5-fold or greater above background (Fig. 1B). Western blot analysis of different tissues using α-mPSCA-1 detected mPSCA as an Mr ~36,000 band in prostate (Fig. 1C). No protein was detected in the other tissues tested. Note that the predicted molecular weight of mPSCA based on the amino acid sequence is Mr ~13,000. The discrepancy between the predicted and apparent molecular weight can be explained by the presence of four putative N-glycosylation sites present on the protein. Western blot analysis also detects PSCA protein in mouse bladder and seminal vesicle (data not shown).

We used immunohistochemical analysis to localize the protein within tissue sections. Immunostaining of paraffin sections detects PSCA in luminal secretory cells of prostatic epithelium (Fig. 1D). Our previous analysis of hPSCA showed that although hPSCA RNA is largely restricted to the basal cells of prostatic epithelium (7), the protein is present in both basal and secretory layers (8). The PSCA protein may be made during differentiation of basal to secretory cells or may be made in the basal cells and then transferred to the secretory cells. We found mPSCA protein in the secretory layer, and a recent study by another group found mPSCA RNA restricted to the secretory cells of prostatic epithelium (14). This difference between the location of hPSCA and mPSCA may also be attributable to species-specific differences in the expression pattern of the two genes.

The α-mPSCA monoclonal antibodies we generated can be used for quantification of PSCA expression, analysis of the molecular size, and tissue and cellular localization of the protein. In addition, our α-mPSCA antibodies detect expression in at least three different strains of mice, FVB, BALB/c and C57BL/6, and therefore can be used for the study of PSCA expression independent of genetic background.

mPSCA Expression in the Prostate Decreases in Castrated and Older Normal Animals But Is Elevated in Age-matched TRAMP Animals. hPSCA is expressed in androgen-dependent and androgen-independent cancers, suggesting that both hormone-dependent and independent expression of PSCA is possible (8). We determined whether PSCA expression would be affected by the removal of
androgen from normal male animals. Twelve-week-old male mice were surgically castrated. Two weeks later, a testosterone pellet was placed in the fold of the skin at the back of the neck. One week later, tissues were harvested and analyzed by Western blot. Unmanipulated animals (then 15 weeks of age) expressed a small amount of PSCA that was undetectable in castrated animals (Fig. 2). One week after the addition of exogenous androgen, a high level of PSCA was detected in the prostate (Fig. 2). These data suggest that the expression of PSCA is androgen dependent.

As the animal ages, there is a drop in levels of circulating testosterone (15, 16). We determined whether PSCA expression would be affected in older animals. FACS analysis of lateral prostates from 21- and 25-week-old mice detected approximately 1–3% of cells with surface PSCA expression (Fig. 3A), showing that the percentage of PSCA+ cells is decreased with increasing age of the animal.

We addressed whether mPSCA would be overexpressed in an animal model of prostate cancer that is generated by the defined oncogenic signal of SV40 T antigen. TRAMP transgenic mice express SV40 T antigen under control of the probasin promoter, which is expressed in the dorsal and lateral lobes of the mouse prostate (9). One hundred percent of these mice developed prostatic intraepithelial neoplasia by 12–18 weeks of age and cancer by 19–25 weeks of age. Metastatic disease develops in older mice. We chose to analyze this animal model of prostate cancer because of its high penetrance and relatively short latency period before the onset of disease.

Fig. 1. Anti-mPSCA antibodies generated against a bacterial fusion protein recognize mPSCA in normal mouse tissues. A, 293T/17 cells were transfected with pcDNA3-N-FLG-mPSCA and then stained with control antibodies (left panel) or α-PSCA-1 (X axis) and α-FLAG (Y axis; right panel). B, dorsolateral prostate from an 8-week-old C57BL/6 mouse was dissociated and then stained with control antibody (left panel) or α-mPSCA-1 (right panel), followed by goat antihamster FITC. C, Western blot analysis of normal mouse tissues from an 8-week-old C57BL/6 male mouse. Protein concentration among lysates was compared by staining with Coomassie blue, and equal amounts of protein were loaded on the gel for Western blot. DLP, —; S.I., —. D, immunohistochemical analysis of paraffin sections from the lateral prostate of an 8-week-old male C57BL/6 mouse. Sections were stained with control antibody (left panel) or α-mPSCA-1 (right panel).

