The Presence of Prostate-specific Antigen-related Genes in Primates and the Expression of Recombinant Human Prostate-specific Antigen in a Transfected Murine Cell Line

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ABSTRACT

Human prostate-specific antigen (PSA) has been shown as an aid in the early detection of prostate cancer (W. J. Catalona et al., J. Am. Med. Assoc., 270: 948–954, 1993) and was approved in 1994 by the Food and Drug Administration for early detection of prostate cancer. Immunotherapies directed against PSA have been suggested in patients with metastatic prostate cancer. One of the essential questions is to define which nonhuman species express PSA for experimental studies. Using Southern blot analyses, genes related to human PSA have been detected in several nonhuman primate species, including chimpanzee, orangutan, gorilla, macaque, and rhesus monkey, but not in other mammalian species, including rabbit, cow, pig, dog, rat, or mouse. Immunohistochemical staining with anti-human PSA antiserum detected strong staining in both human and monkey prostatic epithelial cells with no reactivity to rat prostate cells. Because the PSA gene is not present in the murine genome, a matched set of murine cell lines has been developed that may be useful to study the biochemical functions of PSA and as an experimental target for PSA-directed immunotherapy. To establish such cell lines, a C57BL/6 murine colon adenocarcinoma cell line, MC-38, was transfected with a retroviral vector containing cDNA encoding the human PSA gene. Genetic analysis of a PSA-secreting clone, PSA/MC-38, demonstrated that the PSA gene had been stably integrated into the MC-38 genome. The PSA/MC-38 cell line was found to secrete PSA into tissue culture medium, producing a protein of approximately Mr 30,000. In vivo, PSA/MC-38 grew as a s.c. tumor in male and female mice. PSA/MC-38 tumors grew more rapidly in athymic mice than in syngeneic C57BL/6 mice, and in both mouse strains, the PSA/MC-38 tumors grew more slowly than control vector-transduced tumors. PSA was detected in the serum and tumors of PSA/MC-38 tumor-bearing mice. It is proposed that PSA/MC-38 cells may be used as a murine tumor model to test potential therapeutic vaccines and other experimental therapies directed against PSA.

INTRODUCTION

PSA2, currently used for early detection of prostate cancer (1), is a Mr 33,000 glycoprotein first characterized by Wang et al. (2). A chymotrypsin-like serine protease that is secreted almost exclusively by the epithelial cells that line prostatic acini and ducts, PSA is produced by normal human prostatic tissue and secreted into the prostatic fluid (3–6).

PSA is also released into the blood at low concentrations. In healthy males without clinical evidence of prostate cancer, the concentration of PSA detected in the serum is usually less than 4 ng PSA/ml (7, 8). In contrast, elevated levels of serum PSA are frequently associated with primary, locally recurrent, or metastatic prostate carcinoma and benign prostatic hyperplasia (7, 9–11). The concentration of PSA is greater than 4 ng/ml in the serum of 79% of prostate cancer patients with Stages A through D carcinoma and 39% of patients with benign prostatic hyperplasia (12). Not only is serum PSA elevated in such patients, but higher serum levels of PSA have been correlated with more advanced clinical stages of prostatic disease (7, 9). Furthermore, prostatic carcinoma metastases have been detected by radioimmuno- logical imaging using an indium-111 radiolabeled anti-PSA mAb, indicating that although PSA is a secretory protein, enough PSA remains cell associated to allow tumor targeting (13). For these reasons, PSA is the most widely used serum marker for prostate cancer and may be considered a tumor-associated antigen in certain settings. In such settings, PSA could also be considered as a potential target for active specific immunotherapy approaches of prostatic cancer.

Aspects of prostatic carcinoma tumorigenesis, metastasis, hormonal regulation of prostatic tumors, and effects of chemotherapeutic reagents on prostate cancer have been studied in animal models, including those developed in dogs, rats, and mice. However, with the exception of human prostatic adenocarcinoma cell lines grown in athymic mice (14–16), these animal models do not express human PSA (17, 18). Because dogs develop spontaneous prostatic adenocarcinoma, with increasing incidence associated with advancing age (19), prostatic disease in dogs has been used frequently as a model for studying prostate cancer in man. One dog prostatic protein, arginine esterase, shares a 58% amino acid homology at the NH2-terminus with the related human serine protease, PSA (18). Despite this low level of homology, McEntee et al. (20) reported that only 2 of 31 canine adenocarcinomas were positive for PSA by immunohistochemical examination, using a prolonged incubation of polyclonal rabbit anti-PSA serum to stain the dog prostatic tissues. The results of these experiments suggest that there are few shared antigenic epitopes between PSA and dog prostate arginine esterase.

In an effort to define PSA-related genes in mammalian species and to identify those species that might be suitable for future in vivo experiments involving PSA, hybridization studies were conducted using genomic DNA from a range of mammalian species. The results of those studies are described here. The goal of additional studies was to develop a matched pair of murine tumor cell lines that are positive or negative for expression of human PSA, which may be useful in determining the immunological properties of PSA, as a model for the study of the biological roles for PSA and for potential use in an experimental rodent model as a target for anti-PSA therapies. The development of such cell lines, the characteristics of their growth rates, the level of PSA expression, and potential applications for these cell lines are also described here.

