Alternative Pathways to Prostate Carcinoma Activate Prostate Stem Cell Antigen Expression

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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 prostatic 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 tumor suppressor 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 on the 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 neovascularature 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 (mPSA) that is 70% identical to hPSA has been identified (7).

The distinct advantage 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 mPSA 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 PSCA 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 mPSA 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-mPSCA was generated by a two-step PCR and contains the entire murine PSCA coding sequence. The FLAG tag sequence was inserted between the signal sequence and the S′ end of the mature protein.

Overproduction of GST-mPSCA Fusion Protein. The pGEX-2T-GST-mPSCA 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). 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.
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 testes. 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, timed-release 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 [0.1M Tris (pH 6.8), 20% glycerol, 2% SDS, 5% N-ethylmaleimide (NEM), 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 1–2 h. The single cell suspension was filtered through a 100 μm nylon mesh, washed thoroughly, and stained with antibodies.

FACS Analysis. 293T/17 (2 × 10⁵) 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(ab′)₂, 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 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 0.02% Tween 20 [pH 7.8]) for 30 min at room temperature. For α-FLAG staining, sections were incubated overnight at 4°C with 2 μg/ml α-FLAG antibody and 145–2C11 antibody. After one wash with TBST, the sections were incubated with biotinylated goat antihamster antibody (1:400) for 30 min at room temperature, washed once, and then incubated with the StreptABC kit (DAKO) for 20 min at room temperature. The peroxidase reaction was developed with diaminobenzidine (Fluka, Steinheim, Germany). The sections were counterstained with hematoxylin, dehydrated, and mounted.

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 prostate, 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 Mₘ ~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 Mₘ ~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. 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. 3. mPSCA expression decreases in the normal prostate with increasing age but is elevated in the prostates of TRAMP mice. Tissues from 21- and 25-week-old male mice were dissociated and stained with control antibody (left panel) or α-mPSCA-1 (right panel), followed by goat antihamster FITC. A, lateral prostates from normal control animals. B, lateral prostates from TRAMP mice. C, extraprostatic tumors from the same TRAMP mice in B.

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Immunohistochemical analysis of tissue sections from the lateral prostates and tumors of these mice showed increased cellularity and disorganization of the ductal structures. The same gradation in level of PSCA expression that was detected by FACS analysis was also seen by immunohistochemistry. Fig. 4 shows analysis of lateral prostates from 21-week-old animals. Weak staining is detected in normal lateral prostate (Fig. 4A), with strong staining in the majority of cells in the TRAMP lateral prostate (Fig. 4B) and tumor (Fig. 4C). 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.

Fig. 4. Immunohistochemical analysis detects PSCA overexpression in the tumors of TRAMP mice. Tissue sections from 21-week-old mice stained with control antibody (left panels) or α-PSCA-1 (right panels). A, lateral prostate from the normal mouse. ×40. B, lateral prostate (B) and tumor (C) from the TRAMP mouse are shown. ×40. Inset, ×100.
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 wild-type mice of the same age. The 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

PSCA 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-

Fig. 5. Elevated levels of mPSCA in the dorsal prostate of a PTEN +/- mouse. A, the dorsal lobe of an 18-month-old male PTEN +/- mouse was dissociated into a single cell suspension and stained with α-mPSCA-1 or control antibody. B, tissue section from the dorsal prostate of a 1-year-old wild-type mouse (left panel) and PTEN +/- mouse (right panel). Sections were stained with α-mPSCA-1. ×40. No staining was detected with control antibody (data not shown).
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 invasion (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).

Acknowledgments
We are grateful to Dr. Susan Ou of the Monoclonal Antibody Facility at California Institute of Technology for immunization of hamsters and generation of hybridomas. We thank Lyly Nguyen and Kabir Singh for excellent technical assistance. We also thank Shirley Quan and James Johnson for help in the production of purified anti-PSCA antibody and Dr. Giriya Sulor for help in immunohistochemical staining. We appreciate the assistance of Adam Mogil and Maria Avina of the Human Tissue Research Center at UCLA for processing and sectioning of tissues. We would also like to thank Dr. Mike Teitell for critical review of the manuscript.

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