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Diagnostic Value of Molecular Forms of Prostate-Specific Antigen for Female Breast Cancer

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Objectives: To examine the diagnostic value of prostate-specific antigen (PSA) subfractions in the serum of female breast cancer patients.
Design and Methods: PSA subfractions (free PSA, F-PSA; PSA bound to α_1-antichymotrypsin, PSA-ACT) were determined in the serum of patients with breast cancer and in the serum of healthy women. Serum was injected into a high-performance liquid chromatography column and all fractions were analyzed for PSA using a highly sensitive PSA immunofluorometric assay. We studied 3 normal male sera, 3 sera from prostate cancer patients who underwent radical prostatectomy (all for comparative purposes), 3 sera from healthy women, 3 sera from women with breast cancer obtained presurgically, and 7 sera from women with breast cancer, postsurgically.
Results: All male sera contained mostly PSA-ACT complexes and very little free PSA. Sera from all healthy women also contained mostly PSA-ACT complexes and nondetectable or traces of free PSA. All 3 presurgical sera from patients with breast cancer contained predominantly free PSA. Patients who had surgical resection of the breast tumor and were in remission had postsurgical serum PSA subfractions similar to those of healthy women (*i.e.,* mostly PSA-ACT complexes).
Conclusion: The serum PSA subfractions of breast cancer patients are substantially different from serum PSA subfractions of male patients, healthy females, and females who have apparently been treated successfully for breast cancer. These findings may form the basis for a serological diagnostic test for breast cancer.

KEY WORDS: breast cancer diagnosis; prostate-specific antigen; PSA subfractions; molecular forms of PSA.

Introduction

Breast cancer is a leading cause of mortality and morbidity among women. One of the priorities of breast cancer research is to define new biochemical markers that could be utilized to enhance the prognostic ability of available markers, and to facilitate treatment strategies on the basis of the probable likelihood of response.

Prostate-specific antigen (PSA), a 33 KDa serine protease, is found at high levels in seminal fluid and prostate epithelial cells (1). Currently, PSA is a highly valuable marker for prostate cancer screening, diagnosis, and postsurgical monitoring of prostate cancer patients, as well as for the detection of micrometastases (2).

The presence and production of PSA by nonprostatic tumors and tissues has been reported as an extremely rare phenomenon (3). We have demonstrated that PSA is not male- or prostate-specific. We have recently reported PSA immunoreactivity in breast tumors (4), in some other tumors, such as lung (5), in healthy female breast (6,7), in amniotic fluid (8), and in maternal serum (9).

Although PSA is not present in all breast tumors, the PSA immunoreactive species in female breast cancer cytosolic extracts has a molecular weight identical to PSA from seminal plasma (4). PSA mRNA was identified by polymerase chain reaction in PSA protein-positive breast tumors, but not in PSA protein-negative ones (10). Also, the PSA cDNA from breast tumors was identical in sequence to PSA cDNA from prostatic tissue (10). We have documented that PSA concentrations $\geq$ 0.03 ng/mg of total protein could be detected in 30% of cytosolic extracts from female breast tumors (11).

PSA production in the prostate is regulated by androgenic steroids, which bind to androgen receptors and upregulate transcription of the PSA gene (1,2). Tissue culture experiments with female breast cancer cell lines have shown that PSA production in these cell lines was mediated through the action of progesterone, androgen, mineralocorticoid, and glucocorticoid receptors, but not the estrogen receptor (12). Moreover, clinical studies have also indicated that PSA in breast cancer is associated with the presence of the progesterone receptor (11).

Normal male serum PSA levels are usually below 4 μg/L (1,2) and are detectable in two molecular

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forms for both healthy and prostate cancer subjects: (a) as free PSA (F-PSA) with a molecular weight of ~33 KDa, and (b) as complex with α_1-antichymotrypsin (ACT), a proteinase inhibitor, with a combined total molecular weight of ~100 KDa (ACT-PSA) (13,14). Previous studies have shown that PSA is undetectable in the serum of most women. In a recent study involving 1161 healthy female sera, we have reported that < 5% of the samples had PSA concentrations ⩾ 50 ng/L (15).

