Biochemical Characterization and Immunolocalization of Prostate-specific Antigen in Human Term Placenta,

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Prostate-specific antigen (PSA) has been biochemically and molecularly characterized as a 33-kDa androgen-dependent glycoprotein related to the kallikrein family of serine proteases, with chymotrypsin- and kallikrein-like enzymatic activity *(1-4)*. PSA has long been thought to be produced exclusively by the prostate cells and has been used as a tumor marker for diagnosis and monitoring of prostate cancer *(5, 6)*. Recently, it was found in several nonprostatic tissues and body fluids *(7)*, although no physiologic role of PSA is known in these tissues *(8)*. Immunoreactivity and gene expression studies have characterized human PSA as a steroid hormone-regulated serine protease *(9-12)*. Considering the studies carried out in amniotic fluids and in healthy endometrium *(13-15)*, we undertook the present study on PSA characterization and immunolocalization in the term placentas collected from five women (ages, 25–37 years) undergoing routine deliveries (40 ± 2 weeks). After the membranes were stripped, each placenta was weighed and placed on ice in a sterile solution of 9 g/L NaCl and 5 mmol/L glucose, transported to the laboratory, and processed within 30 min. Samples of entire placentas were minced and homogenized as described *(16)*. After sonication, the lysates were centrifuged at 9000*g* at 4 °C for 30 min, and the supernatants were stored at −80 °C until analysis. n-Butanol-extracted fractions were prepared from cytosolic extracts, according to a previously described method *(17)*. Blood was also drawn from healthy pregnant women (n = 15; ages, 23–38 years); after the blood clotted, the sample was centrifuged at 500*g* for 10 min and the serum stored at −30 °C until assay. Free and total PSA concentrations were determined in serum and cytosolic extracts of placentas, using an automated enzyme immunoassay with a detection limit of 0.01 μg/L (AxSYM® PSA, Abbott Laboratories) *(9, 16, 18)*. Placental extracts were serially diluted in PSA-negative female serum and reanalyzed to exclude the possibility of matrix artifacts. The analytical recovery of purified PSA added to placental extracts was also tested. Decidual extract components were separated on a 600 × 9 mm column of Sephacryl S-200 (Pharmacia Biotech) and eluted *(16)*. Reagents and equipment for Western blotting were purchased from Bio-Rad. Our protocols were followed throughout, using anti-human PSA monoclonal and polyclonal mouse antibody (Dako) *(16, 18)*. For thermal inactivation studies, placental extract was incubated in a thermostable water bath for different lengths of time at 55 °C or at different temperatures (45, 55, 65, and 75 °C) for 30 min. Five repeated freeze-thaw cycles were also performed using placental extract aliquots stored in cryotubes at −30 °C. Moreover, we tested PSA stability at 25 °C both in different buffer systems (phosphate, HEPES, 1,4-piperazinediethanesulfonic acid, Tris-HCl, and borate-citrate-phosphate-HCl) as well as at different pH values. The results obtained from five different placental specimens (mean ± SE) were statistically analyzed using the StatView, Ver.4. package (Abacus Concepts) on a Macintosh Power PC (Apple).

For the electron microscopic analysis, term placenta was fixed in a mixture of 40 g/L paraformaldehyde and 5 g/L glutaraldehyde in 0.1 mol/L Sörensen phosphate buffer, pH 7.4, at 4 °C for 2 h. After the placenta was washed in Sörensen phosphate buffer, the free aldehydes were blocked in 0.5 mol/L ammonium chloride in phosphate-buffered saline at 4 °C for 45 min; the placenta was then dehydrated through graded concentrations of ethanol and finally embedded in LRWhite resin (MultiLab). Polymerization was carried out under ultraviolet light at room temperature. For immunocytochemical analyses, ultrathin sections were placed on nickel grids, floated for 3 min on normal goat serum, and incubated for 17 h at 4 °C with rabbit anti-human PSA antiserum (Biomeda). After the sections were rinsed, they were reacted for 30 min at room temperature with the secondary gold-conjugated antibody (Jackson ImmunoRes Laboratories). Finally, sections were rinsed, air-dried, and stained with uranyl acetate. As controls, some grids were treated with the incubation buffer without the primary anti-PSA antibody.

The present work was carried out in accordance with the ethical standards of Helsinki Declaration of 1975, as revised in 1983.

The average serum PSA content of the women examined (n = 15) was 0.15 ± 0.03 μg/L. The linearity and interference studies revealed a good correlation between PSA concentration and dilution (serum of pregnant women, n = 15, r^2 = 0.98; placental extracts, n = 5, r^2 = 0.99), demonstrating that "placenta matrix" (constituted of lipids, hemoglobin, hormones, and proteins) did not affect the performance of PSA assays. Analytical recovery of purified PSA added to cytosolic placental extract was 97% ± 4%. Assay reproducibility (CV) was determined by assaying placenta samples in replicates of two or three in at least four independent analyses; the within-run CV was 2.5% and the between-run CV was 4.4%.

