Immunoreactivity, Ultrastructural Localization, and Transcript Expression of Prostate-specific Antigen in Human Neuroblastoma Cell Lines

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Prostate-specific antigen (PSA) is considered a highly specific biochemical marker of the human prostate gland, and it currently is used for prostate cancer diagnosis and monitoring. Recently, PSA production and secretion were found in nondiseased and diseased cells, tissues, and fluids from women. In this study, we characterized the presence of PSA in two human neuroblastoma cell lines with biochemical, ultrastructural, and molecular approaches. Using reverse transcription-PCR, we identified PSA mRNA, and Western blotting revealed a substantial amount of complexed form of PSA protein, which is localized mainly in free ribosomes. Although the role of PSA in human neuroblastoma cell lines is still unknown, our study supports the hypothesis that this serine protease may be involved in controlling the growth of human brain tumor cells, adding more support to the notion that PSA is a widespread kallikrein-like protease with biological functions much more complex than recently thought.

Several authors have studied PSA immunoreactivity in nondiseased and pathological cerebrospinal fluid (CSF), but the data reported are discordant. In fact, Mencel et al. (12), Morote et al. (13), and Schaller et al. (14) found substantially higher PSA concentrations in the CSF of patients with prostatic carcinoma and metastases to the skull or meningeal membranes than in nondiseased CSF. On the other hand, Wolff et al. (15) also detected high PSA immunoreactivity in the nondiseased CSF of male patients, whereas Melegos et al. (16) found high PSA positivity in only ~7% of the CSF samples from subjects with various neurological disorders, without sex differences and with PSA positivity depending on patient age; the authors hypothesized that PSA may be produced by brain tissue (16). Recently, we presented a preliminary report on PSA expression in neuroectodermal tumor-derived cell lines (SK-N-BE-2 and SK-N-MC), which typically are composed of heterogeneous cellular subpopulations including neuroblastic and nonneuronal epithelial-like cells (17), and indicated that these brain tumor cell lines can produce and secrete this kallikrein-like serine protease (18).

To detail the presence of PSA in these human neuroectodermal tumor-derived cell lines, we undertook the present study on the biochemical characterization, ultra-
structural immunocytochemical localization, and molecular detection of a PSA transcript in SK-N-BE-2 cells, derived from a bone marrow biopsy specimen (19), as well as in SK-N-MC cells, derived from a metastatic tumor mass (20).

Materials and Methods

Cell Lines and Controls
SK-N-MC and SK-N-BE-2 neuroblastoma cell lines, all mycoplasma-free, were cultured and maintained in a 5% CO₂ humidified incubator at 37 °C according to conditions reported previously (18). Briefly, both BE-2 and MC neuroblastoma cell lines were cultured and routinely maintained in Eagle’s minimum essential medium supplemented with nonessential amino acids, 150 mL/L fetal bovine serum, and antibiotics (10⁵ units/L penicillin and 100 mg/L streptomycin), according to the protocols described by Biedler and co-workers (19, 20). The supernatants of the prostate carcinoma cell line LNCaP were used as positive control, and blood from PSA-negative healthy control women was used as negative control.

Biochemical Determinations
After sufficient cell growth, the cell culture supernatants were removed at day 5 for PSA analysis, whereas the cell pellets, obtained after scraping, resuspension in the culture media, and centrifugation at 5000g for 15 min at 4 °C, were immediately lysed for 30 min on ice with 1 mL of lysis buffer containing 50 mmol/L Tris, pH 7.5, 150 mmol/L sodium chloride, 5 mmol/L EDTA, 10 g/L nonidet NP-40 surfactant, 10 g/L Triton X-100, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mg/L each aprotinin and leupeptin as protease inhibitors (21). The suspensions were then frozen at −80 °C, thawed at 37 °C three times, and sonicated on ice for 5 cycles at 40 W of output (Heat-System Ultrasonic Inc.); the cell debris was pelleted at 9000g for 30 min at 4 °C, and the supernatants (5 mL) were collected and tested immediately in all biochemical assays.

