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Advances in Brief

Molecular Characterization of Prostate-specific Antigen Messenger RNA Expressed in Breast Tumors[1]

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Abstract

Prostate-specific antigen (PSA) is considered a highly specific biochemical marker of the prostate gland and is currently used for prostate cancer diagnosis and monitoring of patients with prostate adenocarcinoma. We recently demonstrated, however, that about 30% of female breast tumors produce a M_r 33,000 protein that has striking similarities to seminal PSA. In this study we characterized the presence of PSA in 6 breast tumors and in the testosterone-stimulated T47D breast cancer cell line at the mRNA level. Using reverse transcriptase-polymerase chain reaction and DNA sequencing techniques we identified PSA mRNA in immunoreactive PSA-positive breast tumors but not in immunoreactive PSA-negative breast tumors. The sequence of the generated polymerase chain reaction products was identical to the sequence of the PSA complementary DNA derived from prostate tissue. The data presented here support the notion that breast tumors produce a M_r 33,000 protein which is identical to PSA produced by the prostate gland. Our study suggests that the presence of PSA in breast tumors may be used as a new additional biochemical marker for breast cancer prognosis, for the spreading of hematogenous micrometastases, and/or for response to adjuvant treatment.

Introduction

PSA[3] is a M_r 33,000 glycoprotein produced almost exclusively by the epithelial cells of the prostate and is currently used as a marker for diagnosis and monitoring of prostate cancer (1–3). PSA detection in tumors of nonprostatic origin has been reported as a rare event, in a few instances, using immunohistochemical techniques (4).

We have recently reported that PSA immunoreactivity can be detected in about 30% of breast tumors and that breast cancer cells in culture can produce IRPSA after stimulation by steroid hormones (5–7). This immunoreactive PSA was shown to be associated with the presence of steroid hormone receptors, early disease stage, and younger patient age (5). Although the results from previous studies suggested that IRPSA in breast cancer cells seems identical to the seminal PSA, a final proof has not yet been provided. In this study we report the molecular characterization of the PSA detected in breast tumors and in the testosterone-stimulated T47D breast cancer cell line. Six breast tumors were tested for PSA immunoreactivity and then analyzed for PSA gene expression by reverse transcriptase-PCR and DNA sequence was determined on the PCR fragments. DNA sequence analysis demonstrated that PSA mRNA derived from breast tumors is identical to the PSA from prostate tissue.

The PSA production by 30% of breast tumors underscores the similarities between breast and prostate tissues and holds promise of being used in breast cancer for prognosis, in selection of therapy, or for devising new therapeutic interventions.

Materials and Methods

Tissue Specimens. Six breast cancer specimens were obtained from women undergoing surgery for primary breast cancer. The breast tumor tissue was stored in liquid nitrogen immediately after surgical resection, transported to the laboratory, and subsequently stored at −70°C until protein and RNA extraction was performed. Frozen surgical specimens from various other tumors were obtained from the Toronto Hospital, Toronto, Ontario, Canada (3 ovarian and 2 lung carcinomas), from the Hospital of Padova, Padua, Italy (1 gastric and 1 colon carcinomas), and from the Jefferson Hospital, Philadelphia, PA (1 melanoma). All tumors were primary lesions except for one ovarian carcinoma, which was metastatic from breast cancer, and one lung carcinoma, which was metastatic from prostate cancer. One normal breast tissue specimen was obtained from a woman undergoing breast cosmetic surgery, and peripheral blood leukocytes obtained from a healthy volunteer were prepared from 10 ml venous blood.

Tissue Culture and Stimulation with Steroids. The steroid hormone receptor-positive breast carcinoma cell line T47D was obtained from the American Type Culture Collection, Rockville, MD. The cell line was grown in flasks at 37°C and 5% CO_2 in RPMI 1640 supplemented with 0.2 IU of bovine insulin/ml, 29 g/liter glutamine, and 10% FCS. When the cells were grown to 90% confluency they were washed with isotonic saline and maintained in the medium as above but without the presence of FCS. Stimulation experiments with steroids were performed by adding in the culture medium various steroids in alcohol at final concentrations of 10^{-7} M. The final concentration of alcohol was always <0.1% of the total volume. Control experiments with alcohol alone were performed in parallel. In this work we used T47D cells stimulated with either solvent or testosterone.

T47D cells stimulated with testosterone produce PSA which is secreted in the tissue culture supernatant and which is measurable by the immunofluorometric procedure. After 4 days of stimulation the cells were detached by trypsin-EDTA treatment and were kept frozen at −70°C until RNA extraction was performed.