The mean concentration of PSA in term placenta tissues (n = 5) was 56 ± 8 μg/L, with ~30% in the free, noncomplexed form (16.8 ± 2.3 μg/L).

n-Butanol extraction of term placental homogenates revealed that the major proportion (~91%) of PSA immunoreactivity was in the aqueous phase, but the altered recovery of PSA subfractions made it an unsuitable method for isolation of PSA from biological samples.

The PSA immunogram of a Sephacryl S-200 column of placental extracts revealed that the major immunoreactivities were in fractions 35–80, where the antichymotrypsin (ACT)-PSA complex and free PSA were expected *(19)*; in

fact, the high molecular weight PSA fraction (presumably ACT-bound) showed a molecular mass of ~100 kDa and constituted ~70% vs the minor immunoreactive fraction (~28%) of the free, uncomplexed form (molecular mass ~33 kDa) (Fig. 1A).

Western blot analysis of placental extracts revealed the specific PSA protein bands in the position of the ACT-bound serum PSA (100-kDa form) and near the free PSA secreted from the LNCaP prostatic carcinoma cell line (Fig. 1B). However, placental extracts showed a free PSA immunoreactive protein band with a slightly higher apparent molecular mass, probably because of greater glycosylation of this serine protease.

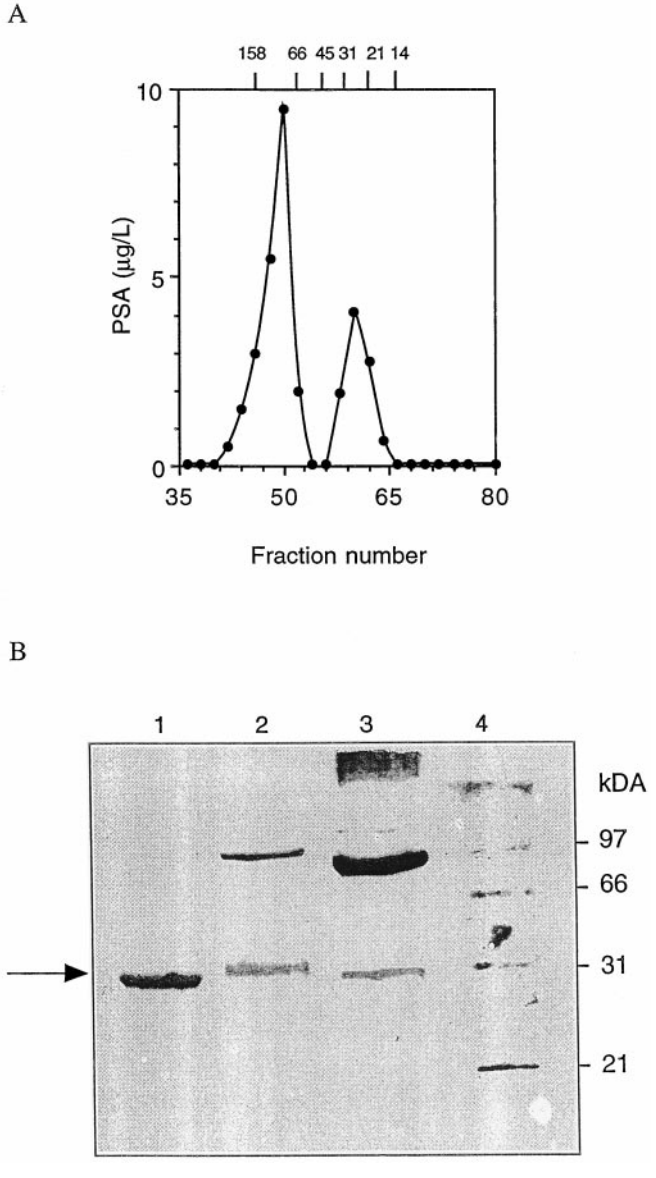

Fig. 1. Separation and identification of immunoreactive PSA.

(*A*) Immunoreactive PSA in Sephacryl S-200 column fractions 35–80 of placenta homogenate, analyzed on IMx® (●); the position of the molecular mass markers, expressed in kDa, are indicated at the top: hen egg white lysozyme (14 kDa), soybean trypsin inhibitor (21 kDa), bovine carbonic anhydrase (31 kDa), hen egg white ovalbumin (45 kDa), bovine serum albumin (66 kDa), and immunoglobulin G (158 kDa). (*B*) Western blot analysis using a monoclonal anti-PSA antibody and alkaline phosphatase detection. *Lane 1*, LNCaP cell line supernatant (5 ng); *lane 2*, cytosolic extract of at-term placenta (7 ng); *lane 3*, serum of prostate cancer patient (10 ng); *lane 4*, biotinylated molecular weight markers (rabbit muscle phosphorylase b, 97 kDa; rest as specified in *A*).