The total protein content was determined with the bicinchoninic method, using a commercially available kit (Pierce). Total and free PSA were measured in an automated microparticle enzyme immunoassay (22), using a monoclonal mouse anti-human PSA antibody (AxSYM®, Abbott Laboratories). The detection limits of the assays, defined as the concentrations at 2 SD above the zero calibrator, were 0.02 µg/L for total PSA and 0.01 µg/L for free PSA. To exclude the possibility of matrix artifacts, neuroblastoma cell extracts and cell culture supernatants were serially diluted in PSA-negative female serum and reanalyzed for response linearity. The analytical recovery of at least two concentrations of purified PSA (Sigma Chemical Co.) added to the cellular extracts (3.5 and 7.0 µg/L) was also tested (23).

All assays performed on the samples were carried out in triplicate in at least four independent experiments, and the results were expressed as mean ± SE.

Gel Filtration
Neuroblastoma cell extract components were separated on a 600 × 9 mm column of Sephacryl S-300 (Pharmacia Biotech) and eluted with 50 mmol/L Tris-HCl, pH 7.7, containing 9 g/L of NaCl, 7.7 mol/L NaN₃, 5 g/L bovine serum albumin, and 0.15 g/L bovine globulin (Sigma). The flow rate was 15 mL/h, and 1-mL fractions were collected, according to the previously described method (21, 24, 25). The recovery of PSA immunoreactivity in the fractions was calculated and compared with the amount of total PSA loaded onto the column, as determined by the assay. The column was roughly calibrated by measuring the absorbance of the fractions at 280 nm to identify the elution volumes of human IgG (150 kDa) and albumin (69 kDa) (25).

Western Blotting
Reagents and equipment for Western blotting were purchased from Bio-Rad Laboratories. Our protocols were followed throughout (26): briefly, samples were electrophoresed under reducing conditions on 10% minislab gels, and separated proteins were transferred to Sequi-blot PVDF membrane (Bio-Rad). After saturation for 1 h at 37 °C in blocking solution (20 g/L nonfat dry milk in Tris-buffered saline, pH 7.5, containing 0.5 mL/L Tween 20); the strips were then incubated for at least 6 h at room temperature with a 1:500 dilution (in blocking solution) of the primary anti-human PSA monoclonal mouse antibody (Dako). After several washes in buffer containing 0.5 mL/L Tween 20, the membranes were incubated for 3 h at room temperature with a 1:1500 dilution of a mixture of high- and low-molecular weight alkaline phosphatase-conjugated goat anti-mouse IgG (Vector Laboratories) in blocking solution. Antibody binding was revealed by exposure to 100 mmol/L Tris-HCl, pH 9.5, containing 4 mmol/L MgCl₂, 0.1 g/L nitroblue tetrazolium chloride, and 0.05 g/L 5-bromo-4-chloro-3-indolyl phosphate, according to the procedures detailed in the amplified alkaline phosphatase immun-blot assay kit from Bio-Rad; this method detected as little as 10 pg of membrane-bound protein. Biotinylated molecular mass markers and PSA from LNCaP prostate cancer cell line culture supernatants were used as calibrators and positive control, respectively.

Electron Microscopy
For the electron microscopic analysis, the neuroblastoma cells were fixed after removal of the culture medium, using a mixture of 40 g/L paraformaldehyde and 5 g/L glutaraldehyde diluted in 0.1 mol/L Sörensen phosphate buffer, pH 7.4. Fixation was performed for 1 h at 4 °C, after which the cells were washed in Sörensen phosphate buffer, scraped, suspended in the same buffer, and then centrifuged at 340g at 4 °C for 10 min. The supernatant was removed, and the cell pellets were embedded in 15 g/L agar-agar. After the embedded cells were washed in 0.1 mol/L phosphate-buffered saline, the free aldehydes...
were blocked with 0.5 mol/L NH₄Cl in phosphate-buffered saline at 4 °C for 45 min. After the specimens were washed in phosphate-buffered saline, they were dehydrated through graded concentrations of ethanol and embedded in LRWhite resin (MultiLab). Resin polymerization was carried out under ultraviolet light to avoid the thermal denaturation of PSA (24). Ultrathin sections were placed on nickel grids coated with a Formvar-carbon layer (MultiLab) and then processed for immunocytochemistry using a rabbit anti-human PSA antiserum (Biomeda) and a secondary gold-conjugated antibody (Jackson ImmunoRes Laboratories), as described in detail elsewhere (24). Control grids were incubated under the same conditions as the experimental samples except that the primary antibody was omitted. All specimens were observed in a Zeiss EM 902 electron microscope operated at 80 kV. To identify eventual differences in PSA expression between SK-N-MC and SK-N-BE-2 neuroblastoma cells, we quantitatively evaluated the labeling distribution on sections treated in the same immunolabeling experiment. The cytoplastic area of the cells was measured on 15 randomly selected electron micrographs (final magnification ×12,000) from each cell line, using the computerized image analysis system Image-Pro® Plus, Ver. 1.3, for Windows 95® (Media Cybernetics). For background evaluation, we examined the resin outside the cells. The gold grains present in the measured areas were counted, and the labeling density was expressed as number of grains/μm². Mean values ± SE were calculated.

