Prostate-Specific Antigen Expression by Various Tumors

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There is a growing body of evidence indicating that prostate-specific antigen (PSA) may be present in many steroid hormone-stimulated epithelial tissues other than that of the prostate. In particular, breast tumor cell lines treated with steroid hormone receptor agonists, breast tumors, and normal human breast have recently been found by our group to contain PSA. To investigate whether PSA may also be present in other human tumors, we employed a highly sensitive immunofluorometric assay technique to quantify PSA immunoreactivity in tumor extracts. Using a PSA-positivity cutoff value of 0.005 ng per mg of protein, 23 of 43 diverse tumors tested positive for PSA protein. Confirmatory analyses for PSA by a commercially available method (IMx) on six samples demonstrated a high degree of concordance between the two methods. To establish the molecular weight of the immunoreactive species, the most highly positive tumor extracts of each tumor type were fractionated by high performance liquid chromatography. Whereas the majority of tumors had immuoreactivity eluting at both 100 KDa and 33 KDa, corresponding to PSA bound to α1-antichymotrypsin and free PSA, respectively, the colon and parotid tumors displayed immunoreactivity only at the 33 KDa fraction. We conclude that in addition to breast tumors and normal breast, colon, ovarian, liver, kidney, adrenal, and parotid tumors can also produce PSA. The physiological role of PSA in these tumors is currently under investigation. © 1995 Wiley-Liss, Inc.

Key words: prostate-specific antigen, cancer, growth factors, serine proteases, prognostic factors, tumor markers

INTRODUCTION

Prostate-specific antigen (PSA) is a 33 KDa serine protease present at high levels in sperm. It was originally thought that this protein is secreted exclusively by the epithelial cells of the prostate gland (1,2). However, more recently it has been demonstrated that PSA could also be found in the periurethral and perianal glands (3–5) and very rarely in tumors of the salivary glands (6). We found that PSA is present in 30% of female breast tumors and that its production in these tumors is associated with the presence of steroid hormone receptors (7,8). We observed a significant advantage in both disease-free and overall survival of breast cancer patients whose tumors are PSA-positive (unpub. data). In addition, we were able to reproduce the phenomenon of PSA production by breast tumors using breast cancer cell lines stimulated by various steroid hormones (9). Additionally, we demonstrated that normal breast can also produce PSA and secrete it into the milk during lactation, postpregnancy (10).

In this report we have examined if tumors other than those of the breast also have the ability to produce PSA. We studied 43 tumors, including 7 breast (as positive controls), 5 ovarian, 9 liver, 5 kidney, 2 adrenal, 7 colon, 1 melanoma, 2 esoph-ageal, 3 parotid, 1 thyroid, and 1 lymphoma. For PSA analysis we have used a highly sensitive and specific procedure described in detail elsewhere (11). Our data suggest that PSA could be produced at relatively low levels by several types of tumors.

The current belief that PSA is produced exclusively by normal or cancerous prostatic epithelial cells must be modified in view of our new and previous findings, which demonstrate that PSA is a relatively ubiquitous biochemical marker. PSA is an enzyme belonging to the serine protease family, but its role, if any, in cancer initiation, progression, or regression is still unknown. We and others have recently speculated that PSA is a molecule involved in growth factor and cytokine regulation. The prognostic significance of PSA presence in a subset of tumors of various organs remains to be determined.

MATERIALS AND METHODS

Tumor Specimens and Extraction Procedure

The primary tumors used in this study were collected at the Toronto Hospital (Toronto, Canada). Diagnosis was established in all cases with histologic examination by the Department of Pathology. The tumor tissue was immediately stored in liquid nitrogen after surgical resection, transported to the laboratory, and subsequently stored at $-80°C$ until extraction was performed (up to 8 months).

Approximately 0.2 g of tissue from each tumor was pulverized to a fine powder at $-80°C$, and the cells were lysed for 30 min on ice with 1mL of lysis buffer (50 mmol/L Tris buffer, pH8.0, containing 150 mmol/L NaCl, 5 mmol/L EDTA, 10 g/L Nonidet NP-40 surfactant and 1 mmol/L phenylmethysulfonyl fluoride). The lysates were centrifuged at 15,000g at $4°C$ for 30 min, after which the supernatants were immediately assayed for PSA protein.