Fig. 2. mPSCA expression is undetectable after castration of normal animals and is strongly re-expressed after addition of exogenous androgen. Twelve-week-old male BALB/c mice were castrated by surgical removal of both testicles. Two weeks after castration, a 90-day, slow-release testosterone pellet was placed s.c. at the back of the neck. One week after addition of testosterone, the animals were killed, and tissues were harvested for Western blot analysis. Left, normal 15-week-old animal. Middle, 15-week-old castrated animal, 3 weeks after castration. Right, 15-week-old castrated animal with exogenous androgen added for 1 week. Equal protein loading was assessed by Coomassie blue staining of a parallel gel (data not shown).
We analyzed the expression of PSCA in the lateral prostates of five TRAMP transgenic mice ranging from 17 to 25 weeks of age. The prostate and seminal vesicle of the 17-week-old TRAMP animals were slightly enlarged as compared with age-matched control animals. In contrast, the 19–25-week-old TRAMP animals had grossly enlarged prostates and seminal vesicles as compared with control animals. The lateral prostates of the 21- and 25-week-old animals had developed tumors, and there were also tumors attached to the seminal vesicles in both mice. Fig. 3B shows FACS analysis of the lateral prostates from 21- and 25-week-old animals. Weak staining is detected in normal lateral prostate (Fig. 3A), with strong staining in the majority of cells in the TRAMP lateral prostate (Fig. 3B) and tumor (Fig. 3C). A similar pattern was observed in all of the animals from 17 to 25 weeks of age that were analyzed, with weak staining in the normal age-matched prostate and strong staining in the TRAMP prostate.

Immunohistochemical analysis detects a larger fraction of PSCA+ cells than is detected by FACS analysis. This discrepancy can be explained by the fact that FACS analysis of intact cells only detects the protein present on the outside of the cell, whereas immunohistochemical analysis also detects intracellular protein. In addition, some cells may express a very low amount of PSCA on the cell surface that is below the limits of detection by FACS.
PSCA Is Overexpressed in the Prostate Cancers of PTEN +/− Mice. Mice that have either acquired mutations in or lost an allele of the tumor suppressor PTEN (PTEN +/− mice) develop a variety of malignancies after a latency period of >1 year (10, 11). The tumors that develop are PTEN null. Approximately 14% of the tumors are prostate cancers (11). We analyzed the prostates of twelve 11–18-month-old male mice and found that the seminal vesicle and prostate were enlarged compared with wild-type mice of the same age. The percentage of PSCA + cells ranged from 6 to 23%. A representative experiment is shown in Fig. 5A. A wild-type mouse of the same age had approximately 1–3% PSCA + cells in the prostate (data not shown). Immunohistochemical analysis of prostate tissue sections from a 12-month-old male mouse showed elevated levels of mPSCA in the dorsal prostate, compared with an age-matched wild-type mouse (data not shown). These sections also show disorganization of the normal prostatic ductal structures and the presence of malignant cells. Together, these data show that mPSCA levels were elevated in the prostates of PTEN +/− mice where prostate cancer can develop. 

Discussion

hPSCA is expressed in all stages of prostate cancer development from prostatic intraepithelial neoplasia to frank carcinoma (8), suggesting that PSCA may be marking the cells that will become malignant. If this is the case, then increased numbers of PSCA + cells in the prostate may be used as a prognostic marker to predict the potential onset of cancer. This issue can be addressed in an animal model where genetically identical animals of different ages can be analyzed. The reagents we have developed that are specific for the murine homologue (mPSCA) of an antigen expressed on human cancers (hPSCA) will allow us to assess whether expression of the marker is elevated during initiation and progression of the disease. If PSCA expression is elevated in animals that will develop cancer even while the prostate is phenotypically normal, it may be possible to use increased percentages of PSCA + cells in the prostate as a predictor for the onset of prostate cancer. In the TRAMP model, there is a very small window (about 2–3 weeks) from the time that PSCA expression is decreased in the normal prostate and the onset of cancer in the prostates of TRAMP mice. In contrast, in the PTEN +/− model, there is a window of 6 months to 1 year from the time that the numbers of PSCA + cells are reduced in the normal prostate and carcinoma develops in the prostates of PTEN mutant mice. Thus, we may be able to make a correlation between elevated numbers of PSCA + cells and the development of prostate carcinoma over a longer time span.

TRAMP transgenic mice develop prostate cancer because of the oncogenic activity of SV40 T antigen. Large T antigen binds to and prevents the activity of negative regulators of cell growth such as Rb and p53, leading to overproliferation and transformation (17). In contrast, cancers develop in PTEN +/− mice because of loss of function of one allele of the tumor suppressor PTEN. The tumors that develop in these mice likely also suffer mutations in or lose the other allele. PTEN is a lipid phosphatase, the activity of which regulates the apoptosis and cell migration and adhesion pathways by negatively regulating the function of proteins such as Akt. In the absence of PTEN, activated Akt levels remain elevated, leading to an antiapo-
ptotic effect (18). In addition, PTEN also directly dephosphorylates FAK and Src, which leads to suppression of cell migration, adhesion, and invasion. Loss of PTEN activity promotes the detachment of cells from the extracellular matrix and increases cell migration and inva-
sion (19). Thus, two very different signaling pathways that cause
cancer up-regulate expression of the same target antigen, PSCA.

Monoclonal antibodies specific for hPSCA may find utility in the
diagnosis and treatment of cancers that may develop from multiple
tumorigenic mechanisms. Monoclonal antibody therapy is currently in
use for the treatment of several human cancers such as non-Hodgkin’s
lymphoma (20) and breast cancer (21).

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