Stability was drastically reduced under pH 5 and over pH 10 and was optimum at pH 7.5. The thermal inactivation profile showed a linear temperature-dependent thermolability (n = 5, r^2 = 0.98), with residual immunoreactivity ~45% after 30 min at 55 °C and only 6% after 75 °C for 30 min. PSA decreased exponentially with time at 55 °C to immunoreactivity ~45% after 30 min and ~8% at 3 h (n = 5, r^2=0.95). In this respect, we can conclude that higher temperatures (e.g., 50 °C) should be avoided in processing of tissues for immunohistochemical localization of PSA.

The recovery of the PSA in term placenta specimens (n = 5), assayed after five freeze-thaw cycles, was 88–105% (mean, 96%). The freeze-thaw procedure did not markedly affect either the immunoreactivity or the PSA fraction concentration.

The addition of NP-40 to sample homogenizing buffer allowed us to detect the immunoreactive PSA in all placental samples, whereas, if the surfactant was omitted, the PSA content was underestimated, thereby suggesting that PSA in placental tissue may be bound to several cellular components. The same biochemical behavior has been characterized in amniotic fluid, using common detergents in sample buffer (i.e., Tween-20 and Triton X-100) *(13)*.

The electron microscopic examination of immunolabeled sections revealed that PSA occurs in the cytoplasm of all placental cells. Prominent immunogold labeling was observed in the epithelial cells facing the maternal blood vessels; in free ribosomes, rough endoplasmic reticulum and Golgi complex; and especially in the cytoplasmic apical region of these cells and in microvilli spreading out into the blood vessel lumen. Mithochondria, cytoplasmic vacuoles, lysosomes, cell nuclei, and the blood vessel lumina were devoid of specific gold particles; the control specimens showed only a negligible signal.

These results shed light on recent findings of PSA in amniotic fluid *(13, 14)* and endometrium *(15)*, where the physiological role and the source of this serine kallikrein-like protease are unknown *(8)*.

Apart from the potential clinical usefulness in breast cancer *(10, 16)*, PSA immunoreactivity could also be important in basic studies as a biochemical marker of gene regulation by steroid hormone receptors *(11, 12)*. The expression of PSA in female nonprostatic sources suggests a new important biological role of this serine protease, i.e., as a potential sensitive molecular marker of hormone responsiveness *(9, 10, 12, 16)*. The concomitant presence of the steroid hormones and receptors in human term placenta *(20)* and the substantial PSA concentration found in placental extracts suggest the possibility of modulation of PSA in the placenta by steroid hormones.

Recent studies support the hypothesis that extra-prostatic PSA may be involved in growth and in translational/posttranscriptional protein regulation of mammalian tis-

sues *(7, 8)*. The proteolytic activity of PSA on different biological substrates detected in term placenta *(21)* could in part explain the potential role of placental PSA as an initiator of the protease cascade, an important biological mechanism for tissue remodeling (i.e., in the breast and uterus).

To our knowledge, this is the first characterization and immunolocalization of PSA in human term placenta, which may be a source of PSA found in amniotic fluids.

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PCR has the potential of extreme sensitivity, specificity, and diversity. As a consequence, transition of this procedure from research to routine application has been relatively rapid in recent years. This transition has, however, been hampered by the labor-intensive, high-skill requirements of existing protocols. Furthermore, as a number of quality-assessment exercises have demonstrated *(1–8)*, many of the existing "in-house" developed test procedures are of poor reproducibility in routine application. The introduction of a commercially produced test procedure has improved the reliability of testing, but the procedure has remained a relatively high-skill test method with only certain steps becoming semiautomated.

The COBAS AMPLICOR™ automated PCR system (Roche Molecular Systems) represents the first approach to full automation of the amplification and detection of nucleic acid targets *(9)*. Evaluations of this instrument *(10, 11)* suggest that the sensitivity and specificity of the automated method are equivalent to those of manual methods, that intraassay sample carryover does not occur *(9, 10)*, and that intra- and interassay variation using the automated system is low *(9)*. However, there remains a need to evaluate the ability of the instrument, which is limited to 24 samples per thermal cycle amplification and 48 samples for the detection phase, to cope with both the work load and test diversity of a routine diagnostic laboratory. Moreover, the ability of the instrument to decrease the high labor and skill requirements of PCR and to decrease the overall cost of PCR needed to be further evaluated. We conducted a multicenter, international evaluation of the instrument from the standpoint of automation, work flow, and labor savings in laboratories differing in size, diversity of testing, and laboratory working practices.

Five laboratories, chosen to represent a wide range of working hours and practices, were studied in Italy, Germany, The Netherlands, England, and the United States. Each laboratory used the COBAS AMPLICOR instrument and test kits according to the manufacturer's instructions. Operators received on-site training by the manufacturer's