Protein concentration of the tumor extracts was determined by the bicinchoninic acid method commercially available by Pierce Chemical Co. (Rockford, IL). The PSA content of each tumor was expressed as ng of PSA per mg of total protein to compensate for the amount of tissue extracted.

PSA Measurements

PSA in the tumor extracts was analyzed by a highly sensitive and specific immunofluorometric procedure described in detail elsewhere (11). Briefly, the PSA assay uses a mouse monoclonal anti-PSA capture antibody coated to polystyrene microtiter wells, a biotinylated polyclonal rabbit detection antibody, and alkaline phosphatase-labeled streptavidin (SA-ALP). In the assay, 50 µL of sample is first incubated with the coating antibody in the presence of 50 µL of assay buffer containing protein, salts, and surfactant (11). After 3 h incubation and washing × 6, the biotinylated polyclonal anti-PSA antibody is added and incubated for 1 h. After washing × 6, the SA-ALP conjugate is added for 15 min, followed by another washing × 6. The activity of ALP is then measured by adding the substrate 5-fluorosalicylphosphate, incubating for 10 min, and then by adding an EDTA-Tb^{3+} solution to form a ternary fluorescent complex between the released 5-fluorosalicylate, Tb^{3+}, and EDTA. The fluorescence is measured in the time-resolved fluorometric mode. Selected tumor extracts were also analyzed by the IMx automated PSA assay (Abbott Laboratories, Abbott Park, IL).

High Performance Liquid Chromatography (HPLC)

High performance liquid chromatography was performed with a Shimadzu system (Shimadzu Corp., Kyoto, Japan), isocratically, using a mobile phase of 0.1 mol/L Na_2SO_4 and 0.1 mol/L NaH_2PO_4, pH 6.80. Flow rate was 0.5 mL/min. The column used was a Bio-Sil SEC-250, 600 mm × 75 mm (BioRad Labs, Richmond, CA). The column was calibrated with a molecular weight standard solution from BioRad. Fractions of 0.5 mL each were collected after injecting 50–300 µL of the sample and analyzed with the PSA assay. In order to achieve the maximum possible PSA assay sensitivity during the analysis of the HPLC fractions, we used 100 µL fraction volumes and 100 µL assay buffer instead of 50 µL used in the regular assay. All other steps of the PSA assay remained the same.

Because of the very low levels of PSA present in some HPLC fractions, all graphs presented in this report were plotted as fluorescence, in arbitrary units, versus the fraction number. A fluorescence value of 6,000 units is equivalent to ~0.025 µg/L of PSA. Typical CVs for PSA concentrations in the range of 0.01 µg/L or higher were ≤5%. All measurements were performed in duplicate.

RESULTS

Our ultrasensitive PSA assay has an analytical detection limit of 0.002 µg/L and a biological detection limit of 0.01 µg/L (11). In our previous studies with breast tumor cytosols, we had arbitrarily selected as a cutoff level a PSA concentration of 0.05 µg/L in order to categorize tumors as being PSA-positive or PSA-negative (7). As the mean total protein concentration of breast tumor cytosols was 1.64 mg/mL, the equivalent cutoff point, expressed as ng of PSA per mg of total protein, was 0.030 ng/mg (8). However, our PSA assay is capable of accurately detecting much less than 0.05 µg/L of PSA. In this study, we classified tumors as negative if the PSA content in the tumor extracts was below 0.005 ng/mg of total protein. All other tumors were classified according to their PSA content using four cutoff levels of PSA, i.e., 0.005, 0.010, 0.020, or 0.030 ng/mg. The obtained results with the 43 tumors studied are shown in Table 1. Only 4/7 breast tumors and 1/7 colon tumors contain PSA ≥ 0.030 ng/mg, the cutoff point used in our previous clinical studies (8). PSA ≥ 0.020 ng/mg was present in 5/7 breast tumors, 1/7 colon tumors, and 1/3 parotid tumors. Many more tumors had PSA content ≥ 0.010 ng/mg, i.e., 5/7 breast, 3/9 liver, 2/5 kidney, 2/2 adrenal, 2/7 colon, and 1/3 parotid. At the level of PSA of 0.005 ng/mg, 23/43 tumors were classified as positive for PSA protein (Table 1). From all tumors studied, 20 had a PSA content of <0.005 ng/mg.

