

Detection and Cloning of a Protein Recognized by Anti-human Prostate-specific Antigen (PSA) Antibody in the Rat Ventral Prostate

Mizuki Onozawa,^{1,2} Kazunori Fukuda,^{1,3} Masahiko Watanabe,¹ Mikinobu Ohtani,² Hideyuki Akaza,² Takashi Sugimura¹ and Keiji Wakabayashi^{1,4}

¹Cancer Prevention Division, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045 and ²Department of Urology, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Tennodai, Tsukuba City, Ibaraki 305-8575 and ³University of Gifu, 40 Tsukasa-cho, Gifu City, Gifu 500-8705

Prostate-specific antigen (PSA), a 33 kDa glycoprotein produced in the epithelium of the human prostate, has become established as a useful tumor marker for prostate cancer in man. Since reports of homologous proteins in animals other than primates have been lacking, the present investigation was carried out to identify any PSA-like protein in rats. Immunoblot analysis using a specific monoclonal anti-human PSA antibody detected a 32 kDa immunoreactive protein in the ventral lobe of the rat prostate, but not in other lobes or in other tissues. Positive immunostaining was observed only for the luminal surface of the glandular epithelium and the intraductal fluid in the ventral prostate. Sequence analysis of a cDNA for the rat PSA-like protein, cloned by immunoscreening of an expression cDNA library prepared from the ventral lobe, revealed identity to the rat submaxillary gland S3 kallikrein. Human PSA also belongs to the kallikrein family. Thus, this protein produced in the rat ventral prostate was suggested to be a possible counterpart of human PSA.

Key words: Prostate-specific antigen — Rat — Ventral prostate — Kallikrein

Prostate cancer is one of the leading causes of cancer death in males in Western countries. For many years, although every effort has been made toward earlier detection and treatment, the mortality rate has been steadily increasing.¹ In most cases, prostate cancers are initially very sensitive to orchiectomy or hormonal therapy with antiandrogens, estrogens and luteinizing hormone-releasing hormone (LHRH) agonists.^{2–4} However, treatment failures for hormonal therapy generally occur, and progression is usually drastic thereafter.⁵ Unfortunately, no well established treatments for hormone-refractory prostate cancer are yet available.⁶ Therefore, more emphasis has recently been focused on prevention of prostate carcinogenesis.⁷

Several reports concerning modulation or chemoprevention of prostate carcinogenesis using rat models have been published.^{8–11} For example, finasteride (a 5- α reductase inhibitor, which inhibits the conversion of testosterone to dihydrotestosterone) and casodex (an anti-androgen agent) have been demonstrated as useful chemopreventive agents in a rat prostate carcinogenesis model induced by the treatment with 3,2'-dimethyl-4-aminobiphenyl and testosterone propionate.⁹ For efficient evaluation of the effects of chemopreventive agents, development of a monitoring system of prostate carcinogenesis using a specific serum marker in experimental animals will be very useful.

Prostate-specific antigen (PSA), a 33 kDa glycoprotein produced predominantly in the epithelium of the human prostate,¹² belongs to the human kallikrein family, and has a chymotrypsin-like serine protease activity which is responsible for the liquefaction of clots formed immediately after ejaculation.^{13,14} Because of increased serum concentrations in prostate cancer patients, PSA has been widely used as a tumor marker to detect early-stage cancer, to monitor disease progress, and to evaluate therapeutic response in humans.^{15,16} However, few investigations have been carried out for identification of PSA-like proteins in animals other than primates.^{17,18}

It is feasible that rodents also possess a PSA-like protein, and the present study was aimed at any rat counterpart. Immunological methods using a monoclonal antibody specific to the human PSA indeed allowed an immunoreactive protein to be detected in the ventral lobe of the rat prostate gland. The protein was found to be identical to the rat submaxillary gland S3 kallikrein, on cDNA cloning analysis.