MATERIALS AND METHODS

Cell Lines. MC-38, a murine colonic adenocarcinoma cell line (21), was obtained from Dr. Bernard Fox (National Cancer Institute, NIH, Bethesda, MD). The LNCaP human prostate adenocarcinoma cell line (22) was obtained from the American Type Culture Collection (Rockville, MD). The Moloney murine sarcoma virus retroviral vector pLNSX (23) was obtained from Dr. A. Dusty Miller (Fred Hutchinson Cancer Research Center, Seattle, WA). The murine ectopic packaging cell line GP+E-86 (24), MC-38, PSA/MC-38, and pLNSX/MC-38 were maintained in DMEM (GIBCO-BRL, Gaithersburg, MD)

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2 The abbreviations used are: PSA, prostate-specific antigen; FBS, fetal bovine serum; hGK-1, human glandular kallikrein-1.
with 10% FBS (GIBCO-BRL). PSA/MC-38 and pLNSX/MC-38 cell lines were kept under continuous selective pressure in 1 mg G418 sulfate/ml (GIBCO-BRL). LNCaP was maintained in RPMI 1640 (GIBCO-BRL) containing 10% FBS.

Cloning of PSA cDNA. cDNA was synthesized from total RNA from the human prostate adenocarcinoma cell line LNCaP using the GeneAmp RNA PCR kit (Perkin-Elmer Corp., Norwalk, CT). Human PSA-specific oligonucleotide primers were selected based on the human mRNA sequence (GenBank accession number X07730) using the MacVector 4.1.4. computer program (Kodak Co., Rochester, NY). The 5' (5'-AGA GAG AGC GTC AAG TTG CCA GCT AAC GCT CAT CTC CGA-3') and 3' (5'-AGA GAG AGC CTT AGT CCT CCC TCT CCT TCT TTC AT-3') primers, containing HindIII restriction enzyme sites, were used to synthesize full-length PSA cDNA by PCR. The 1.5-kb PSA gene was ligated to HindIII restriction endonuclease-digested pLNSX DNA and was used to transform competent DH5α Escherichia coli cells (GIBCO-BRL). Ampicillin-resistant colonies were tested for orientation of the cDNA insert by PCR using a vector-specific 5' oligonucleotide primer (5'-TTT GGA GCC CCA GCT GTC TGT AAA-3') and the PSA-specific 3' primer described above. Transformants were selected with PSA cDNA in the sense orientation, characterized by restriction endonuclease digestion, and sequenced by the dideoxy method using Sequenase (United States Biochemical Corp., Cleveland, OH). Sequence analysis of the gene recovered by PCR confirmed that the gene was identical to the human PSA gene sequence in GenBank.

Transfection and Transduction of DNA. The PSA/pLNSX plasmid (5 μg) was transfected into MC-38 cells using Transfection-reagent (DOTAP; Boehringer-Mannheim Biochemica, Indianapolis, IN) according to the manufacturer's instructions. At 24 h, selection medium containing 100 μg/ml (w/v) of G418 was added to the cells. Selective pressure was maintained by continuous culture in DMEM containing 10% FBS and increasing concentrations of G418 (to 1 mg/ml). Drug-resistant cells were cloned by limiting dilution. Conditioned medium from the cloning wells was tested using the solid phase, double-determinant Tandem-R PSA immunoradiometric assay (Hybritech, Inc., San Diego, CA). The highest producers of secreted PSA were cloned twice by limiting dilution. One clone, designated PSA/MC-38, produced approximately 10 ng PSA/ml and was selected for further studies. Vector-transduced, PSA-negative MC-38 cells were developed as follows. GP+E-E-86 cells, an ecotropic murine packaging cell line, were transfected with 2 μg of pLNSX vector DNA using DOTAP transfection-reagent, as described above. At 24 h, the transfected cells were replated and grown in selection medium (1.0 mg G418/ml). Cells surviving G418 selection and containing pLNSX were grown in medium without G418, and this medium was added to MC-38 cells in the presence of 8 μg/ml of polybrene (Sigma Chemical Co., St. Louis, MO). The transduced MC-38 cells were grown in the presence of G418 for 3 weeks. Individual drug-resistant colonies were isolated by sterile cloning rings and characterized by PCR for the presence of pLNSX. One clone, pLNSX/MC-38, was used for further study.

