Prostate-specific antigen expression in a case of intracystic carcinoma of the breast: characterization of immunoreactive protein and literature surveys

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A case is presented of female breast intracystic carcinoma with prostate-specific antigen (PSA) expressed in high amounts in aspirated cystic fluid (55 μg/L). Tumor extract analysis revealed the presence of both estrogen and progesterone receptors (0.38 and 1.87 nmol/L, respectively) and high quantities of PSA too (19.52 μg/L). Chromatographic analysis of cystic fluid revealed two peaks of PSA, at the expected positions for free and bound serine protease. A major proportion of 33-kDa free form was also confirmed by Western blotting analysis. Free PSA was heat-stable at 56 °C and displayed no change after freezing-thawing. These findings are discussed in the context of a detailed literature survey. Our data support the contention that PSA immunoreactivity in intracystic fluid of breast carcinoma is partly the result of secretory activity by the neoplastic cells and that the steroid receptors can also modulate its expression.

INDEXING TERMS: α1-antichymotrypsin • breast cyst fluid • fibrocystic disease • serine proteases • estrogen receptor • progesterone receptor

Prostate-specific antigen (PSA), first identified in 1970 [1], is a 33-kDa androgen-dependent glycoprotein, structurally and functionally related to the kallikrein family of serine proteases [2], with a chymotryptic-like and kallikrein-like enzymatic activity and substrate specificity [2–4]. PSA has long been thought to be produced exclusively by the prostate epithelial cells but, recently, it has been found in female breast tumors, in normal breast, and in breast gross cysts [5–11]. The physiological role of PSA production by the breast cells and its role, if any, in cancer initiation and progression are currently unknown and under investigation. PSA has been characterized in breast cancer cells and tissues as a hormone-regulated serine protease [6, 8, 12]. Recently, several reports documented the immunoreactivity and expression of PSA serine protease in several biological fluids [9–11, 13–18] and in human normal and tumoral tissues [5, 19–40]. In blood, complexes form between PSA and serine protease inhibitors such as α1-antichymotrypsin (ACT) and α2-macroglobulin [3, 41]. Apart from these complexes, free PSA is also present in serum, even though the increased concentration in female serum is a matter of debate [3, 42–50].

Intracystic breast tumor arising from the cyst wall, especially intracystic carcinoma, is a rare event in a typical clinical patient subset, its incidence ranging from 0.29% to 2% of all carcinomas of the breast [51]. Intracystic carcinoma of the breast (ICB) is distinguished from cystic degeneration, which is caused by central necrosis of the tumor or carcinomatous invasion into benign microcystic diseases or gross cystic breast disease [52]. ICB has a predilection for older women (mean age 67 years, compared with 54.5 years for all breast carcinomas), black women, and those with a long clinical history. ICB occurs as a large, grossly evident tumor confined to a solitary and cystically dilated duct with internal papillary projections [52]. Aspiration of the breast mass yields a breast cyst fluid (BCF) containing many groups of cells and several biochemical markers [53–55]. BCF, therefore, provides a unique opportunity to study the background of
cystic changes related to the hormonal and biochemical environment, although diagnostic imaging techniques and cytological examination are the most widely used modalities for differential diagnosis [56–58]. Despite the tumor’s rarity and the difficulties encountered in papillary lesions of the breast, the cytological features of ICB have been well characterized [53, 56, 59].

Here, we report the results of the measurement and characterization of PSA immunoreactive protein and steroid hormone receptors status in a case of ICB. Our aim is to improve biochemical knowledge about the expression and hormone-responsiveness of the PSA protein found in needle-aspirated fluid from human breast intracystic cancer.

**Case History**

In May 1996, a 69-year-old woman (nullipara) attended our Center with a 5-year history of a slowly enlarging right breast mass. The patient had noticed no complications. Physical examination revealed a 7 × 8 cm cystic right subareolar mass. Palpable adenopathy was not noted. Routine hematology and serum chemistry data were within the reference ranges for healthy persons. Mammography, ultrasonography, and xeropneumocystography demonstrated a large, well-circumscribed mass with papillary projections from the cyst wall (data not shown).

All the procedures followed in this work were carried out in accordance with the ethical standards of Helsinki Declaration of 1975 (as revised in 1983).

**Materials and Methods**

**Materials**

*Sample collection and morphological features.* A percutaneous thin-needle aspiration rapidly filled the syringe (34 mL) with a murky brown fluid, indicating a mildly hemorrhagic cyst. After collection, BCF samples were centrifuged at 500g for 5 min to collect the cellular components, after which the supernates were centrifuged at 19 000g for 30 min at 4 °C. The final supernate was stored at −30 °C until processed.