Breast cancer patients with PSA-positive tumors have a lower risk of relapse and death in comparison with patients whose tumors are PSA negative. We, therefore, proposed that PSA is a new favorable prognostic indicator in female breast cancer (16). A recent report studying associations between serum PSA levels from healthy women, serum PSA levels from women with breast cancer, and breast tumor PSA levels, indicated that there is no diagnostic or monitoring value of female serum total PSA (17). In this paper, we report substantial differences of PSA molecular forms between sera from healthy women and presurgical sera from women with breast cancer. We propose a hypothetical model to explain the differences and, further, speculate that these differences may form the basis for a biochemical diagnostic test for breast cancer.

Materials and methods

Serum samples

Three presurgical sera with total PSA values ⩾ 50 ng/L were selected from a series of 198 presurgical sera of patients with primary breast cancer. No other criterion was used to select these 3 sera. A total of 7 postsurgical sera with total PSA ⩾ 16 ng/L were selected from another series of 346 breast cancer patients who were treated by surgery. Three normal (from nonbreast cancer subjects) sera with total PSA ⩾ 35 ng/L were also selected from a total of 212 sera from female blood donors. These were provided by the Red Cross Blood Transfusion Service in Toronto. Other clinical samples included sera from 3 healthy male blood donors and sera from 3 males who underwent radical prostatectomy for prostate cancer. All 6 male sera had PSA ⩾ 80 ng/L. All samples were stored at –20 °C.

We selected sera with total PSA ⩾ 16 ng/L to be able to determine the PSA molecular forms by HPLC, followed by PSA immunofluorometry. Samples with total PSA < 16 ng/L are not suitable because the individual HPLC fractions contain very little PSA, which is difficult to measure.

High-performance liquid chromatography (HPLC)

HPLC analysis was performed with a Hewlett Packard 1050 system. The mobile phase was a 0.1 mol/L sodium sulphate and 0.1 mol/L sodium dihydrogen phosphate, pH 6.80. The gel filtration column used was a TSK-GEL G3000SW, 60 cm × 7.5 mm (TosoHaas, Montgomeryville, PA, U.S.A.) and was calibrated with a molecular mass standard solution from Bio-Rad (Bio-Rad Laboratories, Hercules, CA, U.S.A.). The flow rate was 0.5 mL/min and the HPLC was run isocratically. After injection of 100–500 μL of each certrifuged sample, fractions of 0.5 mL were collected and analyzed for PSA using the outlined method below. Sample carry over of < 5% was ensured by between-sample-injection column and injector washings, and by the order of sample injection (*e.g.,* the samples with the highest total PSA were injected last).

PSA immunoassay

PSA determinations were performed using a modified methodology from our highly sensitive and specific immunofluorometric procedure previously established and described in detail elsewhere (18). Briefly, the PSA assay uses a mouse monoclonal antiPSA capture antibody coated to polystyrene microtiter wells, a biotinylated monoclonal antiPSA detection antibody, and alkaline phosphatase-labeled streptavidin (SA-ALP). In this immunoassay, 100 μL of sample is incubated with the coating antibody in the presence of 50 μL of assay buffer containing the monoclonal antiPSA detection antibody. After 1-h incubation followed by washing × 6, the SA-ALP conjugate is added for 15 min, followed by another washing × 6. The activity of ALP is then measured by adding the substrate 5-fluorosalicylphosphate, incubating for 10 min and, then, by adding a Tb^{3+} and EDTA-containing developing solution. After 1 min, the fluorescence is measured in the time-resolved fluorometric mode with the Cyberfluor-615 Immunoanalyzer (Cyberfluor Inc., Toronto, Ontario, Canada). This assay has a biological detection limit of 1 ng/L of PSA. Details are described elsewhere (18). All assays were run in duplicate.