Southern Blot Analysis. High molecular weight DNA was extracted from murine spleens (allogenic mice) or livers (C57BL/6 mice), normal human spleens, normal rhesus monkey spleen, and murine and human tumor cell lines (MC-38, PSA/MC-38, pLNSX/MC-38, and LNCaP) as described by Davis et al. (25). Ten μg of each preparation were digested overnight with the restriction endonuclease HindIII (Boehringer-Mannheim) and separated by electrophoresis at 35 V overnight on 0.8% agarose gels (FMC, Rockland, ME) in Tris-acetate EDTA buffer (0.04 M Tris-acetate-0.001 M EDTA). The DNA was depurinated, denatured, neutralized, and transferred to BioTrans nylon membranes (ICN Biomedicals, Inc., Costa Mesa, CA) by capillary blotting, as described by Sambrook et al. (26). Following transfer, the DNA was cross-linked by UV irradiation at 120 mJ in a UV Stratalinker 2400 (Stratagene Cloning Systems, La Jolla, CA). Nylon blots of HindIII restriction endonuclease-digested mammalian DNA (8 μg species) were prepared commercially (Bios Laboratories, New Haven, CT). PSA or hGK-1 (provided by Dr. Andrew Schrader, University of Sydney, Sydney, Australia) cDNA probes were radiolabeled with [γ-32P]dCTP. Blots were hybridized overnight with the radiolabeled probes at 37°C using 50% formamide in 5X Denhardt's solution with 1% SDS, 3X saline-sodium phosphate-EDTA (3.0 m NaCl, 0.2 m NaH2PO4-H2O, and 0.02 m disodium EDTA), 2.5% dextran sulfate, 1 mm NaPO4 (pH 6.7), and 100 μg/ml salmon sperm DNA, as described previously (26). The final two 15-min washes were performed at 55°C in 0.1X SSC with 0.1% SDS. Autoradiographs were made by exposing the membranes to X-Omat AR film (Eastman Kodak Company, Rochester, NY).

In Vitro Growth Rate. PSA/MC-38 and pLNSX/MC-38 cells were seeded at 1 × 10⁶ cells/well in 16-mm wells and incubated at 37°C in 5% CO2. Conditioned medium was collected from two wells/cell line at each time point (1, 2, 3, 4, or 5 days), after which viable cell counts were determined using trypan blue exclusion.

PSA Quantitation. The concentration of recombinant human PSA in tissue culture conditioned medium, mouse serum, and protein extracts was quantitated by solid phase, double-determinant Tandem-R PSA immunoradiometric assay.

Immunoprecipitation. Fifteen ml of conditioned medium from LNCaP, PSA/MC-38, pLNSX/MC-38, and MC-38 were concentrated 15-fold in Centriprep-10 concentrators (Amicon, Beverly, MA). Sheep anti-rabbit IgG-coated Magnetic Dynabeads M-280 (Dynal, Lake Success, NY) were incubated with polyclonal rabbit anti-human PSA (DAKO Corp., Carpinteria, CA) at a concentration of 200 μg antibody to 5 mg beads for 48 h with rotation at 4°C. Unbound antibody was removed by washing the beads three times for 60 min each at 4°C in PBS with 1% BSA (PBS-BSA). The beads were suspended in 500 μl PBS-BSA. A 100-μl aliquot of these beads was added to 1 ml of concentrated conditioned medium and incubated overnight at 4°C. The beads were then washed three times in PBS-BSA, resuspended in 1 ml PBS-BSA, and stored at 4°C.

Western Blot Analysis. Prestained molecular weight protein markers (GIBCO-BRL), immunoprecipitated PSA (100 μl of the above beads/lane), and 50 ng of purified PSA (Calbiochem-Novabiochem Corp., La Jolla, CA) were separated by SDS-PAGE on 18% Tris-glycine denaturing and reducing gels (Novex, San Diego, CA) at 150 V for 2 h. Proteins were transferred to nitrocellulose membranes (Novex) at 30 V. The membranes were blocked at 4°C overnight in 5% BSA, rinsed once in PBS-BSA, and then incubated at room temperature in a 1:1000 dilution of polyclonal rabbit anti-human PSA (DAKO Corp.) in PBS-BSA for 1 h. The membranes were washed three times in Dulbecco's PBS with calcium and magnesium (Biofluids, Inc., Rockville, MD) with 0.05% Tween 20 and incubated for 1 h in a 1:100 dilution of phosphatase-labeled goat anti-rabbit IgG (heavy chain plus light chain recognition; Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD) in PBS-BSA. Following three washes, protein bands were visualized with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium phosphatase substrate system (Kirkegaard and Perry Laboratories, Inc.).

Protein Extracts of PSA/MC-38 and pLNSX/MC-38 Cells. Extracts of PSA/MC-38 and pLNSX/MC-38 monolayer cultures and tumors were prepared as described previously (27). The protein concentration was determined using the method of Lowry et al. (28).

Immunohistochemical Assays. Immunohistochemical staining was performed on frozen sections of normal prostate tissues and cell pellets that had been formalin fixed for 30 min prior to staining. The primary antibody, polyclonal rabbit anti-human PSA (Biomedica, Foster City, CA), was used at a dilution of 1:500. Polyclonal rabbit anti-horseradish peroxidase (DAKO Corp.) was diluted 1:500 and used as a negative control. Biotinylated, goat anti-rabbit IgG (H+L) secondary antibody (Vector Laboratories, Inc., Burlingame, CA) was used at 1:500. The avidin-biotin-peroxidase reagent (Vectastain Elite ABC; Vector Laboratories, Inc.) was used, followed by 3,3'-diaminobenzidine-imidazole as the chromogen.

