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Differential Distribution of Soluble and Complexed Forms of Prostate-Specific Antigen in Cyst Fluids of Women With Gross Cystic Breast Disease

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Gross cystic breast disease (GCBD) is the most common benign disease of the human female breast, and patients with GCBD have an increased risk of breast cancer. The aim of this study was to evaluate the distribution inside apocrine cells and in breast cyst fluids aspirated from gross cysts of prostate-specific antigen (PSA) molecular forms, and to correlate the different intracystic PSA profiles to the subpopulations of gross cysts. Type I cysts showed a median value of 0.71 μg/L of total PSA and 0.32 g/L of ACT, significantly different to that of Type II cysts (Wilcoxon $P < 0.001$). Although large excesses of ACT were detected in all samples, BCF samples and apocrine cells from Type I gross cysts contained about 70% of free PSA, compared to the higher amounts of complexed PSA found in Type II gross cysts. We demonstrate that in apocrine/secretive Type I breast gross cysts the serine protease PSA was mainly present in its free form, in contrast to a major proportion of complexed PSA found in flattened/transudative Type II cysts. Our results are consistent with the notion that a prolonged exposure of apocrine breast cells lining the Type I gross cysts to the proteolytic activity of PSA could be involved in the etiopathogenesis of GCBD. J. Clin. Lab. Anal. 15:81–86, 2001. © 2001 Wiley-Liss, Inc.

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INTRODUCTION

Gross cystic breast disease (GCBD) is a benign disorder of the human female breast that has been reported to affect about 7% of women in Western countries, with the highest incidence occurring in women in their premenopausal decade (1). Although GCBD is not considered a premalignant lesion per se (2), epidemiological studies indicate that women affected by GCBD are at two- to fourfold greater risk of developing breast cancer than nondiseased females (3,4); however, this is the subject of great debate (5,6).

In recent years, novel approaches to this issue have been offered by studies on the composition of the breast cyst fluid (BCF), in an attempt to understand the mechanism(s) of cystic disease initiation and progression (7), and its relationships to carcinogenesis (8). These studies consistently indicate that the concentration in BCF of a wide variety of substances (including steroids, tumor markers, proteins, and electrolytes) (9) shows a bimodal distribution, leading to the identification of two main types of cysts. Type I cysts, often referred to as secretory cysts, are characterized by a peculiar biochemical profile and lined by metabolically active apocrine cells. Type II cysts, also named transudative cysts, have a biochemical composition similar to that of plasma and are lined by a flattened epithelium (10). Several authors suggest that women bearing Type I cysts have an increased breast cancer risk, probably associated with the higher biosynthetic activity of the lining apocrine epithelium (11,12). On the other hand, epidemiological studies gave contradictory results (13,14).

Recently, several biochemical and molecular studies have demonstrated that the female breast produces and secretes PSA (15) through steroid hormone regulation (16), and some authors suggest PSA as a marker for women with breast cancer (17). However, this argument is still a matter of some debate (18–20).

It has been found that about 60% of BCFs contain detectable levels of PSA (21–24), a 33-kDa serine protease with

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chymotryptic-like activity belonging to the kallikrein family, which was thought to be produced exclusively by the prostate cells. Previously, several authors reported increased levels of immunoreactive PSA in BCFs of apocrine/secretive Type I cysts respect to flattened/transudative Type II cysts (21–24), and a consistent amount of ACT-PSA was found to be detectable in some BCF samples (22). Subsequently, we provided evidence that PSA found in Type I BCFs is a secretory product of the apocrine cells lining breast gross cysts (25).

In the present study we investigated the biochemical and immunocytochemical distribution of PSA molecular forms in BCFs, in an attempt to obtain more insight into the possible involvement of the serine protease PSA in the GCBD development.

MATERIALS AND METHODS

Samples

With informed consent, 136 BCF samples were obtained by needle aspiration from women affected by GCBD; the patients were all premenopausal (ages, 25–44 years). The size of the cysts varied from 1 to 6.5 cm in diameter and the volume of BCFs ranged between 1.5 and 40 mL. Cancer was excluded by clinical, echographic, and mammographic examinations. No patients had taken any hormonal medication for at least six months prior to the study. Women reporting pregnancy, lactation, or clinical symptoms concerning the breast within 3 years prior to this study as well as those who had been medically treated for breast diseases within 6 months prior to the study were excluded from it.