MATERIALS AND METHODS

Preparation of monoclonal antibodies against human PSA Monoclonal antibodies specific to human PSA were prepared by fusion of FO-1 myeloma cells with lymphocytes of inguinal lymphnodes of mice immunized with human PSA. Immunization and cell fusion were carried out according to the procedures of Orlik *et al.*¹⁹ with

⁴To whom correspondence should be addressed.
E-mail: kwakabay@gan2.ncc.go.jp

slight modifications. In brief, 6-week-old male BALB/c mice were immunized with 2 μ g of human PSA (Chemicon International, Inc., Temecula, CA) with adjuvant (RIBI Adjuvant System; RIBI ImmunoChem Research, Inc., Hamilton, MT) into a foot pad on the 1st and the 4th days. On the 16th day, inoculation was repeated without adjuvant. Three days after the final immunization, lymphocytes from the inguinal lymph nodes were fused with murine myeloma FO-1 cells at a ratio of 1:2 using polyethylene glycol 1500 (Boehringer Mannheim Biochemica, Mannheim, Germany). The cells were cultured in RPMI1640 medium (Gibco BRL, Rockville, MD) containing 15% fetal bovine serum, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol, hybridoma fusion and cloning supplement (Boehringer Mannheim Biochemica) and antibiotics. Hybridoma cells were selected with medium containing 1 \times HAT supplement (Sigma, St. Louis, MO) and screened by analyzing antibody activity against human PSA in culture media. The cell lysate of the human prostatic cancer cell line LNCaP (American Type Culture Collection, Rockville, MD), which produces PSA, was used as an antigen source for immunoblotting. Hybridoma cells producing monoclonal antibodies specifically detecting the 33 kDa protein corresponding to PSA were selected and cloned. To obtain anti-PSA antibody-containing ascites, 1 \times 10⁶ cloned hybridoma cells were intraperitoneally injected into BALB/c mice. IgG fractions in the ascites were purified using an AmpureTM PA Kit (Amersham Pharmacia Biotech, Little Chalfont, UK).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot analysis Protein lysates from tissue samples, which were obtained from 4-month-old male F344 rats, or culture cells of LNCaP were separated on 12% SDS-PAGE gels and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore Corp., Bedford, MA). After blocking with 2% nonfat skim milk in TTBS (20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 0.05% Tween 20), the membranes were incubated with the monoclonal antibody developed in this study or anti-human PSA rabbit polyclonal antibody (DAKO, Glostrup, Denmark) in TTBS containing 1% bovine serum albumin, and washed with TTBS. Then they were incubated with horseradish peroxidase-conjugated anti-mouse (Immunotech, Marseille, France) or anti-rabbit (Amersham Pharmacia Biotech) antibodies, washed and developed with an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech).

Immunohistochemical examination Tissues from 4-month-old male F344 rats were fixed in 4% paraformaldehyde, and embedded in paraffin. Examined tissues were the ventral prostate, dorsolateral prostate, anterior prostate, seminal vesicle, testis, liver, kidney, pancreas, spleen, colon, adrenal gland, lung, heart, cerebrum and cerebel-

lum. Endogenous peroxidase and nonspecific protein binding were blocked with 0.3% hydrogen peroxide in methanol and with horse serum, respectively. Specimens were then incubated with the monoclonal or the anti-human PSA polyclonal antibodies, followed by biotinylated anti-mouse or anti-rabbit antibodies, respectively (Vector Laboratories, Inc., Burlingame, CA), and then avidin-biotin reagents (Vectastain Elite ABC Kit; Vector Laboratories, Inc.). Positive staining was visualized with 3,3'-diaminobenzidine. Nuclear counter-staining was achieved using hematoxylin.

Construction and screening of a cDNA library Total RNA was extracted from the ventral prostate of a 4-month-old male F344 rat using ISOGEN RNA isolation reagent (Nippon Gene, Tokyo). The poly(A)⁺ mRNA fraction was recovered using a poly-A SpinTM mRNA Isolation Kit (New England Biolabs, Beverly, MA). For cDNA preparation, a Universal "RiboClone" cDNA Synthesis System (Promega, Madison, WI) was employed following the manufacturer's instructions. Synthesized cDNA was ligated to dephosphorylated λ gt11 arms and packaged using a "Gigapack" III Gold Packaging Extract (Stratagene, La Jolla, CA).