Preparation of Tumor Cytosolic Extracts. Approximately 0.5 g of tumor tissue was weighed out, fragmented with a hammer if necessary, and pulverized in a Thermovac tissue pulverizer at liquid nitrogen temperature. The resulting powder was transferred into 50-ml plastic tubes along with 10 ml of extraction buffer [10 mmol/liter Tris-HCl (pH 7.40), 1.5 mmol/liter EDTA, 5 mmol/liter sodium molybdate]. The suspended tissue powder was solubilized on ice with a single 5-s burst of a Polytron homogenizer. The particulate material was pelleted by centrifugation at 105,000 × *g* for 1 h. The intermediate layer (cytosol extract) was collected without disturbing the lipid or particulate layers. The cytosols were then assayed for PSA with the immunofluorometric procedure.

PSA Assay in Tumor Cytosols and Tissue Culture Supernatants. PSA in the cytosolic breast extracts and tissue culture supernatants was measured with a highly sensitive and specific immunofluorometric technique described in detail elsewhere (7). 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. In the assay 50 ml of sample are first incubated with the coating antibody in the presence of 50 ml of assay buffer. After 3 h incubation and 6

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[3] The abbreviations used are: PSA, prostate-specific antigen; IGFBP, insulin growth factor-binding protein; IRPSA, immunoreactive PSA; cDNA, complementary DNA; PCR, polymerase chain reaction.

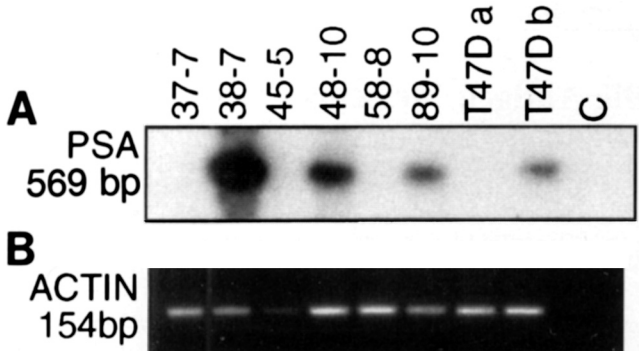

Fig. 1. *A*, PSA reverse transcriptase-PCR from breast tumor samples detected by Southern blotting and hybridization of the filter with a PSA cDNA probe. A PSA hybridization band of 569 base pairs (*bp*) is detected in breast cancer samples 38-7, 48-10, and 89-10 (*Lanes 2, 4, 6*) and in the testosterone-stimulated T47D cells; it is absent in samples 37-7, 47-5, and 58-8 (*Lanes 1, 3, 5*) and in the alcohol-stimulated T47D cells. *B*, ethidium bromide-stained agarose gel of β-actin reverse transcriptase-PCR products, an internal control (*C*) for the integrity of the RNA in all samples. *Lanes* as in *A*.

washings, the biotinylated polyclonal anti-PSA antibody is added and incubated for 1 h. After 6 washings, the alkaline phosphatase-labeled streptavidin conjugate is added for 15 min, followed by another 6 washings. The activity of alkaline phosphatase is then measured by adding the substrate 5-fluorosalicyl phosphate, incubating for 10 min, and then adding a Tb^{3+}-EDTA 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.

RNA Extraction. Total RNA extraction was performed using the RNAzol B method (Tel-Test Inc., Friendswood, TX) following the instructions of the manufacturer.

cDNA Synthesis. One μg of total RNA was used for the synthesis of the first strand of cDNA using the SuperScript II reverse transcriptase (GIBCO-BRL). Briefly, RNA, oligo(dT)$_{12-18}$ primers (100 ng), and random hexamers primers (100 ng) were first denatured for 5 min at 70°C, chilled on ice for 1 min, and then incubated for 1 h at 37°C in 20 ml of a reaction mixture containing 1 × first-strand buffer, 250 mM/liter deoxynucleoside triphosphate mix (Boehringer Mannheim), 10 mM DTT, and 200 units of SuperScript II reverse transcriptase.

Oligonucleotide Primers. Oligonucleotides were synthesized by the Jefferson Cancer Institute Nucleic Acid Facility (Philadelphia, PA) and used without further purification. The PSA oligonucleotide sequences used as primers were: PR5, 5′AGCCCCAAGCTTACCACCT3′; PR6, 5′CACAATC-CGAGACAGGAT3′; and PR2 and PR3 sequences as published previously (8). Previously published (9) actin PCR primer sequences were used to rule out degraded RNA. Amplification yielded a 571-base pair PSA cDNA fragment with PR5 (from exon 1) and PR2 (from exon 4) oligonucleotide primers and a 154-base pair cDNA fragment with the actin oligonucleotide primers.