None of the commerically available PSA assays has the sensitivity of our immunofluorometric procedure. However, a comparison with a few PSA-positive tumor extracts revealed agreement between our assay and the IMx assay (12). Concentrations of PSA by our assay of 0.51 (breast), 1.81 (breast), 0.28 (colon), 5.5 (breast), and 0.18 (breast) µg/L were measured as 0.6, 1.5, 0.3, 5.2, and 0.2 µg/L by the IMx assay. The PSA concentration of the other tumor extracts was too low for the IMx assay to give a meaningful result.

To further strengthen our proposal that various tumors have

TABLE 1. PSA Content of Various Tumors[a]

| Tumor type | Number of tumors | PSA, ng/mg of total protein | | | | |
		≥0.005	≥0.010	≥0.020	≥0.030	Maximum content
Breast	7 (F)	7	5	5	4	0.77 (F)
Ovarian	5 (F)	1	0	0	0	0.005 (F)
Liver	9 (5 M, 4 F)	5 (4 M, 1 F)	3 (M)	0	0	0.014 (M)
Kidney	5 (3 M, 2 F)	3 (2 M, 1 F)	2 (1 M, 1 F)	0	0	0.019 (M)
Adrenal	2 (F)	2	2	0	0	0.015 (F)
Colon	7 (4 M, 3 F)	3 (1 M, 2 F)	2 (1 M, 1 F)	1 (M)	1	0.035 (M)
Parotid	3 (M)	3	1	1	0	0.020 (M)
Melanoma	1 (F)	0	0	0	0	—
Esophagus	2 (1 M, 1 F)	0	0	0	0	—
Thyroid	1 (F)	0	0	0	0	—
Lymphoma	1 (F)	0	0	0	0	—
Total	43 (16 M, 27 F)	24 (10 M, 14 F)	15 (6 M, 9 F)	7 (9 M, 5 F)	5 (1 M, 4 F)	—

[a](M) or (F) indicates tumors from either male (M) or female (F) patients.

the ability to produce PSA, we have established the molecular weight of the immunoreactive species measured by our ultrasensitive assay, using HPLC as previously described (11). For this experiment, the most highly positive tumor extract from each tumor type, i.e., breast, liver, kidney, adrenal, colon, ovarian, and parotid were examined. The results are shown in Figures 1 and 2.

HPLC analysis of the most highly PSA-positive tumor extract, derived from a female breast cancer patient, has shown that PSA immunoreactivity elutes as two distinct peaks at fractions 31±1 (molecular weight ~100 KDa) and 40±1 (molecular weight ~33 KDa). These fractions had been previously shown to represent PSA bound to α_1-antichymotrypsin (PSA-ACT) and free PSA, respectively (7,9–11). In accordance with our previous report (7), the vast majority of PSA in the breast tumors is present in its 33 KDa form; the bound fraction represents about 10% or less (Fig. 1A).

The ovarian tumor extract from a female patient (Fig. 1B) is probably associated with PSA immunoreactivity with a molecular weight of ~100 KDa (this is an ambiguous peak with very low peak area) and 33 KDa (a weak but unambiguous peak eluting at fraction 40-41). The peak heights of this chomatogram are very weak due to the relatively very low concentration of PSA in this extract (0.005 ng/mg, Table 1). The liver tumor extract from a male patient (Fig. 1C) is associated with two peaks with molecular weights of ~100 KDa and ~50–60 KDa. The second peak, eluting at fraction 37 was reproduced repeatedly and presumably represents another form of PSA different from the 33 KDa free PSA. The kidney tumor extract from a male patient (Fig. 2A) was associated with PSA immunoreactivity with molecular weights of ~100 KDa (major peak at fraction 31-32) and 33 KDa (minor peak at fraction 40-41). The adrenal tumor extract from a female patient (Fig. 2B) was associated with a major peak at ~100 KDa and no detectable peak corresponding to free PSA. The colon tumor extract from a male patient (Fig. 2C) was associ-

ated with PSA immunoreactivity with a molecular weight of 33 KDa only. Similar results were obtained with the parotid tumor from a male patient (Fig. 2D).