Tumor Growth Studies. Six-week-old C57BL/6 and NCr athymic (nu/nu) female mice were obtained from Taconic Farms (Germantown, NY). Twenty mice of each strain were injected s.c. in the right hind flank with PSA/MC-38 or pLNSX/MC-38 cells (2 × 10⁶ cells/100 μl/mouse) in HBSS. Tumors were measured twice weekly. Five mice from each group of twenty were sacrificed at 14 and 21 days following inoculation. Tumors in the remaining 10 mice/group were measured until the tumor volume (width² × length/2) reached 2 to 3 cm³, or 12 weeks following injection of the tumor cells.

Collection of Serum from PSA/MC-38 Tumor-bearing Mice. To determine serum PSA levels in tumor-bearing mice, 6-week-old male C57BL/6 mice (10/group) were injected s.c. with PSA/MC-38 or pLNSX/MC-38 cells (1 × 10⁶ cells/100 μl/mouse). Tumors were measured on day 21 following tumor cell injection. Mice were bled by the retro-orbital route at day 22. Serum was diluted 1:2 in PBS and tested for PSA by Tandem-R PSA assay.

To determine the anti-PSA humoral response in tumor-bearing mice, female
C57BL/6 mice (five mice/group) were injected s.c. with PSA/MC-38 or pLNXS/MC-38 cells (1 X 10^6 cells/100μl/mouse). Mice were bled before and on day 21 following tumor cell injection. Serum was tested for anti-PSA antibodies as described below.

**ELISA for Murine Anti-PSA Antibodies.** Mouse anti-PSA antibody responses were assessed by ELISA, essentially as described previously (27). Briefly, 96-well polystyrene microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) were sensitized with purified PSA (Calbiochem-Novabiochem Corp.) at 100 ng/well overnight at 37°C. Serum samples were diluted in PBS-BSA, and 50 μl of each sample were added per well. The remainder of the assay was performed as described (27).

**RESULTS**

**PSA in Mammalian Species.** Since PSA is a member of the kallikrein gene family, which also includes human glandular kallikrein-1 and tissue kallikrein, Southern blot studies were undertaken to compare the presence of the PSA and hGK-1 genes in a range of mammalian species. To prepare the DNA blots, genomic DNA from these species (rabbits, orangutans, chimpanzees, gorillas, macaques, humans, dogs, mice, rats, cows, and pigs) was digested with HindIII restriction endonuclease, blotted, and hybridized with human PSA or hGK-1-radiolabeled nucleic acid probes, as described in “Materials and Methods.” The blots were washed under conditions of high stringency (0.1X SSC-0.1% SDS at 55°C).

The relatedness among mammalian PSA and hGK-1 nucleotide sequences can be seen in Fig. 1. Three fragments of HindIII restriction endonuclease-digested human genomic DNA (20, 11, and 8 kb) hybridized to the full-length human PSA cDNA probe (Fig. 1A; human DNA). When the blot was later stripped and rehybridized to a cDNA probe for exon three of hGK-1, similar fragments were detected (Fig. 1B; human DNA). The 8-kb signal was strongest with the PSA probe (Fig. 1A; human DNA), and the 11-kb signal was strongest with the hGK-1 probe (Fig. 1B; human DNA), suggesting that the 8-kb HindIII fragment represents the PSA gene and the 11-kb fragment represents the hGK-1 gene. Further evidence that the PSA gene is located on the 8-kb HindIII fragment can be seen in Fig. 2. On this genomic DNA blot, the PSA cDNA probe again hybridized to three HindIII restriction endonuclease-digested fragments of human DNA (from the LNCaP human prostate adenocarcinoma cell line and the human spleen). However, only the signal for the 8-kb fragment was intense on this blot, indicating that this fragment had the highest sequence homology with the PSA probe. The 11-kb signal was considerably weaker, and the 20-kb fragment was only detected in conditions of lower stringency (50°C; 0.5X SSC and 0.1% SDS), suggesting that the 20-kb fragment has the least identity to the PSA probe.

Hybridization of the human PSA and hGK-1 probes to human DNA was compared to that of DNA from several other mammalian species. There was a similar pattern of strong hybridization to two fragments of 11 and 8 kb from orangutan (Pongo pygmaeus), chimpanzee (Pan troglodytes), and human DNA, suggesting highly conserved sequences of PSA- and hGK-1-related genes in these closely related species, all of which are in the Hominoidea superfamily (Fig. 1). As compared with results with human DNA, the 8-kb HindIII fragment of both orangutan and chimpanzee DNA was more intense with the PSA probe, and the 11-kb fragment was more intense with the hGK-1 probe. There was also strong hybridization of the human PSA and hGK-1 probes to a 5-kb HindIII fragment of gorilla (Gorilla gorilla, another member of the Hominoidea superfamily) DNA, to two fragments of 18 and 6 kb from macaque (Macaca fascicularis) DNA and two fragments of 20 and 18 kb in rhesus monkey (Macaca mulatta) DNA (Fig. 2), both in the Cercopithecoidae superfamily. There was also a faintly visible 20-kb fragment of chimpanzee DNA (Fig. 1).