Blood was also drawn from the woman and, after clotting, the sample was centrifuged at 300g for 10 min and the serum stored at −20 °C until assay.

Cytological analysis of the above-collected cellular components, performed after Papanicolaou’s stain, showed papillary clusters of monomorphic epithelial cells with hyperchromatic nuclei, irregularly shaped nuclear contours, and cytoplasmic degenerative changes with minimal atypia (data not shown). Microscopic evaluation of a frozen section confirmed the presence of intracystic carcinoma, even though previous diagnostic imaging and cytological examination had not provided clear evidence for the diagnosis of malignancy.

The surgically resected tumor was well circumscribed and did not invade the adjacent soft tissues. Histological examination showed a large cystic ductal carcinoma sur-

**Declaration of 1975 (as revised in 1983).**

**Tumor cytosol extract.** The resected tumor specimen was placed on ice, transported to the laboratory, and processed within 1 h. Portions of breast tumor sample (0.5–1 g) were washed in isotonic saline solution, chopped with scissors, and then resuspended in 2 volumes of extraction/homogenizing buffer (0.01 mol/L Tris, 1.5 mmol/L EDTA, 5 mmol/L sodium molybdate, 10 g/L Nonidet NP-40 surfactant, 1 mmol/L phenylmethylsulfonyl fluoride, pH 7.4). The tissue sample was then homogenized by a small probe of a Labsonic Brown (Thomas Co., Philadelphia, PA) sonifier system for 5 cycles of 30 s at 40 W output on ice. The lysate was finally centrifuged at 15 000g at 4 °C for 25 min, and the supernate (the cytosolic fraction) was analyzed for biochemical determinations.

**Procedures**

**Protein measurement and steroid receptors analyses.** Total protein content was determined in triplicate with either the Coomassie G-250 or the bicinchoninic methods (with commercially available kits from Bio-Rad Labs, Munich, Germany, and Pierce Chemical Co., Rockford, IL, respectively). The assays were calibrated with bovine gamma-globulin and serum albumin, respectively.

For quantitative analysis of estrogen and progesterone receptors (ER, PR), we used a commercially available enzyme immunoassay kit (Abbott Labs., Abbott Park, IL), performing according to the manufacturer’s instructions.

**PSA measurements.** PSA was determined in serum, BCF, and cytosolic extract with two commercially available kits, IMx® PSA (automated enzyme immunoassay from Abbott Labs.) and the CIS PSA-RIACT® (radioimmuno- metric assay from CIS Bio International, Gif-sur-Yvette, France), described in detail previously [9, 17, 18]. PSA immunoreactivity—determined for a minimum of three concentrations and at least in triplicate—was expressed as micrograms per liter.

**Immunogram and electrophoretic separations.** Sample components were separated on a 600 x 9 mm column of Sephacryl S-300 (Pharmacia Biotech, Uppsala, Sweden) calibrated with protein molecular mass markers (hen egg white lysozyme, soybean trypsin inhibitor, bovine carbonic anhydrase, hen egg white ovalbumin, bovine serum albumin, and IgG). The samples (0.5 mL) were applied to the column and eluted with 0.05 mol/L Tris-HCl buffer, pH 7.5, containing 0.15 mol/L NaCl, 7.7 mol/L NaN3, and 1 g/L bovine serum albumin. In all, ~80 fractions of 0.5 mL each were collected and analyzed for PSA content with both the IMx and CIS-RIACT kits.

All necessary reagents and equipment for sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting were purchased from Bio-Rad Labs. (Milan, Italy). Our protocols were followed throughout
Stability studies. Thermal inactivation of PSA was tested by incubating aliquots of BCF in a thermostable water bath, both for different lengths of time at 56 °C as well as at different temperatures (50, 65, 80, and 95 °C) for 30 min. We also studied the effects of repeated cycles of freeze–thawing on BCF aliquots stored in cryotubes at −30 °C, after which the samples were stored at −80 °C until testing; the PSA concentration was expressed as a percentage of residual activity vs controls kept at 4 °C in buffered-physiological saline solution, pH 7.4. Moreover, we tested the PSA stability in different buffer systems and at different pH values (measured at 25 °C); the buffer solutions were 100 mmol/L phosphate, 100 mmol/L HEPES, and 100 mmol/L Tris-HCl at pH 7.6, and 100 mmol/L borate–citrate–phosphate HCl at pH 4–12.

Statistical analyses. All results, reported as the mean ± SE value of at least five independent experiments, were determined with the StatView ver. 4 package (Abacus Concepts, Berkeley, CA) on a Macintosh Power PC (Apple, Cupertino, CA).