Biochemical Determinations

BCFs were centrifuged at 19,000*g* for 30 min at 4°C and the clear supernatants stored at –30°C until assay. In order to characterize and identify the different types of breast gross cysts, Na and K concentrations in BCFs were measured by flame photometry (Perkin Elmer), with and without an internal standard at 589 and 776 nm, respectively (10,25). Gross cysts were classified either as Type I, if the Na/K ratio found in BCF was less than 3, or as Type II when the Na/K ratio was 3 or more, in agreement to previous reports (26). To best separate the populations of breast cysts, log-normalized Na/K ratio values were employed throughout the analysis, because of the skewness of the data (7,8,11).

Total and free PSA content (expressed as μg/L) were determined by a commercially available kit based on a microparticle capture enzyme immunoassay (AxSYM, Abbott Laboratories), using two monoclonal mouse antihuman PSA antibodies according to the procedures detailed elsewhere (21,25,27). The detection limit of the AxSYM PSA assay, defined as the concentration 2 SD above the zero calibrator, was reported to be 0.02 and 0.01 μg/L for total and free PSA, respectively. Complexed PSA was calculated by subtracting free portion from total PSA content. To exclude the possibility of "matrix" artefacts caused by interfering substances present in BCFs (e.g., lipids, proteins, and hormones), the samples were serially diluted in PSA-negative healthy female serum and reanalyzed for the response linearity (range dilution, 0- to 100-fold) (21). Moreover, the analytical recovery of at least two concentrations of purified human PSA (Sigma) added to BCF supernatants was also tested, and the recovered PSA was calculated by subtracting the concentration initially present from the measured concentration after the dilution (27).

Reagents and equipment for SDS-polyacrylamide gel electrophoresis and Western Blotting were purchased from Bio-Rad; our protocols were followed throughout, using an antihuman PSA monoclonal antibody (Dako) (28).

Being that ACT is the main PSA complexing protein in BCFs (22,24), we estimated ACT levels in BCF supernatants by radial immunodiffusion technique (end point method), using commercially available agarose plates (Boehring) and polyclonal specific goat antiserum to human ACT (Dako) (29).

Immunoelectron Microscopy

A double immunolabelling for both PSA and ACT detection was carried out on epithelial cells obtained from BCFs, according to the procedure described elsewhere (25). The sections were immunolabelled on one side with the ER-PR8 clone of mouse antihuman PSA monoclonal antibody (Dako), revealed by a specific secondary antibody conjugated with 6-nm colloidal gold particles (Jackson ImmunoRes Labs), whereas a rabbit antihuman polyclonal ACT antibody (Dako) revealed a secondary antibody conjugated with 12-nm colloidal gold particles (Jackson ImmunoRes Labs). The specimens were observed in a Zeiss EM 902 electron microscope operating at 80 kV.

In order to analyse the colocalization frequency of anti-PSA and anti-ACT signals, labelling distribution was quantitatively evaluated on sections treated in the same immunolabelling experiment. Single and colocalizing gold grains present over cytoplasmic areas were counted for each probes on forty randomly selected electron micrographs (final magnification × 42,000). The percentage of anti-PSA colocalizing with anti-ACT signals was calculated and expressed as mean value ± SE.

Statistical Analyses

The distribution of the various analytes was heavily skewed. Normalization of the data through logarithmic transformation resulted in a satisfactory stabilization of the variance. Nonparametric statistics were therefore used. The results obtained from two independent experiments, carried out at least in triplicate (mean ± SE), were statistically analyzed by the StatView program (version 4.1) (Abacus Concepts) on a Macintosh Power PC (Apple). The comparison of all analyte levels in the different subgroups of cysts was performed by

means of the Mann–Whitney U-test, while Wilcoxon's signed rank test was used to compare means of the various analytes in the two groups of breast cysts, as defined by their Na/K ratios. Correlation coefficients were calculated using the Spearman's rank correlation test (Spearman rho rank correlation coefficient = r_s).