The expression cDNA library was then screened by an immunoenzymatic procedure. The rabbit polyclonal antibody specific to human PSA was used for immunoscreening to improve sensitivity. Phages were adsorbed on *Escherichia coli* strain Y1090^{r-}, plated on LB medium (Gibco BRL) hardened with 1.5% agar and grown at 42°C for 4 h. Plates were then overlaid with a nitrocellulose membrane (NitroPure; Micron Separations, Inc., Westboro, MA) pre-soaked with 10 mM isopropyl- β -D-thiogalactopyranoside (IPTG) (Nippon Gene), and β -galactosidase fusion proteins expressed from cDNA inserts were transferred onto the membrane and screened with the anti-human PSA polyclonal antibody. Plaques producing positive signals were collected and subjected to successive rounds of antibody screening at lower plaque densities. The immunoscreening was repeated until all plaques produced a positive signal. Recombinant phage DNA was prepared from the phage plaques with positive signals, and then subjected to PCR amplification of the insert cDNA fragment using λ gt11 sequencing primers (Promega). Nucleotide sequences of the PCR products were determined by cycle sequencing using the "fmol" DNA Sequencing System (Promega).

RESULTS

Detection of a PSA-like protein in rat ventral prostate by immunoblotting About 800 hybridoma clones were produced by cell fusion of mouse myeloma FO-1 cells with 4.4 \times 10⁸ lymphocytes obtained from 8 immunized mice. Among them, 5 produced antibodies that specifically

detected the 33 kDa PSA in LNCaP cell lysate by immunoblot analysis.

These monoclonal antibodies were further screened for cross-reactivity. One hybridoma clone, named PS-121AA, was shown to produce a monoclonal antibody that reacts with the 33 kDa human PSA and a 32 kDa protein in the rat ventral prostate with high affinity (Fig. 1). The reactivity of the monoclonal antibody was reduced by preincubation with the human PSA (data not shown). However, no immunoreactive bands in the 32 kDa region were detected in the other lobes of the prostate or in other tissues. The lower band that appeared in the lane of the ventral prostate was considered to be a portion of degraded S3 kallikrein because the intact S3 kallikrein is known to be split into two bands with lower molecular weight.²⁰⁾ Although another faint band was observed in the liver, its molecular weight differed from that of PSA (Fig. 1). Rabbit polyclonal anti-human PSA antibody also reacted with the 32 kDa protein in the rat ventral, but not the other lobes of the prostate (data not shown).

Immunohistochemical analysis Immunostaining was performed for samples of various rat tissues using the anti-human PSA polyclonal antibody to ascertain the cellular localization of immunoreactive forms. The antibody stained the luminal surface of epithelium cells and the intraductal fluid in the ventral prostate (Fig. 2, A and B). Cytoplasmic and nuclear staining were not observed. No

significant immunostaining was observed in the other lobes of the prostate (Fig. 2, C, D, and E), or in other 11 tissues, except some renal tubules (Fig. 2F). Although staining intensity was low, immunostaining of the tissues using the monoclonal antibody from clone PS-121AA also gave similar results (data not shown).

cDNA cloning of the 32 kDa protein in the rat ventral prostate We then tried to identify the 32 kDa immunoreactive protein expressed predominantly in the ventral prostate. Fourteen positive clones were identified on screening of 1×10^5 independent recombinant phage clones with the polyclonal antibodies specific to human PSA. Most clones were clearly shown to be immunoreactive with the monoclonal antibody from PS-121AA. Insert cDNA fragments from the positive clones were isolated using PCR amplification and the nucleotide sequences were determined. Database analysis revealed the sequences of all positive clones to be identical to parts of that of rat submaxillary gland S3 kallikrein.²¹⁾ Among the 14 positive clones, the longest one contained the entire coding region of the cDNA, from nucleotide positions 66–859, that appeared in Genbank (accession no. M11566).