PCR Procedure and Southern Blot Analysis. One μl of cDNA was added to 39 μl of PCR mix containing 1 × PCR buffer (Boehringer Mannheim), 18 pM concentrations of each primer, 250 mM/liter of deoxynucleotide triphosphates, and 1.25 units of Taq DNA polymerase (Boehringer Mannheim). PCR was performed with primers PR5 and PR2 for 30 cycles according to the following program: 94°C for 1 min (2 min for the first cycle); 56°C for 1 min; and 72°C for 2 min (5 min for the last extension). Actin fragments were amplified from 1 μl of the cDNA preparation under the same conditions used for PSA PCR. Twenty μl of each PCR reaction were electrophoresed in 2% agarose gel and visualized by ethidium bromide staining.

After electrophoresis the PSA cDNA PCR products were transferred onto Hybond nylon membranes (Amersham, Amersham, United Kingdom) and hybridized with a PSA cDNA probe (kindly provided by Dr. José Moreno). Southern blotting, probe radiolabeling, hybridization, and autoradiography were performed by standard techniques (10). A second PCR amplification was performed on 1 μl of the remaining PCR reaction to provide sufficient material for subsequent analysis. Finally, the fragments were purified by ion exchange columns (Qiagen, Dusseldorf, Germany) according to the instructions of the manufacturer.

Sequencing. An automated 373A DNA sequencer (Applied Biosystems) and dye terminator kits from the same manufacturer were used for direct sequencing of the PSA cDNA fragments by the dideoxynucleotide chain termination method using fluorescent labels (11). The coding and noncoding strands of each fragment were sequenced with primers generating overlapping sequence data. Sequences were reassembled and analyzed using the sequence assembly and editing program (12).

Results

We have extensively studied six tumors from female patients with primary breast cancer. These patients were selected after screening of 100 breast tumor cytosols for the presence of PSA with a highly sensitive and specific immunofluorometric procedure (7). The three tumors with cytosols containing the highest PSA concentration (coded tumors 38-7, 48-10, and 89-10) and three tumors with cytosols containing no detectable PSA (coded tumors 37-7, 47-5, and 58-8) were selected for molecular studies. The PSA concentrations in the three positive cytosols were 6.59 mg/liter (38-7), 6.29 mg/liter (48-10), and 20.5 mg/liter (89-10). Tumors 37-7, 47-5, and 58-8 had PSA concentrations <0.005 mg/liter.

Total RNA extracted from the six breast tumors and the T47D breast carcinoma cell line (stimulated with either testosterone or alcohol solvent) was reverse transcribed to cDNA and then amplified by the PCR with primers derived from exons 1 and 4 of the PSA gene. Agarose gel electrophoresis and ethidium bromide staining of the PCR products revealed that tumors 38-7, 48-1,0, and 89-10 which are positive for PSA protein immunoreactivity and the testosterone-stimulated T47D cells produced the expected 569-base pair band. None of the tumors 37-7, 47-5, and 58-8, which are negative for PSA protein immunoreactivity or the alcohol-stimulated T47D cells, produced a PCR product. The PCR products were then transferred to a nylon