RESULTS

Electrolyte, PSA and ACT Concentrations in BCFs

Intracystic concentrations of electrolytes varied widely; a regression analysis revealed the existence of a significant inverse correlation between Na and K contents (n = 136, r_s = –0.98) ($P < 0.001$). According to their Na/K ratios, the gross cysts were subdivided into two main subpopulations: Type I (~ 48 %) had low Na and high K levels (Na/K < 3), Type II (~ 52 %) had high Na and low K concentrations (Na/K < 3) (Fig. 1).

About 65% of BCFs examined had a detectable total PSA concentration (PSA > 0.02 μg/L) with a mean value of 0.65 ± 0.1 μg/L (Table I). We found a statistically significant difference between PSA values in apocrine Type I cysts respect to flattened Type II cysts (Wilcoxon $P < 0.01$). The highest PSA concentration observed was 125 μg/L, referred as apocrine Type I cyst. The distribution of free-PSA was ~75 % (0.42 ± 0.1 μg/L) in apocrine Type I vs. 37% (0.03 ± 0.01 μg/L) in Type II flattened breast cysts (Wilcoxon $P < 0.001$). In our series of BCF samples we found a free/bound PSA ratio of 36.1 ± 3.2 (median of 15.5, range 0.5–120), with a statistically significant association between apocrine Type I cysts and higher BCF free/bound PSA ratio (r_s = 0.85, Wilcoxon P = 0.012). We found no association between gross cyst types and age, cyst size, and cyst volume.

The linearity and interference studies revealed a good correlation between PSA concentration and dilution (n = 12; r_s = 0.95), demonstrating that BCF matrix did not affect the performance of PSA assay—there was no significant difference between gross cyst subtypes (data not shown).

The analytical recovery of purified PSA added to BCF samples was 97 ± 4%. Assay reproducibility (CV) was determined by assaying BCFs in triplicate in at least two independent analyses; the within-run CV was 2.5%, and the between-run CV was 3.7%.

Western blotting analysis of apocrine Type I BCFs confirmed the previous finding of immunoreactive PSA in two main forms, free PSA (~33 kDa) and ACT-complexed PSA (~100 kDa) (25,27). No glycosilated protein and/or cross-reacting fragments were detected in our samples. The control specimens (PSA-negative female serum) showed no immunoreactive bands with the monoclonal mouse antihuman PSA antibody used (data not shown).

In BCFs the content of ACT was significantly higher ($P < 0.01$) in flattened Type II cysts than in apocrine Type I cysts (Table 1).

Immunoelectron Microscopy

Type I BCF apocrine cells only showed detectable levels of anti-PSA immunolabelling. Ultrastructural observations on these cells demonstrated that both PSA and ACT showed a cytoplasmic distribution; the quantitative evaluation of immunolabelling revealed that 33.35 ± 3.25 % of total anti-PSA labelling colocalized with anti-ACT probe. In particular, immunolabelling signals for both proteins were distributed singly as well as in association on RER, free ribosomes (Fig. 2a), cytosolic regions, and small vesicles containing homogeneous, slightly electron-dense material (Fig. 2b); such vesicles were sometimes found to be in exocytosis. The two signals were also found along the cell surface, especially where many elongated cytoplasmic protrusions occurred (Fig. 2c). Cell nuclei appeared almost devoid of gold grains. Control samples showed only negligible labelling.

DISCUSSION

GCBD is thought to represent an advanced form of fibrocystic disease and it is not considered a premalignant lesion per se (2), even though several epidemiological studies have demonstrated that bearers of apocrine Type I gross cysts are at higher risk of developing subsequent breast can-

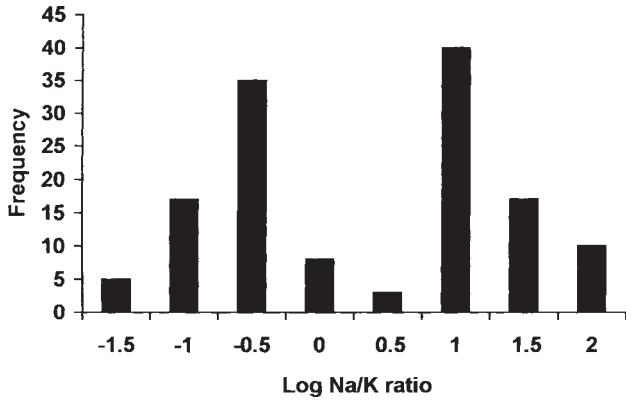

Fig. 1. Frequency distribution of $\log_{10}$ Na/K ratio distribution in 136 BCF samples.