Results

The serum sample was neither lipemic nor hemolytic, and electrophoretic and laboratory data were within relevant reference ranges. Serum PSA was 0.04 ± 0.01 μg/L. The murky brown BCF sample, characteristic of a hemorrhagic/lipemic cyst, showed a PSA content of 55 ± 1.62 μg/L. The linearity and interference studies revealed a good linear correlation between PSA concentration and dilution (y = −0.133 + 65.2x, r² = 0.907, SD_intercept = 0.08, SD_slope = 3.79), demonstrating that BCF matrix (principally constituted of lipids, pigments, hormones, and proteins) did not affect the performance of PSA assays (Table 1).

Analytical recoveries of purified seminal plasma PSA added to BCF and cytosolic tumor extract were 95.33% ± 1.76% and 94.66% ± 2.33%, respectively (Table 2). Assay reproducibility (CV), determined by assaying sample in replicates of three or four in at least five independent runs, was 3.25% within-run and 5.15% and between runs. Results of the PSA assays on the IMx (y) and the CIS-RIACT (x) agreed well, giving respective values of 55 ± 1.62 and 52 ± 0.97 μg/L (n = 20, y = 1.08x + 0.24, r² = 0.93, SD_intercept = 0.14, SD_slope = 0.62). The concentration of PSA in tumor breast cytosolic extracts was determined to be 19.52 ± 4.4 μg/L. ER and PR concentrations in tumor cytosolic extracts were 0.38 and 1.87 mmol/L, respectively.

The PSA immunogram of Sephacryl S-300 column fractions 40–72 of BCF from the patient (Fig. 1) demonstrated immunoreactivity in the fractions where the ACT-PSA complex and free PSA are expected, without any additional peaks. The fraction of ACT-bound PSA (molecular mass 100 kDa) constituted ~15% of the major immunoreactive fractions, the remaining being free PSA (molecular mass ~33 kDa).

We further characterized the presence of PSA in BCF as well as in tumor cytosolic extracts with Western blotting.

### Table 1. PSA dilution linearity for serum, BCF, and cytosolic tumor extract.

<table>
<thead>
<tr>
<th>Dilution factor</th>
<th>Serum, μg/L</th>
<th>BCF, μg/L</th>
<th>ICB, μg/g protein</th>
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<tbody>
<tr>
<td>None</td>
<td>2.25</td>
<td>55.00</td>
<td>238.00</td>
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<tr>
<td>2</td>
<td>1.14</td>
<td>31.51</td>
<td>134.62</td>
</tr>
<tr>
<td>5</td>
<td>0.43</td>
<td>9.94</td>
<td>45.25</td>
</tr>
<tr>
<td>10</td>
<td>0.24</td>
<td>5.8</td>
<td>21.9</td>
</tr>
<tr>
<td>20</td>
<td>0.11</td>
<td>2.4</td>
<td>11.6</td>
</tr>
<tr>
<td>40</td>
<td>0.06</td>
<td>1.6</td>
<td>5.7</td>
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### Table 2. Analytical recovery of PSA added to BCF and cytosolic extract.

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Initially present</th>
<th>Added</th>
<th>Recovered</th>
<th>% recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>55.0</td>
<td>2.5</td>
<td>2.4</td>
<td>96</td>
</tr>
<tr>
<td>1:2</td>
<td>22.5</td>
<td>5.0</td>
<td>4.9</td>
<td>92</td>
</tr>
<tr>
<td>1:10</td>
<td>5.5</td>
<td>10.0</td>
<td>9.8</td>
<td>98</td>
</tr>
<tr>
<td>ICB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>238.00</td>
<td>2.5</td>
<td>2.35</td>
<td>94</td>
</tr>
<tr>
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<td>23.8</td>
<td>10.0</td>
<td>9.10</td>
<td>91</td>
</tr>
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</table>

* Measured minus initial concentration.
analysis. At the same position of the band present in culture supernate from LNCaP prostatic carcinoma cell line, a specific PSA immunoreactive protein at 33 kDa was observed (Fig. 2).

The three profiles of PSA stability in BCF (time-dependence at 56 °C, temperature-dependence after 30 min of preincubation, and pH optimum) are shown in Fig. 3. Recovery of the PSA in the BCF sample, assayed after five freeze–thawing cycles, was 89–103% (mean 96%) and did not substantially affect either the immunoreactivity or the total PSA concentration measured; neither did the use of different buffer solutions at pH 7.6 (data not shown). The pH-dependent stability profile showed a drastically reduced immunoreactivity below pH 5 and over pH 10; optimum pH was determined to be 7.5. The thermal inactivation profile clearly showed a typical curve of thermolability, with residual immunoreactivity being reduced to ~34% after incubation for 30 min at 65 °C. After heating at 56 °C, the PSA immunoreactivity decreased ~50% by 30 min of preincubation and even more drastically within 4 h.
Discussion

Until recently, PSA was thought to be a highly tissue-specific biochemical marker for prostate cells [1, 3], but recent biochemical and molecular evidence has shown that PSA is a widespread protein [4, 61]. The physiological role of this serine protease in the breast cyst compartment is unknown at present [9–11], even though some have suggested that breast epithelium surrounding the apocrine “active” cysts is able to produce, secrete, and accumulate large amounts of PSA with a mechanism that could initiate events leading to proliferative breast disease [9].

