Prostate-specific Membrane Antigen Is Produced in Tumor-associated Neovasculature


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Abstract
Prostate-specific membrane antigen (PSMA), a type II transmembrane protein, was originally thought to be strictly expressed in prostatic tissue, but recent studies have demonstrated PSMA protein expression in nonprostatic tumor neovasculature as well. Using immunohistochemistry, reverse transcription-PCR assays, and in situ hybridization, we have demonstrated PSMA mRNA transcripts and protein expression in the endothelium of tumor-associated neovascularization of multiple nonprostatic solid malignancies. In addition, we found no PSMA mRNA or protein expression in the vascular endothelial cells of corresponding benign tissue examples. Our findings expand the possible therapeutic role of PSMA and establish it as a unique biomarker specifically produced and expressed by tumor-associated neovasculature but not produced or expressed by normal vessels.

Introduction
PSMA, initially defined by the 7E11 IgG mAb, is a 100,000 transmembrane glycoprotein highly expressed in prostatic tissues including benign secretory acinar epithelium, high-grade prostatic intraepithelial neoplasia, and prostate cancer (1–9). PSMA is located on the short arm of chromosome 11 (10) and functions both as a folate hydrolase and a neuropeptidase (11, 12). As PSMA was becoming a more attractive target in prostate cancer, researchers developed different anti-PSMA mAbs that, unlike 7E11, which binds an internal PSMA epitope, bind the external portion of the PSMA protein (13–15).

PSMA expression is not confined solely to the prostate. It has also been reported in select benign tissues (3, 4, 13, 16), and recent immunohistochemical studies have demonstrated PSMA protein expression in the endothelial cells of solid tumor neovasculature (4, 13, 16). None of these prior studies, including our own, have examined PSMA mRNA expression in these neovascular endothelial cells; thus, it remains unclear whether these angiogenic endothelial cells take up PSMA or produce PSMA. We sought to demonstrate PSMA production by the endothelial cells of tumor-associated neovasculature by using IHC, RT-PCR, and in situ hybridization in a variety of benign and malignant tissues.

Materials and Methods
Tissue Specimens and Antibodies. Fresh frozen tissues samples from male and female patients were randomly obtained from the Tissue Procurement Service, Memorial Sloan-Kettering Cancer Center. Tumor tissues included conventional (clear cell) renal cell carcinoma, transitional cell carcinoma of the bladder, pancreatic carcinoma, lung carcinoma, melanoma, sarcoma, and vascular neoplasms. For each tumor, a corresponding benign tissue sample was studied. We also studied benign prostate as a positive control. The 7E11 mAb was provided by Cytogen, Inc. (Princeton, NJ). The J591 and J415 antibodies have been described previously and were graciously donated by Dr. Neil H. Bander (Cornell University, New York, NY; Ref. 13). The antiendothelial cell mAb CD34 was obtained from Immunotech, Coulter Company (Opa Locka, FL).

IHC. At least four examples of the different tissue samples were snap-frozen in OCT compound, placed in isopentane, and stored at −70°C. Multiple 5-μm cryostat tissue sections were then cut and fixed in cold acetone (4°C) for 12 min. The primary antibody (5 μg/ml) incubations were performed in all specimens with the anti-PSMA mAbs 7E11, J591, and J415, and additionally, in tumor specimens, with the antiendothelial cell mAb CD34 for 60 min at room temperature. The remainder of the immunohistochemical reaction was completed using the streptavidin-biotin method. Briefly, the sections were washed thoroughly in PBS. Biotinylated secondary antibody (horse antimouse IgG) was added for 60 min. After washing with PBS, the specimens were incubated with streptavidin for 60 min, and the slides were washed again in PBS. The specimens were then immersed for 5 min in a solution of 0.06% diaminobenzidine tetrachloride and 0.01% hydrogen peroxide in PBS. After washing, the sections were lightly counterstained with hematoxylin, dehydrated, and mounted. In tissue with known significant quantities of endogenous biotin, the immunoperoxidase method using rabbit antimouse immunoglobulin-peroxidase as the secondary antibody (DAKO Corp., Carpinteria, CA) was used. Appropriate negative controls were performed with each specimen.