TABLE 1. Concentrations of continuous variables in breast cyst fluid samples

	Apocrine Type I cysts (n = 65)	Flattened Type II cysts (n = 71)	Wilcoxon *P*
Analytes			
Na/K ratio	0.11 ± 0.04	16.58 ± 3.13	0.001
Total PSA (μg/L)	0.63 ± 0.06	0.09 ± 0.03	0.009
Free/bound PSA	2.82 ± 0.41	0.43 ± 0.05	0.012
ACT (g/L)	0.28 ± 0.05	1.18 ± 0.21	0.008

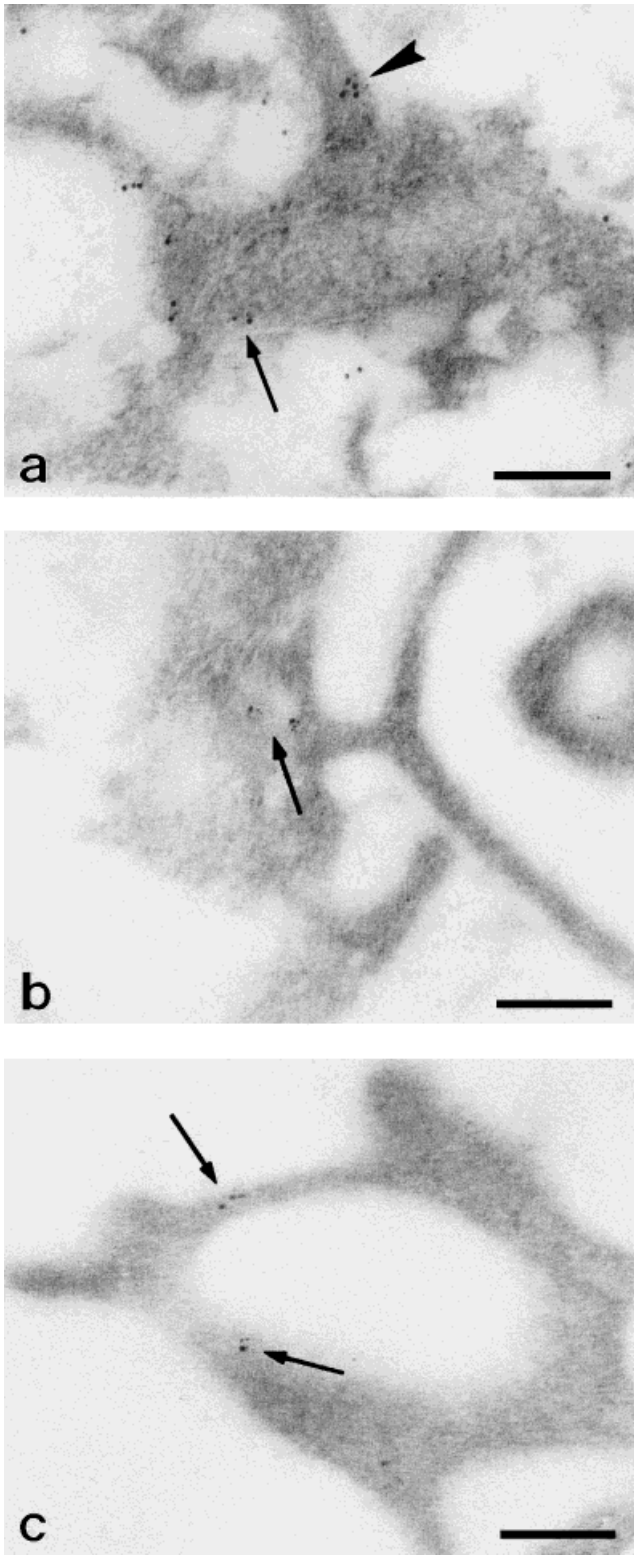

Fig. 2. Electron micrographs showing apocrine cells immunolabelled with anti-PSA (6-nm gold grains) and anti-ACT (12-nm gold grains) antibodies. **a**, the two probes appear to be singly distributed in the cytoplasm and to colocalise on RER (arrow) and on free ribosomes (arrowhead); **b**, a vesicle (arrow) contains both PSA and ACT proteins; **c**, PSA and ACT signals co-localise (arrows) on the cytoplasmic protrusions along the cell surface; bars, 0.2 μm.