Our findings in this case report clearly suggest that PSA in BCF from ICB is produced and (or) secreted from cells lining the cysts, as well as from the breast carcinoma tissues [5, 6, 23, 37]. The high concentration of the PSA serine protease in BCF and in cytosolic tissue extract characterizes the tumor of the patient examined as a “highly PSA-positive tumor,” according to a previous classification protocol [40].

Results of the cytological examination of the present ICB case widely agree with previous extensive, specific, and reviewed morphological studies [51–53, 55, 56, 59].

The characteristic steroid hormone status of the patient examined (particularly the high proportion of PR) is probably correlated to high PSA expression in cytosolic tissue extract and BCF, largely in agreement with the previously reported association between PSA immunoreactivity in breast cancer and the presence of steroid hormone receptor [6, 8, 12, 40].

The biological functions of PSA remain unknown at present [4]; however, our studies support the hypothesis that PSA does not act as an IGFBP-3/IGF-I regulator in breast cyst compartments (Mannello et al., submitted for publication). The functions of PSA in the female breast may thus converge with other proposed functions currently under investigation [4, 62].

Even though ICB is an unusual breast lesion, constituting 0.7% of all breast carcinomas, clinical studies and cytological evaluations illustrate the importance of the characteristic clinical setting and natural history of the disease [51–59, 63–67].

After an extensive bibliographic search, we can demonstrate that PSA is also a component of aspirated fluid an ICB. PSA concentration in needle-aspirated BCF was higher than that in serum but lower than the ICB cytosolic extract value, suggesting that the production or secretion (or both) of this serine protease is probably from the neoplastic cells surrounding the cyst. The very low proportion of ACT-bound PSA tends to favor this hypothesis and refutes the possibility of PSA accumulation in the breast cyst compartment from plasma through a transudative mechanism. This is partly confirmed in previous reports concerning the lack of PSA expression in sera of patients with breast cancer, even though this argument is a matter of recent debate [37, 41, 48–50].

According to reported studies with serum samples,

different buffer systems (phosphate, HEPES, and Tris-HCl) at pH 7.6 did not substantially affect the stability of PSA in BCF or the real-time stability at −20 °C and the freeze–thawing cycles [68]. Moreover, free PSA in BCF from ICB was heat-stable during 30 min of pretreatment at 56 °C, whereas PSA-IV complex immunoreactivity was heat-sensitive, according to preliminary studies performed in serum samples [69]. That the residual activity was −50% after heat-treatment at 56 °C is in agreement with literature data, showing equal concentrations of free and ACT-bound PSA in breast cyst fluids [9, 10].

The concomitant expression of steroid receptors (in particular PR) and high PSA in ICB tumor extract suggests the possibility of PSA modulation by steroid hormones, as has been reported in breast carcinoma and other hormone-dependent tissues [6, 27, 30, 38, 62].

Although PSA values have previously been considered a new favorable prognostic indicator for women with breast cancer [70], other reports on PSA immunoreactivity in breast cancer patients suggest a more cautious evaluation of the utility of PSA [37, 71] and of the possibility for utilizing PSA as a marker for early identification of the hormone responsiveness of breast tumors [4, 50, 62].

In summary, the expression of PSA in nonprostatic sources, particularly in the female breast, suggests a new important biological role of this serine protease, certainly more complex than those reported previously, i.e., as a potential sensitive molecular marker of hormone responsiveness of the glandular cells [4, 41, 62, 72]. It would therefore be interesting to study in detail the biochemical processes of PSA associated with breast cyst formation to provide an insight into the etiology of breast cancer evolution.

PSA immunoreactivity could also be important in basic studies as a biochemical marker of gene regulation by steroid hormone receptors and perhaps as indicative of increased cancer risk. Because PSA is not a single molecule of uniform features [3, 4, 62, 72], further studies will be conducted on its biochemical-molecular characteristics [i.e., microheterogeneity (from glycosylation), epitopes, inhibitors, and specific substrates] to provide knowledge of the mechanism of the appearance of this serine protease in the breast cyst compartment and of its biological role in the normal female breast, in breast cystic disease, and in breast cancer.

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References