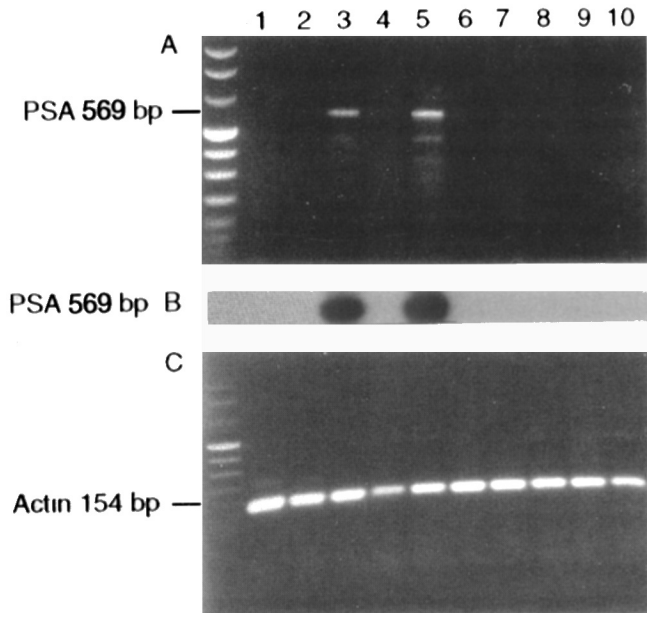

Fig. 2. PSA expression in various human solid tumors (*Lanes 1-8*) and normal tissues (*Lanes 9* and *10*). *Lanes 1* and *2*, primary ovarian carcinoma; *Lane 3*, ovarian carcinoma metastatic from breast cancer; *Lane 4*, primary lung carcinoma; *Lane 5*, lung carcinoma metastatic from prostate adenocarcinoma; *Lane 6*, gastric carcinoma; *Lane 7*, colon carcinoma; *Lane 8*, melanoma; *Lane 9*, normal breast tissue; *Lane 10*, peripheral leukocytes. *A*, ethidium bromide-stained agarose gel of the PSA-PCR products; *B*, Southern blot hybridization with a PSA cDNA probe of the gel shown in *A*; *C*, ethidium bromide-stained agarose gel of β-actin reverse transcriptase-PCR products, used as internal control. *bp*, base pair.

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  1  AGCCCCAAGC TTACCACCTG CACCCGGAGA GCTGTGTCAC CATGTGGGTC
 51  CCGGTTGTCT TCCTCACCCT GTCCGTGACG TGGATTGGTG CTGCACCCCT
101  CATCCTGTCT CGGATTGTGG GAGGCTGGGA GTGCGAGAAG CATTCCCAAC
151  CCTGGCAGGT GCTTGTGGCC TCTCGTGGCA GGGCAGTCTG CGGCGGTGTT
201  CTGGTGCACC CCCAGTGGGT CCTCACAGCT GCCCACTGCA TCAGGAACAA
251  AAGCGTGATC TTGCTGGGTC GGCACAGCCT GTTTCATCCT GAAGACACAG
301  GCCAGGTATT TCAGGTCAGC CACAGCTTCC CACACCCGCT CTACGATATG
351  AGCCTCCTGA AGAATCGATT CCTCAGGCCA GGTGATGACT CCAGCCACGA
401  CCTCATGCTG CTCCGCCTGT CAGAGCCTGC CGAGCTCACG GATGCTGTGA
451  AGGTCATGGA CCTGCCCACC CAGGAGCCAG CACTGGGGAC CACCTGCTAC
501  GCCTCAGGCT GGGGCAGCAT TGAACCAGAG GAGTTCTTGA CCCCAAAGAA
551  ACTTCAGTGT GTGGACCTC
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Fig. 3. PSA cDNA sequence obtained from breast cancer. Total RNA, isolated from a breast cancer specimen (tumor 38-7), was reverse transcribed to cDNA and 569 base pairs of the PSA cDNA were amplified by PCR, purified, and directly sequenced.

membrane and hybridized with a radiolabeled PSA cDNA probe. The results are shown in Fig. 1. The probe hybridized to PCR products derived from tumors 38-7, 48-10, and 89-10, and from the testosterone-stimulated T47D cells. No hybridization signal was detected in tumors 37-7, 47-5, and 58-8 or in the alcohol-stimulated T47D cells.

PSA production at a very low level has been demonstrated in various human tumors and tissues other than breast cancer by the immunofluorometric procedure (13). We used the reverse transcriptase-PCR and Southern blot hybridization techniques to analyze different human solid tumors and normal tissues for PSA gene expression. Among 7 different tissues examined, we were able to detect the PSA mRNA in 2 tumors, 1 ovarian and 1 lung carcinoma secondary to breast and prostate cancers, respectively (Fig. 2). None of the primary tumors derived from different tissues (ovary, lung, stomach, colon, and skin) or normal breast tissue or peripheral leukocytes showed positivity for PSA mRNA.

To further examine the nature of the 569-base pair PCR fragments, the PCR products obtained from breast cancer were purified and the DNA sequence was determined. Both strands of each PCR product were sequenced. A representative example of the obtained DNA sequence is shown in Fig. 3. When we compared the sequence of the PCR products with the published sequence of PSA cDNA derived from prostatic tissue, we obtained 100% homology. No mutations were identified in the 569-base pair fragments that were sequenced.

Discussion

PSA is a M_r 33,000 protein with serine protease enzymatic activity found at very high concentrations in the sperm. It has been suggested that PSA is involved in semen liquefaction postejaculation. PSA is considered a highly specific biochemical marker of the prostate gland and is currently used for diagnosis, prognosis, and management of patients with prostate cancer (1–3). Presence of PSA in tumors of nonprostatic origin has been demostrated but it appears that this is a very rare event. Recently, Van Krieken (4) reported the presence of PSA in some salivary gland neoplasms.