RNA EXTRACTION, cDNA SYNTHESIS, AND PCR PROCEDURE
Total RNA extraction was performed using the RNAfast kit assay (Biotex), following the instructions of the manufacturer. The quality and quantity of the extracted RNA was checked by spectrophotometric measurements at 260–280 nm. Total RNA (5 μg) underwent reverse transcription (RT) for the synthesis of the first strand of cDNA, using 1 μmol/L deoxynucleoside triphosphates, 10 mmol/L dithiothreitol, and 200 U of SuperScript II reverse transcriptase (Life Technologies). The reaction was performed at 42 °C for 1 h and 5 min at 95 °C. Amplification of the cDNA (5 μL) was based on previously described procedures (9) and performed in a DNA thermal cycler (Minicycler®; Genenco). An initial denaturation step (95 °C for 2 min) was followed by 40 cycles (94 °C for 50 s, 61 °C for 50 s, and 72 °C for 90 s) and a final extension for 10 min. The PSA was amplified in 45 μL of a PCR mixture containing 2.5 U of AmpliTaq DNA polymerase (Promega), 1.5 mmol/L MgCl₂, 200 μmol/L deoxynucleotide triphosphates, and 200 nmol/L of each primer. The product (10 μL) was electrophoresed on a 2% agarose gel and visualized by ethidium bromide staining. The new PSA primer sequences, designed on the basis of sequence data obtained from the European Molecular Biology Gene Bank and used to avoid amplification of the highly homologous human glandular kallikrein gene (9), were as follows:

PSAE-S 5′-CTCTCGTGGCAGGCAGT-3′ Exon 2
PSAAE-AS 5′-CCCCCTGTCGCGGTCCGAG-3′ Exon 4

The predicted PSA primer-amplified product was 485 bp in size. A strict procedure was followed to avoid cross-contamination with PCR products. RNA was extracted in a PCR room remote from the laboratory where cDNA amplification was performed. Each step (i.e., RNA extraction and first amplification) was programmed on different days. For the cell lines and the negative-control sample, mRNA extraction was carried out one sample at a time to avoid cross-contamination between samples. In any series of reactions, contamination at the DNA level was excluded by performing PCR analysis without reverse transcriptase. A water control, containing no cDNA template, was also used to detect carryover, and cDNA isolated from a negative control was amplified in each set of PCR reactions.

STATISTICAL ANALYSIS
All statistical analyses were performed with the StatView, Ver. 4.1, package (Abacus Concepts Inc.) on a Macintosh Power PC (Apple Computer).

**Results**

**PSA BIOCHEMICAL ANALYSES**

The prostate cancer cell line LNCaP produces PSA and secretes it into the culture medium; in our laboratory, the extract from LNCaP cells showed a high total PSA content (25.68 ± 2.36 ng/10⁷ cells), of which ~95% was free.

The linearity studies revealed a good linear correlation between PSA concentration and dilution (r² = 0.98). The average analytical recovery of purified PSA added to cytosolic extracts from SK-N-MC and SK-N-BE-2 neuro-
blastoma cell lines was 97% ± 2% and 93% ± 3%, respectively. The within-run imprecision (CV) was 3.8%, and the between-run imprecision was 4.2%.