DISCUSSION

It has been reported that PSA is not expressed in animals such as rats and mice.^{17, 18)} In the present study, however, immunoblot analysis of rat tissues with an anti-human PSA monoclonal antibody revealed that the rat ventral prostate specifically produces a protein which can be detected as a strong immunoreactive band with an estimated molecular weight of 32 kDa, very similar to the 33 kDa human PSA protein. Although another faint immunoreactive band was observed in the liver, the difference in its molecular weight suggested cross-reactivity or non-specific binding. Thus, the findings point to the 32 kDa protein being a possible rat counterpart of human PSA.

Immunohistochemical examination using polyclonal and monoclonal antibodies also demonstrated a tissue distribution consistent with the results of immunoblot analysis. Thus, with the exception of the kidney tubules, positive immunostaining was limited to the luminal surface of epithelial cells and intraductal fluid in the ventral prostate. In the human prostate gland, PSA positivity is seen diffusely in the cytoplasm of epithelial cells. The reason for this difference in staining pattern is not clear at present.

The rat prostate gland, which consists of several lobes, differs markedly in its anatomical structure from that of man, and anatomical and functional relationships remain controversial. Our finding that the 32 kDa PSA-like protein, a possible rat counterpart of human PSA, is localized only in the ventral lobe of the rat prostate may provide a clue in this respect. Some of the renal tubules were also stained with anti-human PSA antibody, whereas no band

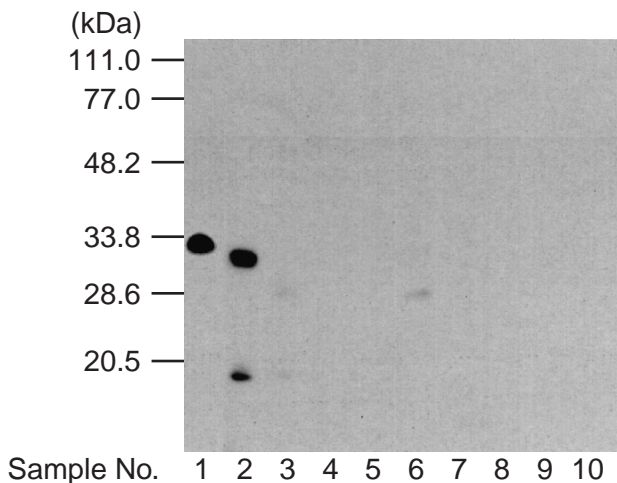


Fig. 1. Immunoblot analysis of the tissue distribution of PSA-like protein in rat. Protein samples ($2.5 \mu\text{g}$) from rat tissues and human PSA ($0.1 \mu\text{g}$) were subjected to immunoblotting using the mouse anti-human PSA monoclonal antibody developed for the present study. A strong immunoreactive band with a molecular weight of 32 kDa was observed in the ventral prostate. Lanes: 1, human PSA; 2, ventral prostate; 3, dorsolateral prostate; 4, anterior prostate; 5, seminal vesicle; 6, liver; 7, kidney; 8, pancreas; 9, lung; 10, brain.