There was very weak hybridization of the PSA and hGK-1 probes to rabbit (Oryctolagus cuniculus), cow (Bovis domesticus), and pig (Sus scrofa) genomic DNA (Fig. 1). In contrast, there was no hybridization of these probes to dog (Canis familiaris), mouse (Mus musculus), or rat (Rattus norvegicus) genomic DNA (Figs. 1 and 2), indicating an absence of the PSA and hGK-1 genes in these species. Less stringent hybridization conditions also failed to detect PSA- or hGK-1-related genes in these species (data not shown). Southern blot studies of mouse genomic DNA from several mouse strains (C58, athymic nude, BALB/c, or C57BL/6) confirmed that PSA or related sequences are not present in the mouse genome (Fig. 2). In summary, the results of these hybridization experiments demonstrate that high sequence similarities exist among PSA and hGK-1 genes in humans and all the nonhuman primate species examined but not in other mammals such as rabbits, dogs, mice, rats, cows, and pigs.

**Prostatic Tissue Immunohistochemistry.** Human, rhesus monkey, and Lobund Wistar rat prostates were stained with polyclonal rabbit anti-human PSA antibody, followed by biotinylated goat anti-rabbit IgG and avidin-biotin reagent. Both human and monkey pros-

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**Fig. 1.** Southern blot analysis of HindIII restriction endonuclease-digested genomic DNA from several mammalian species. Genomic DNA was hybridized with [α-32P]dCTP radiolabeled human PSA (A) or hGK-1 (B) cDNA. High stringency wash conditions were used to detect the bands.
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Fig. 2. Southern blot analysis of PSA homologous sequences in genomic tissue and cell line DNA. Mouse (C58. athymic, BALB/c, and C57BL6), cell line (MC-38. murine colon adenocarcinoma; PSA/MC-38, MC-38 transfected with PSA; pLNSX/MC-38, vector-transduced MC-38; LNCaP, human prostate adenocarcinoma), human, and rhesus monkey DNA were digested with HindIII restriction endonuclease and separated on a 0.8% agarose gel. Following transfer to a nylon membrane, the DNA was hybridized to [a-32P]dCTP-radiolabeled human PSA cDNA and washed in high stringency conditions.

Fig. 3. Immunohistochemistry of human, rat, and monkey prostate tissues. Tissue sections were incubated with rabbit anti-human PSA, followed by biotinylated goat anti-rabbit IgG (H+L) antibodies, ABC, and 3,3'-diaminobenzidine-imidazole.

Development of a Murine Tumor Cell Line Expressing Human PSA. As mouse genomic DNA does not encode PSA or PSA-related proteins, a murine tumor cell line has been used to develop a matched set of cells that are positive or negative for expression of human PSA. PSA cDNA was cloned from the LNCaP human prostate adenocarcinoma cell line by PCR and inserted into the pLNSX Moloney murine sarcoma virus retroviral vector; the resulting construct was used to transfect the MC-38 murine adenocarcinoma cell line, as described in "Materials and Methods." The MC-38 carcinoma cell line was used because it had been shown previously to be transfectable with the gene for the human carcinoembryonic antigen (29) and has also been shown to metastasize (30). Following cloning three times by limiting dilution, the cell line producing the highest level of PSA (9.6 ng PSA/ml conditioned medium) was designated PSA/MC-38. Further studies of this clone, maintained in tissue culture under G418 selection for more than 30 passages, revealed long-term production of PSA ranging from 7 to 14 ng/ml. A vector-transduced cell line, pLNSX/MC-38, was also developed for use as a negative control for in vitro and animal tumor model studies. No PSA was detected in conditioned medium from the pLNSX/MC-38 cell line.

Genomic DNA from the PSA/MC-38 cell line was analyzed by Southern blot hybridization to confirm that the cloned PSA gene was intact and unarranged. The 1.5-kb HindIII restriction endonuclease fragment detected in PSA/MC-38 DNA represents the full-length cDNA that was cloned into the vector pLNSX prior to transfection of MC-38 cells (Fig. 2) and demonstrates the integrity of the PSA gene in the PSA/MC-38 cell line. Moreover, restriction endonuclease mapping using BamHI and EcoRI enzymes showed identical patterns for the human PSA cDNA clone and the DNA in the PSA/MC-38 cells. Although there was strong hybridization of the human PSA probe to PSA/MC-38 and LNCaP human prostate adenocarcinoma cell line DNA, no hybridization was detected to negative control DNA from the untransfected MC-38 and pLNSX/MC-38 cell lines (Fig. 2).

PSA/MC-38 cell line protein extracts were then tested for cell-associated PSA expression using the Tandem-R PSA assay. While protein extracts of PSA/MC-38 cells grown in tissue culture had low levels of PSA (less than 1.0 ng PSA/mg protein), extracts of pLNSX/MC-38 and MC-38 cells had no detectable PSA (data not shown). The presence of intracellular PSA in the PSA/MC-38 cell line was confirmed by immunohistochemistry on frozen sections of cell pellets (Table 1). Using a polyclonal rabbit anti-PSA antibody, the PSA/MC-38 cells were shown to be positive for PSA expression. In comparison, LNCaP cells were strongly positive, while pLNSX/MC-38 and MC-38 cells were negative.