An esophageal tumor extract from a male patient that contained <0.005 ng of PSA per mg of total protein was also subjected to high performance liquid chomatography as a negative control. No peaks associated with PSA immunoreactivity were detected corresponding to either 100 or 33 KDa (data not shown).

In all chomatograms but that of Figure 1A, a peak around fractions 21–24 was also seen, corresponding to molecular weights of 400–700 KDa. We do not know the nature of this peak. However, in previous studies with prostate cancer sera, we have seen a similar peak in the same chromatographic area and speculated that it may represent PSA bound to α_2-macroglobulin (11). This PSA complex is weakly recognized by our assay.

DISCUSSION

Prostate-specific antigen was, until recently, considered a very specific biochemical marker of normal and cancerous prostatic tissue (1,2). More recently, PSA was localized in other tissues using immunohistochemical techniques. In both females and males, it is now known that PSA is present in the periurethral and perianal glands, structures that have many similarities with prostatic tissue (3–5). PSA presence in nonprostatic tumors has also been reported, but only as a very rare event (6, 13).

We have recently reported PSA presence in 30% of female breast tumors (7) and have demonstrated that such an expression is mediated by steroid hormone receptors (8). We have also shown that PSA is produced by metastatic breast tumors (14) and by the normal breast during pregnancy. PSA enters the milk and serum of lactating women (10). It appears that the presence of PSA in breast
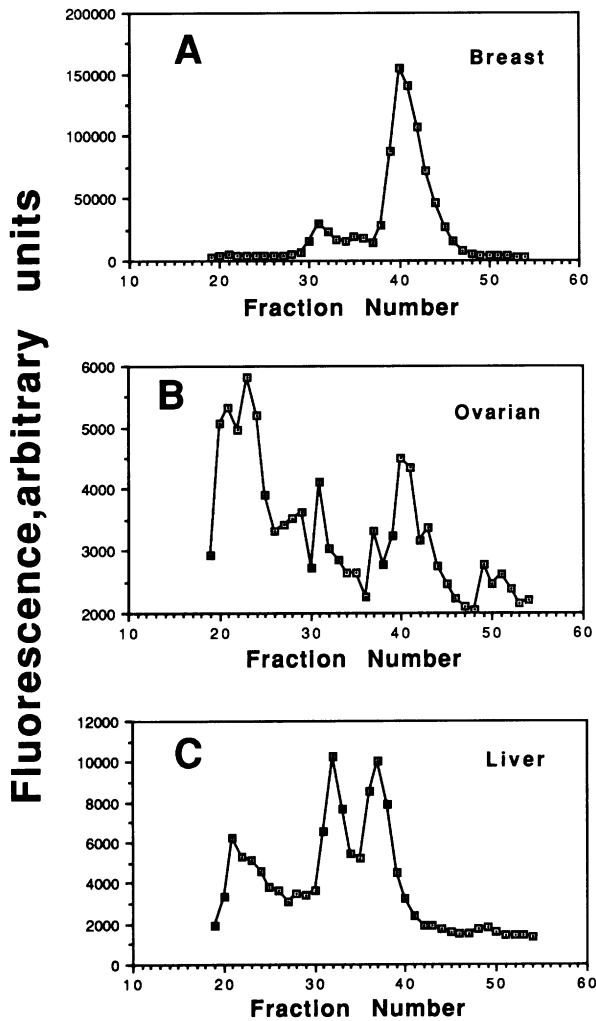
Fig. 1. High performance liquid chromatography (HPLC) of extracts of breast (**A**), ovarian (**B**), and liver (**C**) tumors. After injecting 300 μL of tumor extract, fractions were collected and analyzed for PSA. The PSA-α_1-antichymotrypsin complex elutes at fractions 31±1 and free PSA at fractions 40±1 (data established with male serum and purified seminal PSA, but not shown). The HPLC column was calibrated with a molecular weight standard from BioRad Labs eluting at fraction 21 (660 KDa), 29 (160 KDa), 38 (44 KDa), 43 (17 KDa), and 52 (1.4 KDa). Reproducibility was at ±1 fraction in all cases. Flow rate was 0.5mL/min. For more discussion, see text.

tumors is associated with improved patient disease-free and overall survival (in prep.).