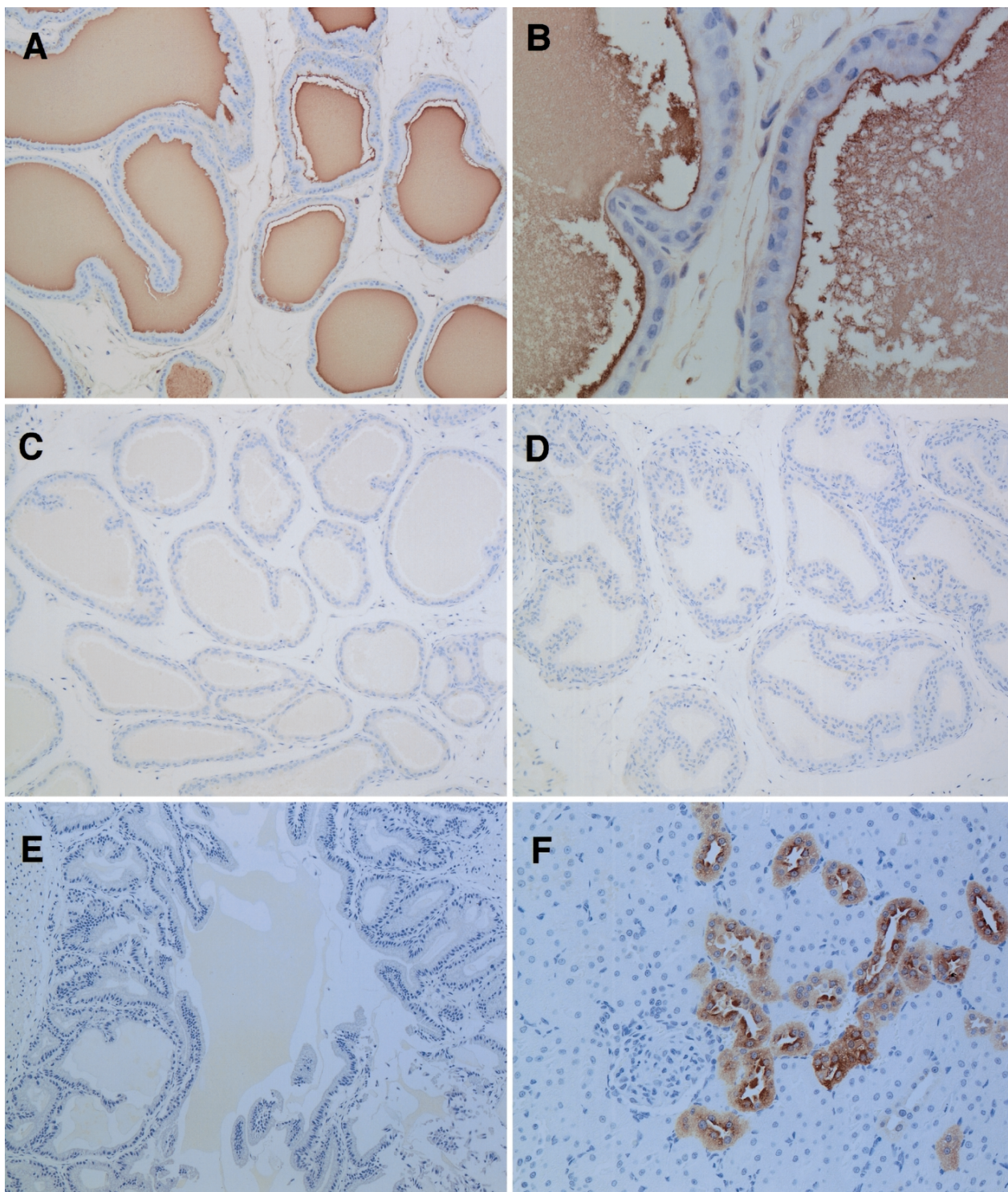


Fig. 2. Immunohistochemical staining of PSA-like protein in rat tissues, with anti-human PSA polyclonal antibody. Positive staining was apparent in the luminal surface and the intraductal fluid of the rat ventral prostate, and in the epithelium of some renal tubules. (A) Ventral prostate ($\times 100$). (B) Ventral prostate ($\times 400$). (C) Dorsolateral prostate ($\times 100$). (D) Anterior prostate ($\times 100$). (E) Seminal vesicle ($\times 100$). (F) Kidney ($\times 200$).