Growth of PSA/MC-38 Murine Adenocarcinoma Cell Line and Quantitation of Secreted PSA. To establish whether PSA expression affected the growth of transfected cells, the in vitro growth rates...
Table 1  Detection of cellular PSA protein by immunohistochemistry

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<tr>
<th>Cell line</th>
<th>Description</th>
<th>Rabbit anti-human PSA</th>
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<tr>
<td>LNCaP</td>
<td>Human prostate adenocarcinoma</td>
<td>++/++</td>
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<tr>
<td>PSA/MC-38</td>
<td>PSA-transfected MC-38</td>
<td>10/0</td>
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<tr>
<td>pLNSX/MC-38</td>
<td>Vector-transduced MC-38</td>
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a Immunohistochemistry was performed using polyclonal rabbit anti-human PSA, as described in "Materials and Methods.”

b++, strong staining; +, weak staining. Numbers represent the percentage of cells scored at each staining intensity.

The growth rates and quantitation of secreted PSA for PSA/MC-38 and pLNSX/MC-38 were compared. Cell lines were seeded at $1 \times 10^5$ cells/well, and viable cells were counted at 1-day intervals. No substantial difference was observed between the growth rates of the two cell lines, with each having doubling times of approximately 12 h (Fig. 4A). Both cell lines grew logarithmically until each had reached plateau phase at approximately $10^5$ cells/well. Each day for 5 days, conditioned medium was collected, and the amount of PSA was quantitated by immunoradiometric assay. The concentration of accumulated PSA after 5 days was 2.8 ng per $10^5$ PSA/MC-38 cells. PSA was not detected in conditioned medium from the pLNSX/MC-38 cells (Fig. 4B).

Conditioned tissue culture medium from PSA/MC-38 cells was collected and analyzed by Western blotting using polyclonal rabbit anti-human PSA to determine if full-length PSA was being produced. A protein of approximately $M_r$ 30,000 was detected for PSA/MC-38 and LNCaP, the human prostate adenocarcinoma cell line (Fig. 5). No PSA was detected for either pLNSX/MC-38 or untransfected MC-38 cells. A $M_r$ 47,000 protein was detected in all four samples (LNCaP, PSA/MC-38, pLNSX/MC-38, and MC-38) and was shown to be gamma heavy chain of the rabbit anti-human PSA antibody used for immunoprecipitating PSA.

Growth of PSA/MC-38 Tumors. The potential of the PSA/MC-38 cells to serve as an in vivo model for syngeneic tumors expressing human PSA was evaluated by growth in C57BL/6 mice. It was not known if the human PSA would be highly immunogenic in mice, causing C57BL/6 mice to reject syngeneic tumor cells bearing this antigen. To address these issues and to establish the growth characteristics of PSA/MC-38 in vivo, $2 \times 10^5$ PSA/MC-38 and pLNSX/MC-38 cells were each injected s.c. into two separate groups of 20 C57BL/6 mice. All mice were evaluated for the time of onset of tumor formation, tumor volume, and the rate of tumor growth.

The control pLNSX/MC-38 tumors were first detected in 8 of 20 C57BL/6 mice 10 days following inoculation (Fig. 6B). In contrast, PSA/MC-38 tumors first appeared in 5 of 20 mice 14 days following injection (Fig. 6A). Twenty of 20 C57BL/6 mice injected with control pLNSX/MC-38 cells had measurable tumors by day 21, and 19 of 20 had PSA/MC-38 tumors by day 24 (Fig. 6, A and B). At each time point, the average pLNSX/MC-38 tumor volume was larger than that of PSA/MC-38 tumors.

In an attempt to determine whether the different growth rates of the PSA-transfected and vector-transduced tumors described above might be due to host factors of the immunologically intact C57BL/6 mice, PSA/MC-38 and pLNSX/MC-38 cells were also transplanted in immune-deficient athymic (nu/nu) mice at $2 \times 10^2$ cells s.c. Seven days following transplantation of pLNSX/MC-38 cells, tumors were detected in 18 of 20 athymic mice (Fig. 6D). PSA/MC-38 tumors were...
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Fig. 6. Tumor growth studies of PSA/MC-38 and pLNSX/MC-38 in C57BL/6 and athymic nude (nu/nu) mice. Female C57BL/6 (A and B) and athymic nude (C and D) mice were inoculated s.c. with $2 \times 10^5$ PSA/MC-38 cells (A and C) or pLNSX/MC-38 cells (B and D). Tumors were measured (volume = width$^2 \times$ length/2) twice weekly.

first detected in 7 of 20 athymic mice 10 days following inoculation (Fig. 6C). Therefore, onset of PSA/MC-38 tumors is later than onset of pLNSX/MC-38 tumors in athymic mice as well as in C57BL/6 mice.

In athymic mice, pLNSX/MC-38 tumors were larger at each time point than PSA/MC-38 tumors, as was the case in C57BL/6 mice. For example, the average pLNSX/MC-38 tumor volume on day 14 postinjection was 1648 mm$^3$, compared to 386 mm$^3$ for PSA/MC-38 tumors, a 4-fold difference.