In this report, we have examined if tumors other than those of breast and prostate tissue could also produce PSA. These studies were carried out with use of an extremely sensitive time-resolved immunofluorometric assay for PSA that we have recently developed and evaluated (11). We used HPLC to establish the molecular weight of the immunoreactive species measured by the PSA assay. From all tumors studied, breast tumors contain the highest amounts of PSA in accordance with our previous studies (7,8). Among all other tu-
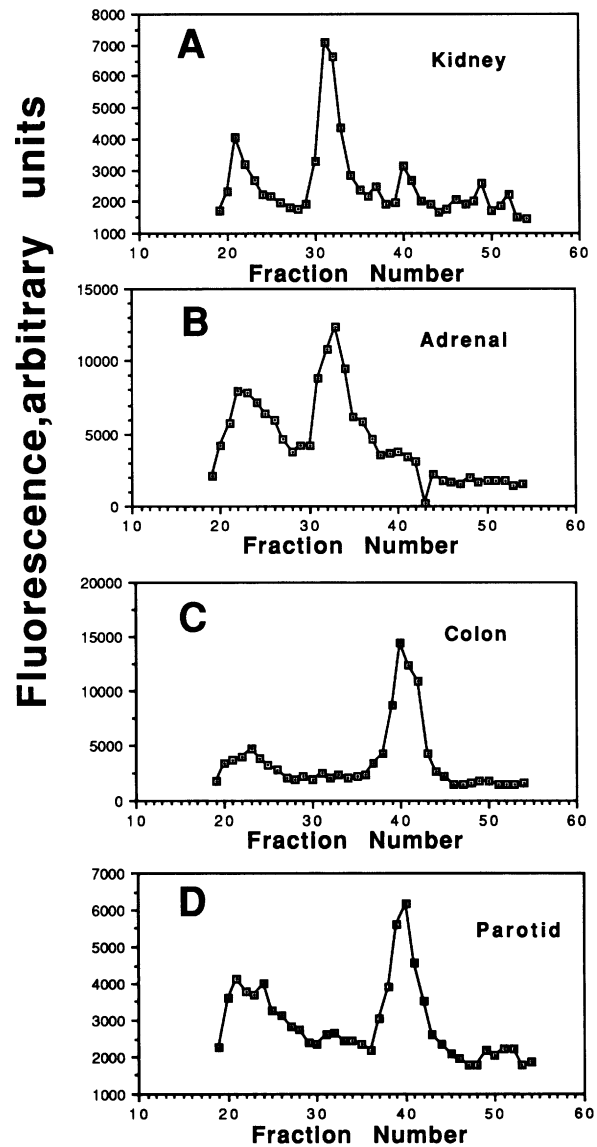

Fig. 2. HPLC as in Figure 1, but for kidney (**A**), adrenal (**B**), colon (**C**), and parotid (**D**), tumor extracts.

mors, one colon tumor had relatively high levels of PSA (0.035 ng/mg) followed by a parotid tumor (0.020 ng/mg). The frequency of PSA-positive tumors increases significantly at the PSA cutoff level of 0.010 ng/mg (Table 1). The overall positivity at a cutoff level of 0.005 ng/mg, considered the limit of detection of our assay, is 53% (Table 1).