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4 The abbreviations used are: PSMA, prostate-specific membrane antigen; mAb, monoclonal antibody; RT-PCR, reverse transcription-PCR; IHC, immunohistochemistry.
**RNA Extraction and RT-PCR.** RNA from the same tumor and benign specimens examined previously by IHC was isolated by taking approximately 15 frozen sections (17 μm thick) and vortexing these sections in 1.5 ml of Trizol (Life Technologies, Inc., Rockville, MD). At least two different benign and malignant examples were examined from each tissue type. The samples were then freeze-thawed once at −20°C and allowed to thaw at room temperature. They were vortexed again, and cell debris was removed by centrifugation at 12,000 × g at 4°C for 10 min. RNA was then extracted from the samples following the protocol provided by the manufacturer, except that 40 μg of RNase-free glycogen (Boehringer Mannheim, Mannheim, Germany) were added as a carrier in the precipitation step. Integrity of the RNA was confirmed by electrophoresis on a 1% agarose gel.

RNA (2.5 μg) was reverse transcribed using random hexamers and the reagents supplied in the SuperScript Preamplification System for First-Strand cDNA Synthesis (Life Technologies, Inc.). One-tenth of the reverse-transcription reaction was then used as target in a PCR reaction to amplify the PSMA gene using the following set of primer sequences for all of the tissue types: (a) primer 1 (sense EX10), 5′-ACAGATATGCAATTCTCCTCT-3′; and (b) primer 2 (antisense EX16), 5′-ACTGCCTCTGAGTTC-3′. In addition to the above-mentioned set of primers, the PSMA gene was then examined in several representative tissue sections with three other sets of primers that covered the entire PSMA genetic sequence. These primers included: (a) sense EX 1, 5′-CTCTCTCTCTGCTCCTGGATTG-3′; (b) antisense EX 3, 5′-AATTGCTTGGCAAGCTGAAG-3′; (c) sense EX 2, 5′-GTTTATATCATCCCTCAATGAA-3′; (d) antisense EX 10, 5′-TTTCAGTGTTCCAAAGCTTCCTAC-3′; (e) sense EX 16, 5′-GGGCTATCCACTGTATCAC-3′; and (f) antisense EX 19, 5′-ACA-TACCAAAATTCAAATCGG-3′.

The PCR reaction was carried out in a total volume of 50 μl containing 0.2 mM of each deoxynucleotide triphosphate and 150 ng of each primer with 1 unit of Expand Long-Template polymerase in the accompanying buffer 2 (Boehringer Mannheim). The PCR conditions were an initial denaturation at 94°C for 3 min 30 s, followed by 32 cycles of 94°C for 20 s, 61°C for 20 s, and 72°C for 50 s. Electrophoresis was carried out using 10 μl of the amplified product (20 μl for vascular tumors) on a 1.3% agarose gel. The expected product size was 555 bp for the primers covering exon 10–16. To confirm the success of the reverse-transcription reaction, 1 μl of cDNA was used as a target in a 25-μl PCR reaction under the same conditions as described above, except that the regular Taq polymerase was used in the reaction (Fisher Biotech, Pittsburgh, PA), and the annealing temperature was reduced to 58°C. The primers used amplify β2-microglobulin cDNA and are as described previously (17).

**Preparation of a PSMA-specific Transcript Probe for in Situ Hybridization.** PSMA sense and antisense digoxigenin-labeled RNA probes were generated from a plasmid (pSSA) (17).
containing the entire human PSMA cDNA sequence (2.65 kb) in the pSPORT-1 vector (Life Technologies, Inc.). Briefly, the plasmid was linearized with either NotI (sense) or SalI (antisense) and phenol/chloroform-extracted before incubating the cDNA template (1 μg) overnight at room temperature with 5× transcription buffer (Promega, Madison, WI), 100 mM DTT, digoxigenin-labeled nucleotide mix, 40 units/μl RNasin, and SP6 or T7 RNA polymerase, respectively (Fisher Biotech). The probes were then treated with DNase I (Fisher Biotech) for 15 min at 37°C and hydrolyzed by incubating with 80 mM NaHCO₃, 120 mM Na₂CO₃, and 10 mM DTT for 60 min at 60°C before neutralizing with 1% acetic acid and 10 mM DTT. Colorimetric analysis was then performed to estimate the probe yield.

**In Situ Hybridization Technique.** Tissue samples were precooled in isopentane, snap-frozen in OCT compound, placed in isopentane, and stored at −70°C. Multiple 5-μm cryostat tissue sections were then hybridized to the probes. Magnification, ×20. A, pancreas; B, pancreas carcinoma; C, skin; D, skin cancer (melanoma); E, lung; F, lung carcinoma; G, bladder; H, transitional cell carcinoma of urinary bladder; I, kidney; J, conventional clear cell renal carcinoma; K, muscle 7E11 mAb (left panel) and J591 mAb (right panel); L, soft tissue sarcoma.