PSA Levels in PSA/MC-38 Tumors. To confirm the expression of PSA in vivo, protein extracts of PSA/MC-38 tumors were prepared as described in “Materials and Methods” and tested for PSA content using the Tandem-R PSA assay. PSA/MC-38 tumor extracts had low levels of PSA (less than 1.0 ng PSA/mg protein), similar to the level of PSA produced by PSA/MC-38 cells grown in vitro (data not shown). Background PSA levels (less than 0.1 ng PSA/mg protein) were observed for pLNSX/MC-38 tumor extracts.

PSA Levels in Serum of Mice with PSA/MC-38 Tumors. The in vivo production of PSA was quantitated in C57BL/6 mice bearing PSA/MC-38 and pLNSX/MC-38 tumors and was compared to the tumor volume producing the PSA. Tumor volumes were determined on day 21 following s.c. injection of $1 \times 10^5$ cells into C57BL/6 mice; serum was collected from the same mice on day 22. PSA was detected only in the serum of mice bearing PSA/MC-38 tumors (0.5 to 10.2 ng PSA/ml serum). It should be noted that 2 of 10 mice did not have detectable PSA/MC-38 tumors on day 21 but did develop small tumors 7 days later. Therefore, the low serum PSA concentrations in these mice on day 21 probably reflected their small tumor burdens. There was a moderate degree of correlation ($r = 0.76$) between the serum PSA concentrations and PSA/MC-38 tumor volumes (Fig. 7).

Anti-PSA Antibody in Mouse Sera. To determine whether C57BL/6 mice developed a humoral response against recombinant human PSA, serum was collected from mice bearing PSA/MC-38 or pLNSX/MC-38 tumors and tested by ELISA as described in “Materials and Methods.” C57BL/6 mice were injected s.c. with $1 \times 10^6$ PSA/MC-38 or pLNSX/MC-38 tumor cells. Serum samples were collected prior to injection and 21 days following injection. Anti-PSA antibodies were present in the serum of three of four PSA/MC-38 tumor-bearing mice at 21 days (Table 2). Two mice had detectable
anti-PSA serum antibodies with an end point dilution of 1:2,700, and one mouse had detectable serum antibody at an end point dilution of 1:218,700. No anti-PSA antibodies were detected in serum of mice bearing pLNSX/MC-38 tumors.

**DISCUSSION**

Three members of the human kallikrein gene family have been identified previously, including PSA, hGK-1, and human tissue (pancreatic, renal, or salivary gland) kallikrein (31-34). Each of these genes has been sequenced, and each has five exons and four introns (32, 35, 36). According to the nucleotide sequences, there is 82% similarity between genomic human PSA and hGK-1 DNA, whereas the identity between PSA and human tissue kallikrein DNA is approximately 74% (37, 38). In the present studies, PSA and hGK-1 probes have been shown by Southern blot analysis to hybridize with three fragments of HindIII restriction endonuclease-digested human DNA. Based on these hybridization results, it appears that the PSA gene is located on the 8-kb fragment and that the hGK-1 gene is located on the 11-kb fragment. The related tissue kallikrein gene is likely located on the barely detectable 20-kb fragment, as shown in previous studies (33).

The human PSA gene was shown to be related to nucleotide sequences in other animals by hybridization of the human PSA cDNA probe to genomic DNA from other mammalian species, demonstrating that nonhuman primates have the genetic information to encode human PSA-related proteins. PSA hybridization in all the nonhuman primate DNA samples closely matched that of human DNA. It is interesting to note that the rhesus monkey PSA cDNA has recently been cloned and sequenced (39). Based on the cDNA sequence, the recombinant rhesus monkey PSA protein is predicted to share an 89% amino acid identity with its human counterpart. Our results support this finding, as evidenced by the strong immunohistochemical staining of human and rhesus monkey prostatic epithelium with anti-human PSA antibody. Furthermore, Aumuller et al. (40) have also reported staining of human and rhesus monkey prostate tissues with anti-human PSA antibodies. On the basis of these studies, the monkey may serve as a model for immune reactions to endogenous PSA, including tolerance and autoantigenicity. The monkey system may also prove particularly useful to study the potential for anti-PSA immune responses in individuals who have been prostatectomized. The subsequent application for such studies would involve patients with prostate cancer. Following prostatectomy, PSA would only be present in metastatic tissues; therefore, anti-PSA therapies would be specific for neoplasm in such patients.

Although the murine genome does not have the gene for PSA, as described above, the murine system can be used in conjunction with the primate system for studying human PSA as a target for specific therapeutic approaches. To our knowledge, PSA/MC-38 is the first murine cell line to express a human prostatic tumor marker. As part of a matched pair of cell lines, with expression of PSA as the only variable, PSA/MC-38 could also potentially be useful in the study of PSA interactions with α-1-antichymotrypsin (41), α-2-macroglobulin (42), protein C inhibitor (43), and semenogelins I and II (44) or in characterizing other previously unidentified biological activities for PSA. By eliminating the genetic elements that normally control expression of PSA in human cells, expression of recombinant PSA in PSA/MC-38 is independent of hormonal requirements. This is supported by the fact that, in our studies, levels of recombinant human PSA produced in tissue culture and in male and female mice are similar. Furthermore, the level of PSA produced by PSA/MC-38 appears to be stable both in vitro and in vivo.