In order to obtain further evidence that the species monitored with the immunofluorometric assay is indeed PSA, we have established the molecular weight of the immunoreactive compounds in many different tumor extracts using a gel filtration column and HPLC (Figs. 1 and 2). PSA-negative tumor extracts (PSA<0.005ng/mg) did not reveal any PSA-positive immunoreactive HPLC fractions. Extracts positive for PSA at the level of 0.005 ng/mg or higher revealed peaks

corresponding to molecular weights of 100 KDa (PSA-ACT, PSA-$\alpha_1$antichymotrypsin complex) and 33 KDa (Free PSA). Breast, ovarian, colon, and parotid tumors contained mostly free PSA. Kidney and adrenal tumors contained mostly PSA-ACT complexes. A liver tumor was found to contain ACT-PSA (100 KDa) and another immunoreactive species with a molecular weight of 50–60 KDa (Fig. 1C). We believe that this peak may represent PSA bound to a protein present in liver cells, which is different from α_1-antichymotrypsin.

Molecular analysis of PSA mRNA was not performed in this study. However, when this analysis was conducted for PSA mRNA in breast tumors, using reverse transcription-polymerase chain reaction analysis and nucleic acid sequencing, we found 100% homology between breast tumor mRNA and mRNA obtained from prostatic tissue (in prep.). These data unequivocally prove that breast PSA is identical to PSA produced by prostatic epithelial cells.

The reason for the observation that some tumors produce free PSA and others PSA bound to α_1-antichymotrypsin is currently unknown. One explanation may be that some tumors, in addition to producing PSA, may also have the ability to produce α_1-antichymotrypsin.

Contamination of the tumor extracts with serum PSA present in tumor vasculature is unlikely for the following reasons. First, many of the PSA-positive tumors were derived from women whose serum had no or very little PSA (15). Second, in many cases, PSA in the tumor extracts was present mainly in its 33 KDa form; the major form of serum PSA is the PSA-ACT complex with a molecular weight of ~100 KDa (11).

The mechanism of PSA expression in the tumors reported here is not clear. However, we believe that PSA might be expressed through the action of steroid hormone receptors as we have shown to be the case for breast tumors (7–10). Breast, ovarian, adrenal, parotid, liver, colon, and many other tissues are known to possess steroid hormone receptors and to be responsive to steroid hormones (6,16–23).

Subsequent to our reports on breast tumors, Clements and Mukhtar (24) recently reported PSA presence in normal endometrium. Our extensive data previously published (7–10, 14) and those of Clements and Mukhtar (24) strongly suggest that tissues that are steroid hormone receptor-positive have the ability to produce PSA if appropriately stimulated by steroid hormones. Our group has additionally found PSA in amniotic fluid, normal breast, and in breast cyst fluid (in prep.).

The physiological role of PSA production by the breast and other tumors and tissues and during pregnancy is currently unknown. However, recent data on prostatic tissue offers clues that PSA may be involved in growth regulation of mammary and other tissues. The sequence of PSA shows extensive homology with γ-nerve growth factor (56%), epidermal growth factor binding protein (53%), and α-nerve growth factor (51%) (25). PSA can hydrolyze insulin A and B chains and recombinant interleukin-2 (25). In addition, PSA can enzymatically digest insulin growth factor I (IGF-1). This activity is thought to regulate IGF-1 concentration through its release from insulin growth factor binding protein 3 (IGFBP-3) (26). Other findings further support the hypothesis that PSA is a regulator of IGFBP-2 and IGFBP-3 in patients with prostate cancer (27). Killian et al. (28) have recently found that PSA has mitogenic activity presumably due to activation by PSA of latent transforming growth factor-β (TGF-β) and by modulation of cell adhesion. Others have shown that PSA binds and inactivates protein C inhibitor (29). Our findings of PSA presence in breast, colon, ovarian, parotid, kidney, and liver tumors, stimulated normal breast, amniotic fluid, and breast milk, and data presented by others for normal endometrium (24) suggest that PSA can no longer be regarded as a specific prostatic marker and as a physiological molecule associated only with semen liquefaction. Instead, PSA should be regarded as a molecule that could be produced by cells bearing steroid hormone receptors under conditions of steroid hormone stimulation. Given the new evidence that PSA may be a candidate growth factor or growth factor regulator, the biological role of PSA in normal tissues, tumors, and during pregnancy may be much more complex than thought and raises numerous questions that will be answered only by further investigation.

ACKNOWLEDGMENTS

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