PSA	1	MWVPVFLTSLVSWTWIGAAPLILSRIVGGWECEKHSQPWQVLVASRGRAVCGGVLVHPQWV	60
S3	1	..FLIL..A..LGQ..D...PGQ..V...YN..TN.....A.-I-.TTF.....ID.S..	58
		* * * * *	
PSA	61	LTAAHCI RNKSVILLGRHSLFHPEDTGQVFQVSHSFPHPLYDMSLLKNRFLRPGDSSHD	120
S3	59	I.....YSKNYRV...NN.VKD.PFA.RRL..Q..Q..D.IPVFMR.HTRQRAY.HNN.	118
		* * * * *	
PSA	121	LMLLRLSEPAELTDAVKVMDLPTQEPALGTTCYASGWGSIEPEEFLLTPKKLQCVDLHVIS	180
S3	119	...H..K..DI.GG...I...E..KV.SI.L.....MTN.S.MKLSHD...NI.LL.	178
		* * * * *	
PSA	181	NDVCAQVHPQKVTKFMLCAGRWTGGKSTCSGSGGPLVCNGVLQGITSWGSEPCALPERP	240
S3	179	.EK.IETYNIE.DVT...EMD...D..T.....I.D.....L..G.AT...K.KT.	238
		* * * * *	
PSA	241	SLYTKVVHYRKWIKDTIVANP	261
S3	239	AI.A.LIKFTS...KVMKE..	259
		* * * * *	

Fig. 3. Similarity in amino acid sequence of human PSA with that of rat S3 kallikrein. Identical amino acids are marked by asterisks. PSA, human PSA that appeared in Genbank accession no. U17040; S3, rat S3 kallikrein that appeared in Genbank accession no. M11566. Forty-nine percent of the amino acids were identical. The nucleotide sequence homology among the coding region of these proteins was 64%.

was detected in the kidney by immunoblotting. This discrepancy could be due to the small amount of PSA-like protein relative to the total proteins in the kidney.

All of the positive cDNA clones from a cDNA library of the rat ventral prostate found here by immunoscreening with polyclonal antibody demonstrated a nucleotide sequences identical to that of rat submaxillary gland S3 kallikrein. In addition, we found that recombinant S3 kallikrein expressed from the clones reacted with monoclonal antibody produced from PS-121AA on western blotting (data not shown). Western blot analysis and immunostaining also demonstrated that the monoclonal antibody was highly reactive with rat submaxillary gland (data not shown). A cDNA for this protein was first cloned using rat pancreatic kallikrein cDNA as a probe by Ashley *et al.*²¹⁾ They investigated the mRNA expression in various tissues and reported the submaxillary gland S3 kallikrein to be also expressed in the whole prostate in rats,²²⁾ in line with our findings. Moreover, Clements *et al.*²³⁾ showed that castration results in a decrease of immunoreactive prostate kallikrein and that testosterone replacement restores the enzyme levels.

The common characteristics of rat submaxillary gland S3 kallikrein and human PSA include similar isoelectric points and molecular weights,²⁰⁾ as well as androgen-dependent expression. Their nucleotide and amino acid sequence homology is 64% and 49%, respectively (Fig. 3). High-level expression of S3 kallikrein in rat prostate²⁰⁾ might explain the fact of the isolation of all 14 positive cDNA clones as the kallikrein. The expression level of

human PSA in the human prostate is considered to be high,¹²⁾ similar to S3 kallikrein in rat prostate. Human PSA was formerly believed to be expressed exclusively in the prostate,¹²⁾ but several recent reports have demonstrated its presence in other tissues.²⁴⁻²⁷⁾ We have analyzed the expression of PSA in the human salivary gland by reverse transcription-PCR (RT-PCR) and sequencing to exclude the possibility that another member of the human kallikrein family is cross-reactive with anti-human PSA antibody. The results of sequencing of PCR products provided a positive identification (data not shown). While this requires further elucidation, it appears very likely that the 32 kDa rat submaxillary gland S3 kallikrein detected in the rat ventral prostate is the rat counterpart of human PSA, and could be a candidate tumor marker in the prostate carcinogenesis of rats. Using a rat prostate carcinogenesis model, analyses of the S3 kallikrein levels in the serum are under way in our laboratory.

ACKNOWLEDGMENTS

This study was supported by a grant from the Organization for Pharmaceutical Safety and Research (OPSR) of Japan, and by a Grant-in-Aid for the 2nd Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan. M. Onozawa was the recipient of a Research Resident Fellowship from the Foundation of Cancer Research.

(Received February 27, 2001/Revised May 30, 2001/Accepted June 4, 2001)

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