*Fig. 2* PSMA expression by IHC with anti-PSMA mAb 7E11. Positive staining was demonstrated in the neovasculature of malignant tissue, but no reactivity was demonstrated in benign tissue vasculature. Proximal tubule cells in benign kidney expressed PSMA. The anti-PSMA mAbs were similar, except in skeletal muscle, which reacted only with the 7E11 mAb. Magnification, ×20. A, pancreas; B, pancreas carcinoma; C, skin; D, skin cancer (melanoma); E, lung; F, lung carcinoma; G, bladder; H, transitional cell carcinoma of urinary bladder; I, kidney; J, conventional clear cell renal carcinoma; K, muscle 7E11 mAb (left panel) and J591 mAb (right panel); L, soft tissue sarcoma.
sections were then cut, allowed to warm to room temperature, and fixed in 4% paraformaldehyde for 15 min. The slides were then treated with proteinase K (20 μg/ml) for 3 min, followed by washing and postfixation with 4% paraformaldehyde. After rinsing in 1× PBS, the slides were acetylated in freshly prepared 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0). The slides then underwent prehybridization in 50% formamide, 5× SSC, 5× Denhardt’s solution, tRNA (250 μg/ml), and salmon sperm DNA (500 μg/ml) for 3 h at 65°C. The slides were then hybridized in the above-mentioned solution with either 1.5 μg/ml sense or antisense probe in solution for 16 h at 65°C.

After hybridization, the slides were washed in 5× SSC and 20% formamide and digested with RNase A (10 μg/ml) for 30 min at 37°C. After washing with 20% formamide for 30 min at 60°C, 2× SSC for 30 min at room temperature, and 1% Boehringer blocking agent for 30 min at room temperature, the sections were analyzed using an alkaline phosphatase-conjugated antidigoxigenin antibody (Boehringer Mannheim) at a
1:5000 dilution and overnight incubation at 4°C. The color reaction was visualized with BM purple (Boehringer Mannheim). Slides were then dehydrated and mounted.

**Results**

**IHC.** To confirm PSMA protein expression in nonprostatic tumor-associated neovascularity, we performed IHC with three different anti-PSMA mAbs: (a) 7E11; (b) J591; and (c) J415. The 7E11 mAb binds a 6-amino acid intracellular epitope of PSMA (1, 18, 19), whereas the J591 and J415 anti-PSMA mAbs bind different portions of the PSMA external domain (13). These anti-PSMA mAbs reacted in a similar manner to each other (Fig. 1), except in skeletal muscle, where a subset of cells reacted only with the 7E11 mAb and not with the J591 and J415 mAbs (Fig. 2).

Benign kidney (proximal tubule cells) was the only benign tissue type that reacted with the anti-PSMA mAbs. In contrast, the angiogenic microvessels associated with all six types of cancerous tissue reacted with all of the anti-PSMA mAbs (Fig. 2). Among the tumor specimens, we also examined vascular tumors. Hemangiomas are benign vascular lesions that proved negative for all of the anti-PSMA mAbs. However, mAb CD34 stained positive in these lesions.

**RT-PCR Demonstrates PSMA mRNA Transcripts in Malignant Tissue.** To demonstrate PSMA production by tumors, we performed and compared RT-PCR assays on the same benign and tumor tissue specimens on which we had performed IHC. The RT-PCR results using primers that span the enzymatically active site of PSMA, exons 10–16, paralleled our immunohistochemical findings. The benign kidney tissue, the only benign tissue type that expressed PSMA protein immunoreactivity, had PSMA mRNA transcripts detected by RT-PCR assay; all other benign tissues did not demonstrate PSMA mRNA transcripts. However, all malignant tissue samples were strongly positive for PSMA mRNA transcripts (Fig. 3). RT-PCR assays with the other sets of primers that included the entire PSMA gene sequence confirmed PSMA mRNA transcripts (data not shown).

As with IHC, the benign hemangioma specimens were negative, but the single hemangioendothelioma displayed a faint PSMA mRNA band by RT-PCR (Fig. 4).