We have recently demonstrated, using an ultrasensitive immunofluorometric procedure for PSA quantification, that approximately 30% of female breast tumors produce a M_r 33,000 protein that has striking similarities to seminal plasma PSA (5, 6). This IRPSA was shown to be associated with the presence of steroid hormone receptors, early disease stage, and younger patient age. Preliminary data suggest that IRPSA may be a new favorable prognostic indicator in breast cancer.[4]

PSA immunoreactivity has been detected in various tumors including ovarian, colon, lung, and parotid tumors. However, PSA expression in these tumors is infrequent and occurs at very low levels, much lower than in breast tumors (13). In agreement with this observation, we were not able to detect the PSA mRNA in primary tumors other than breast cancer.

IRPSA is present in breast tumors at relatively low concentrations and we have not as yet purified enough IRPSA protein sufficient for sequencing. To establish if IRPSA is indeed identical to the PSA produced by the prostate gland, it was important to characterize the presence of IRPSA at the mRNA level. The data presented here have shown that only tumors which are positive for the IRPSA protein contain PSA mRNA. Furthermore, sequencing of the entire 569-base pair PCR product, spanning exons 1–4 of the PSA cDNA, has shown that this mRNA species in the breast tumors has 100% sequence identity to PSA cDNA derived from prostate tissue. Furthermore, we were able to produce PSA *in vitro* by stimulating breast carcinoma cell lines with steroid hormones (6). We here also demonstrate that the mRNA isolated from the stimulated cells has an identical sequence to the PSA cDNA derived from prostatic tissue. We have further found that IRPSA could also be produced by normal breast tissue after stimulation by progestin-containing oral contraceptives (14), that it is secreted into the milk of lactating women, and that it is present in amniotic fluid (15).

The data presented here support the notion that breast tumors produce a M_r 33,000 protein which is identical to PSA produced by

[4] H. Yu, M. Giai, E. P. Diamandis, D. Katsaros, D. J. A. Sutherland, M. A. Levesque, R. Roagna, R. Pouzone, and P. Sismondi, Prostate-specific antigen is a new favorable prognostic indicator for women with breast cancer, submitted for publication.

the prostate gland, and these data provide for the first time a biochemical link between the two most common cancers in males (prostate) and females (breast).

The mechanism of *PSA* gene expression in breast cancer is unknown. We have provided evidence that this gene is under the control of the steroid hormone receptors and that glucocorticoids, progestins, and androgens, but not estrogens, could derepress the gene in cells bearing the appropriate receptors (6). We have speculated that adrenal or ovarian steroids or tumor-derived molecules derepress the *PSA* gene through the steroid hormone receptors or that the *PSA* gene is constitutively overexpressed due to post-steroid hormone receptor complex defects. Alternatively, the gene may be expressed due to loss of physiological repressors or to defects in the hormone response elements. Recently, Zou *et al.* (16) identified a protein, maspin, which is a serine protease inhibitor and a candidate tumor suppressor gene. Maspin expression is lost in many breast tumors. However, no studies have been conducted to show whether there is any relation between loss of maspin expression and PSA, a serine protease, in breast cancer. The physiological role of PSA in breast tissue and tumors is currently obscure. However, recent evidence offers some clues. PSA is an active serine protease involved in the process of semen liquefaction (17). The sequence of the PSA protein has shown extensive homology with kallikrein (57%), γ nerve growth factor (56%), tonin (54%), epidermal growth factor-binding protein (53%), and α nerve growth factor (51%) (18). Recently, it was shown that the PSA can enzymatically digest IGFBP-3 and may thus increase the availability of insulin growth factor I, an important peptide which is thought to be involved in normal and malignant cellular proliferation (19). Other data presented by Kanety *et al.* (20) further support the hypothesis that the PSA is a regulator of IGFBP-2 and IGFBP-3 concentrations in patients with prostate cancer. Killian *et al.* (21) have recently reported that the PSA, at a relatively low concentration, could cause proliferation of cultured osteoblasts; they also found that the mitogenic activity was due to activation by PSA of latent transforming growth factor β. They also demonstrated that PSA could proteolytically modulate cell surface receptors with temporary contact inhibition. These data, in combination with our findings of PSA in normal breast, milk, and amniotic fluid, suggest that PSA could be produced by nonprostatic tissue and that it may be involved in growth control by regulating growth factors and growth factor-binding proteins.

In conclusion we demonstrate that PSA, a serine protease thought to be produced only by prostatic epithelial cells, could also be produced by breast tumors and breast cancer cell lines after steroid hormone stimulation. Our finding of the PSA in breast tumors may have potential application for breast cancer prognosis, selection of therapy, and design of new treatments. Further investigations are needed to examine the biological role of this protein in cancer initiation and progression.

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