cer (1,3,5,11,12). This argument is still a matter of recent and consistent controversy (13,14) and, in spite of numerous epidemiological and clinical studies, there is a significant lack of univocal interpretation in a subject so worthy of attention as the relationship between GCBD and breast cancer.

Type I BCFs are characterized by a high content of several metabolically active compounds, including proteases, which are thought to be involved in cyst development and premalignant transformation (21,30–32). In this respect, the relationships between proteases and antiproteases could shed light on the mechanism(s) proning Type I gross cysts to higher breast cancer risk (29).

PSA, a serine protease belonging to the kallikrein family thought until recently to be "specific" to the prostate gland, has been found in the normal breast, as well as in benign and malignant breast diseases (15). Moreover, several authors have demonstrated that PSA presence in the female breast is hormone dependent (16), suggesting PSA as a new prognostic indicator for women with breast cancer (17), although there are discordant data (18–20).The extraprostatic expression of PSA reveals new biological roles for this serine protease in the female breast, where it may be involved in both growth factor modulation and in translational-transcriptional protein regulation (33). In fact, the proteolytic activity of PSA on different biological substrates—e.g., insulin-like growth factor binding proteins (34), transforming growth factor-β (35), single-chain urokinase-type plasminogen activator (36), and plasminogen (37)—all detected in BCFs from women affected by GCBD (8,9,38), would indicate new role(s) of PSA in the female breast.

The presence of immunoreactive PSA in BCFs was previously demonstrated in both free (33 kDa) and complexed form (100 kDa) (21–25,27). It has been also demonstrated that Type I BCFs contain higher amounts of PSA than Type II (21,24), and that the apocrine cells occurring in Type I cysts are able to synthesize and actively secrete PSA in BCF (25). Recently, it has been reported that Type I cysts have proportionally more free PSA than Type II cysts and that ACT represents therein the major complexing molecule for PSA (24).

In the present study we have matched the biochemical data of PSA molecular forms present in BCF samples and the intracellular distribution of this serine protease, as well as of its major complexing protein ACT, inside the apocrine cells responsible for PSA synthesis and secretion. The higher resolution power of immunoelectron microscopy with respect to the light microscopic immuno-histochemistry allows us to reveal not only the presence of the labelled proteins, but also the fine localization and quantitative distribution inside the intracellular organelles, giving information about their possible molecular interactions.

We show that most of the PSA present in Type I BCFs is in its free form, whereas complexed PSA represents the main isoform found in Type II BCFs. In fact, in agreement with previous observations (24), the free/bound PSA ratio is sig-

nificantly higher in Type I than in Type II cysts. Accordingly, quantitative evaluation of immunoelectron analysis showed that in apocrine cells found in Type I BCFs about 30% of PSA was colocalized with ACT, suggesting that only a minor proportion of PSA was intracellularly complexed.

Further bindings of PSA with other proteins (e.g., α_2-macroglobulin) can not be excluded, even though it has been demonstrated that molecular bindings to inhibitory proteins other than ACT represent only a minor percentage of complexed-PSA (39,40). In addition, there are no current data about the presence of complexing-PSA polypeptides such as α_2-macroglobulin, c-reactive protein or α_1-protease inhibitor in BCF samples.

Although there is a large excess of ACT in all BCF samples, we showed that in secretive/apocrine Type I cysts, PSA occurs mainly in free form (both inside apocrine cells and in cyst fluids), whereas a major proportion of complexed PSA was detected in flattened Type II cysts. However, the mechanism by which this different distribution of PSA molecular forms takes places in breast cyst compartment is currently unknown (41).

The presence of PSA in the female breast is consistent with the hypothesis that this serine protease could be involved not only in hormone-responsiveness (16,33), but also in the proteolytic pathways related to the etiopathogenesis of GCBD (7,31).

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