Prior to using PSA/MC-38 cells in animal studies, experiments were conducted to evaluate the recombinant protein secreted by these cells. PSA was immunoprecipitated from PSA/MC-38 and LNCaP tissue culture medium and then analyzed on a Western blot. The proteins detected by this method were approximately M, 30,000, compared to the M, 33,000 protein described by Wang et al. (2). The LNCaP PSA gene encodes a 261-amino-acid prepeptide, of which 17 amino acids form a hydrophobic leader sequence, followed by 7 amino acids that are part of the zymogen (propeptide) form of the molecule. These short peptides are removed by cleavage, leaving 237 amino acids in the mature protein (31). PSA/MC-38 also encodes the complete 261-amino-acid prepeptide. However, PSA produced by the recombinant PSA cell line appears to be of a slightly higher molecular weight than that of LNCaP. There are two possible explanations for this phenomenon. The PSA molecule synthesized by MC-38 cells may not be completely processed to the mature protein, leaving some of the 24 amino terminal residues attached to the recombinant PSA molecule. For example, if the PSA molecule produced by PSA/MC-38 was only processed to the zymogen form, leaving an extra 7 amino acids attached to the amino end of the molecule, this zymogen form would be 876 daltons larger than the mature protein produced by LNCaP. This small increase in size is detectable by gel electrophoresis. A second possible explanation for the slight size variation between the two PSA molecules is that PSA may be differentially glycosylated by murine MC-38 and human LNCaP cell lines.

PSA production by PSA/MC-38 tumor cells was quantitated both in vitro and in vivo. When grown in tissue culture for 5 days, the amount of PSA in the tissue culture medium increased with cell number, up to 2.8 ng PSA/10^6 cells. In vivo, serum was collected from PSA/MC-38 tumor-bearing mice, and PSA concentrations were generally higher in serum from C57BL/6 mice with larger PSA/MC-38 tumor volumes. These results are similar to those reported for human LNCaP cells grown in athymic mice of BALB/c origin (45). There was a high degree of correlation between serum PSA concentration and LNCaP tumor volume in the BALB/c mice, indicating that the PSA concentration was dependent on cell number, as it appears to be for the PSA/MC-38 cells. However, the amount of serum PSA secreted by LNCaP cells was reported to be 672 ng/ml in a mouse with a tumor volume of 2855 mm^3 (45), in striking contrast to the 10 ng/ml serum PSA in a mouse with a 5244 mm^3 PSA/MC-38 tumor. In an attempt to quantitate cell-associated PSA, protein extracts from the PSA/MC-38 cell line and tumors were assayed by immunoradiometric analysis and found to have less than 1 ng PSA/mg cellular protein (data not shown).

The growth of PSA/MC-38 and pLNSX/MC-38 cells in syngeneic C57BL/6 and athymic (nu/nu) mice were evaluated. Although the PSA/MC-38 cell line grows at the same rate as the control pLNSX/MC-38 cell line in tissue culture, it is apparent that there are differences in the growth of these cells in vivo. Three conclusions can be drawn from these experiments: (a) PSA/MC-38 tumors are established later, grow slower, and are generally smaller than control pLNSX/MC-38 tumors; (b) both PSA/MC-38 and pLNSX/MC-38 tumors grow faster and larger in athymic mice than in C57BL/6 mice;
(and c) growth of the pLNSX/MC-38 tumors is relatively uniform in C57BL/6 and athymic mice compared to the growth of PSA/MC-38 tumors. One possible explanation for the in vivo growth differences is that the human PSA is acting as an immunogenic foreign antigen, stimulating an anti-PSA immune response that slows the growth of PSA/MC-38 cells in both C57BL/6 and athymic mice. Based on the delayed appearance of PSA/MC-38 tumors in C57BL/6 mice (at day 14), this response is apparently stronger in the immunologically competent C57BL/6 strain.

The results reported here confirm that it is possible to induce production of anti-human PSA antibodies in C57BL/6 mice. Anti-PSA antibodies have also been detected in the serum of “normal” women and prostate cancer patients, indicating that humans can respond to PSA in a like manner (46–48).

In conclusion, using hybridization analysis of genomic DNA from several mammalian species, it has been demonstrated that PSA-related genes exist in nonhuman primates and those species that might be suitable for future in vivo experiments involving PSA have been identified. Also described is the development of a matched pair of murine cell lines, differing only by expression of PSA, which may be useful in determining the immunological and biological properties of PSA. These cell lines may also have potential use as targets for anti-PSA therapies in an experimental rodent model.

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The Presence of Prostate-specific Antigen-related Genes in Primates and the Expression of Recombinant Human Prostate-specific Antigen in a Transfected Murine Cell Line

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