**In Situ Hybridization Confirms Tumor-associated Neovascularity PSMA Expression.** We performed in situ hybridization to localize PSMA mRNA production to a certain cell type. Using prostate as a positive control for our probes, we examined lung carcinoma and neuroendocrine carcinoma of the pancreas, two different types of cancers that in benign examples of these tissues did not express PSMA and had never been noted in previous studies to express PSMA. The in situ staining with the antisense PSMA probe demonstrated positive PSMA mRNA expression in angiogenic vessels associated with the tumor but showed no expression in the tumor cells (Fig. 5).

**Discussion**

Our results demonstrate that PSMA is produced and expressed in the endothelial cells of tumor-associated neovascularity but not in the endothelial cells of normal vessels in benign tissue. We and others have examined PSMA expression by IHC and shown this neovascular activity. (4, 13, 16) Because the epithelial cells of prostate cancer strongly express PSMA,
we focused in this study on nonprostatic tissue and specifically on tumor-associated neovasculature. We again found consistent PSMA protein expression in the neovasculature, because all of the examples of different tumors, except for hemangiomas, had PSMA-positive neovasculature.

Whether or not PSMA circulates in the serum is controversial (20–24). Thus, we performed RT-PCR and in situ hybridization in a variety of nonprostatic, malignant tissue types that were PSMA positive by IHC to confirm PSMA mRNA transcripts in the angiogenic endothelial cells. The sequencing of the PCR products generated from the tumor sections provided more definitive evidence of PSMA synthesis by these angiogenic endothelial cells. In tumor specimens, this sensitive method confirmed PSMA mRNA transcripts encoding for the PSMA protein and showed no PSMA mRNA in all corresponding benign tissue specimens except benign kidney. This latter finding was expected because several studies have reported PSMA expression in benign kidney proximal tubules by IHC (1, 4, 13). Using in situ hybridization, we further confirmed our immunohistochemical findings and localized PSMA production specifically to the endothelial cells of tumor-associated neovasculature. The endothelial cells of tumor-associated neovasculature, not the tumor cells, contained PSMA mRNA transcripts. These results strongly supported our hypothesis that the endothelial cells of tumor-associated neovasculature synthesize the PSMA protein and do not sequester the protein from the serum or the surrounding stromal cells.

In contrast to tumor-associated neovasculature, benign vascular tumors did not express PSMA. This is not surprising, given that in these tumors, the endothelium itself is altered and presumably not stimulated by angiogenic factors. The hemangiogendothelioma, a lesion intermediate between hemangioma (benign) and angiosarcoma (malignant), demonstrated a weak PSMA band by RT-PCR, suggesting the presence of small amounts of PSMA mRNA. Another interesting tissue type was benign skeletal muscle, which reacted only with the 7E11 mAb. The lack of other mAb reactivity and PSMA mRNA expression makes it highly unlikely that PSMA is produced or expressed by muscle fibers. The 7E11 binding may be due to nonspecific binding unique to this mAb.

Angiogenesis, essential for the growth and development of both primary and metastatic tumors, is a complex process involving a dynamic interrelationship between stimulators and inhibitors (25–28). Antiangiogenic strategies have been successful in the laboratory setting, and a single angiogenic target can be useful in treating a variety of diverse tumors (29–33). PSMA

Fig. 5 PSMA expression by IHC and in situ hybridization with digoxigenin. In situ hybridization confirmed PSMA mRNA transcripts in neovasculature of nonprostatic malignancies. Prostate served as a positive control. Magnification, ×20. Prostate tissue: A, 7E11 mAb IHC; B, antisense probe in situ; and C, sense probe in situ. Pancreas carcinoma: D, 7E11 mAb IHC; E, antisense probe in situ; and F, sense probe in situ. Lung carcinoma: G, 7E11 mAb IHC; H, antisense probe in situ; and I, sense probe in situ.
is a unique antiangiogenesis target because it is selectively and consistently expressed in nonprostatic tumor-associated neovascular but not in normal vessels in benign tissue. This is in contrast to other endothelial cell targets such as vascular endothelial growth factor receptors, integrin αβ3, CD34, and Tie receptors are normally expressed in normal vasculature and are up-regulated in tumor neovascularization (34–36). In addition, PSMA has folate hydrolase activity that can and has been used in a pro-drug strategy with cytotoxic agents (37).

Our findings imply that the PSMA promoter and PSMA gene or the surrounding sequence contains transcriptional enhancer regions that selectively activate PSMA transcription in tumor neovascular but not in normal vessels. Isolating the specific enhancer regions of the PSMA gene that drive expression in tumor neovascular may form the basis for a very specific antiangiogenic gene therapy construct.

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References


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