In agreement with our preliminary data (18), in the SK-N-MC cell line, we found a higher expression of total PSA (2.69 ± 0.35 ng/10⁷ cells) with respect to the cellular content of total PSA found in the SK-N-BE-2 cell line (0.18 ± 0.02 ng/10⁷ cells; \( P < 0.0001 \)); a statistically significant difference was also found for free PSA concentration (0.28 ± 0.03 ng/10⁷ cells for SK-N-MC and 0.04 ± 0.01 ng/10⁷ cells for SK-N-BE-2; \( P < 0.0001 \)).

CHROMATOGRAPHY AND WESTERN BLOTTING
In both neuroblastoma cell lines examined, the PSA immunogram of a Sephacryl S-300 column of cellular extracts revealed that the major immunoreactivities were in fractions 100–150, where the \( \alpha_2 \)-antichymotrypsin (ACT)-complexed PSA and free PSA were expected (Fig. 1) (1, 21, 25); in fact, Western blotting of SK-N-MC cellular extract confirmed that the high-molecular-mass PSA fraction (immunoreactive band of ~100 kDa) was ~70% of the total PSA vs 30% for the minor immunoreactive fraction of the free uncomplexed form (Fig. 2).

ULTRASTRUCTURAL IMMUNOLocalIZATION
The electron microscopic examination of immunolabeled sections revealed that both SK-N-MC and SK-N-BE-2 cells displayed a cytoplasmic PSA distribution localized mainly on free ribosomes, whereas the rough-surfaced endoplasmic reticula cisternae and Golgi complexes were weakly labeled, and the cell nuclei and mitochondria were devoid of gold grains (Fig. 3). In SK-N-MC cells, the signal was stronger than that found in SK-N-BE-2 cells; moreover, it appeared to be concentrated in the peripheral region of the cytoplasm as well as along the cell surface, especially where many elongated cytoplasmic protrusions occurred. Control samples showed only a negligible signal.

Quantitative evaluation of PSA labeling confirmed the above observations. In fact, the labeling density in SK-N-MC cells (2.30 ± 0.16 grains/\( \mu \)m²; \( n = 15 \)) was significantly higher than in SK-N-BE-2 cells (0.59 ± 0.04 grains/\( \mu \)m²; \( n = 15 \); \( t = 10.31; P < 0.0001 \)). Moreover, SK-N-BE-2 cells showed a signal significantly higher than that of background (0.13 ± 0.02 grains/\( \mu \)m²; \( n = 15 \); \( t = 11.31; P < 0.0001 \)).

PSA mRNA RT-PCR
The results of the PSA RT-PCR are illustrated in Fig. 4. The PSA signal for the negative controls (control without RT and water control) remained consistently undetectable.
PSA is a 33-kDa glycoprotein with chymotrypsin-like enzymatic activity that belongs to the kallikrein family of serine proteases (1, 2). Although PSA is used currently as a marker for screening, diagnosing, and monitoring prostate cancer (3), several research groups recently reported the detection of PSA in several nonprostatic tissues and fluids (4, 6) that are under steroid hormone control (5–8).

Previous reports detected PSA immunoreactivity in both nondiseased and pathological CSF collected from patients affected by various diseases (12–16). Melegos et al. (16) hypothesized that PSA could originate from brain tissue.

In a previous study, our preliminary findings showed that the PSA is also expressed in SK-N-MC and SK-N-BE-2 neuroblastoma cell line extracts as well as in cell culture media after 5 days of vitro culture (18).

To detail our previous study and to evaluate the hypothesis of the novel expression of PSA by human brain tumor cells, we performed the present study using biochemical, morphological, and molecular approaches. We found that PSA occurs in neuroblastoma cell lines mainly as a complexed protein, probably bound to the serine protease inhibitor ACT; this peculiar situation is different from any other cell line examined up to now [the other cell lines produce and secrete mostly free PSA (4)] and might be correlated with the high amount of ACT found in nondiseased and diseased brain tissue (27–29). A more detailed study, performed through additional methodological approaches (biochemical and ultrastructural colocalization of immunoreactive PSA and ACT) in the neuroblastoma SK-N-MC cell line is ongoing.

The present immunocytochemical study revealed that PSA is synthesized in the cytoplasm of neuroblastoma cells, mainly in free ribosomes. However, the presence of some labeling also on rough-surfaced endoplasmic reticula cisternae and Golgi complexes would indicate an additional, although minor, synthesis pathway. Because no secretory granules containing PSA were observed, this serine protease is probably secreted as discrete molecules, as is also suggested by the presence of significant amounts of labeling on SK-N-MC cell surfaces and cytoplasmic protrusions; on the other hand, SK-N-BE-2 cells do not show an evident peripheral signal, thereby indicating a very low secretory activity. These observations are in agreement with the biochemical data on PSA content in these neuroblastoma cell lines and in their culture media (18).

The different patterns of PSA expression in neuroblastoma cells could reflect the peculiar characteristics of these cell lines; in fact, these cells are considerably different in both morphological structures and biochemical properties (17–20, 30).

A very sensitive procedure for in vitro amplification of DNA sequences, PCR has gained widespread acceptance in many areas of molecular biology, particularly in tumor biology and clinical medicine and more recently in routine diagnostic applications (31). RT-PCR-based methods for analyzing mRNA specific to neoplastic cells have been applied to the unequivocal detection of PSA expression in several cells and tissues (2, 4, 6, 8–11).

In this study, we utilized the most sensitive RT-PCR method for detecting PSA mRNA, according to a previously published procedure (9). The detection of a PSA transcript reveals the presence of PSA at the mRNA level in human neuroblastoma cell lines. To evaluate and compare the entire coding sequence of PSA cDNA from neuroblastoma cells with the known sequence of prostate PSA (32), a more focused study is in progress.

The present multidisciplinary results, together with our previous observations (18), add support to the notion that PSA is a widespread kallikrein-like serine protease and focuses attention on the novel PSA expression by human neoplastic brain tissue. The PSA immunoreactivity in human neoplastic neuroblastoma cell lines gives additional evidence of the distinctiveness of this biochemical marker in nonprostatic sources. Our results are in close agreement with the hypothesis that brain tissue might play an important biological role in the increased PSA content described in serum and CSF during nonpathological and pathological conditions (12–16). The presence of detectable amounts of PSA might indicate involvement in growth regulation; PSA might act both as a growth factor modulator and as a translational/posttranscriptional protein regulator. In fact, PSA hydrolyzes the insulin chains and interleukin-2 (33), enzymatically digests insulin-like growth factor binding proteins (34), activates latent transforming growth factor (35), inactivates protein C inhibitors (36, 37), and regulates the hormonal bioactivity of parathyroid hormone-related proteins.
protein (38, 39). On the other hand, it has no kininogenase activity (40), and studies of PSA activity on the single-chain urokinase-type plasminogen activator disagree (41, 42). The proteolytic activity of PSA on these different biological substrates, all detected in brain tissue (43, 44), could explain in part the potential role of PSA in the brain tumor cells, not only as a sensitive molecular marker implicated in hormone-responsiveness but also as an initiator of the protease cascade. Although the biological effects and the physiologic role of PSA in neuroblastoma cells are still obscure, our data indicate that the human neoplastic brain tissue may actively produce/secrete the PSA protease. Interestingly, the SK-N-MC cell line, which is derived from a metastatic tumor mass (20), expresses and secretes a higher amount of PSA than does the SK-N-BE-2 cell line, suggesting that this serine protease could explain in part the potential role of PSA in the brain tumor cells, not only as a sensitive molecular marker but also as an initiator of the protease cascade. Although the biological effects and the physiologic role of PSA in neuroblastoma cells are still obscure, our data indicate that the human neoplastic brain tissue may actively produce/secrete the PSA protease. Interestingly, the SK-N-MC cell line, which is derived from a metastatic tumor mass (20), expresses and secretes a higher amount of PSA than does the SK-N-BE-2 cell line, suggesting that this serine protease might be involved in brain tumor growth and/or in proteolytic cascade regulation (44), similar to other tumor types (2, 6, 8).

This work was supported by a grant of the Assessorato alla Sanità, Regione Marche, Italy. We thank Antonella Cardinali and Francesco Marcheggiani for skillful technical assistance.

References