Results

All types of serum samples, with the exception of male sera, were selected on the basis of their total PSA level (⩾16 ng/L), and the availability of sufficient sample volume (>100 μL) for HPLC analysis (Table 1). In general, they approximately represented samples from the upper pentile of their respective serum type.

Separation of serum immunoreactive PSA by HPLC and immunofluorometric analysis of their corresponding fractions revealed for 3 normal female sera, that the molecular form of immunoreactive PSA is the complexed form; PSA bound to ACT (PSA-ACT; ~100 KDa), which peaks at fraction 30 ± 1 (Figure 1, panels A,B,C). Free PSA was not detectable (see below). Immunofluorometric analysis of serum fractions from 3 preoperative females with primary breast cancer demonstrated that the predominant molecular form of PSA is free PSA (F-PSA; ~33 KDa), which peaks at fraction 39 ± 1 (Figure 1, pan-

TABLE 1
Clinical Samples Used in This Study

Case ID*	Serum type	Gender	PSA Level (ng/L)	Time of collection
A	normal; non-breast cancer	female	36	Random
B	normal; non-breast cancer	female	50	Random
C	normal; non-breast cancer	female	80	Random
D	breast cancer; presurgical	female	54	<1 month before surgery
E	breast cancer; presurgical	female	59	<1 month before surgery
F	breast cancer; presurgical	female	82	<1 month before surgery
G†	breast cancer; postsurgical	female	61	163 months postsurgery
H	breast cancer; postsurgical	female	65	92 months postsurgery
I	breast cancer; postsurgical	female	63	36 months postsurgery
J	breast cancer; postsurgical	female	53	104 months postsurgery
K	breast cancer; postsurgical	female	50	1 month postsurgery
L	breast cancer; postsurgical	female	16	36 months postsurgery
M	breast cancer; postsurgical	female	101	1 month postsurgery
N	normal; non-prostate cancer	male	413	Random
O	normal; non-prostate cancer	male	554	Random
P	normal; non-prostate cancer	male	544	Random
Q‡	post-radical prostatectomized	male	84	7 months postsurgery
R	post-radical prostatectomized	male	132	10 months postsurgery
S	post-radical prostatectomized	male	420	21 months postsurgery

* The volume injected into the HPLC column was ~500 μL for all samples, with the exception of cases D, E, F, and J, which were 200 μL, 100 μL, 100 μL, and 460 μL, respectively.
† Patients I to M are still in remission; for patients G and H no current clinical status was available.
‡ Patients Q, R, S are still clinically asymptomatic but biochemically relapsed.

els D,E,F). PSA-ACT complex constitutes a minor molecular form in the presurgical serum of the 3 females with breast cancer. Fractions from 7 postsurgical sera were also analyzed in the same manner (Figure 2). Our results show that the predominant molecular form of PSA in the 6 of 7 postoperative sera is a complex with ACT. The present clinical status for cases G and H of Table 1 is unknown, but all other subjects are in remission for the times indicated. The predominant molecular form of PSA for case G is F-PSA.

Immunofluorometric PSA determination of serum fractions from 3 normal male sera and 3 sera from post-radical prostatectomized subjects with prostate cancer indicated that the major PSA species in all of these serum samples is the PSA-ACT complex. Representative data are shown in Figure 3. F-PSA is the minor molecular form of PSA in these sera.

Discussion

PSA is primarily produced and secreted by the columnar epithelial cells of the prostate (1,2). Briefly, PSA is translated as a 261 amino acid pre-proPSA precursor. It enters the secretory pathway when the signal peptide represented by the pre-region (17 residues) is removed in the endoplasmic reticulum. The resulting inactive proPSA (zymogen) is exocytosed into the lumina of the prostate ducts. The release of 7 N-terminal residues results in the 237-amino acid mature extracellular form, enzymatically active PSA. The protease(s) responsible for the formation of the active PSA *via* proPSA cleavage has not yet been identified. The primary biologic role of PSA is to increase sperm motility *via* the cleavage of the major seminal gel-forming proteins semenogelin I, II, and fibronectin in seminal fluid (SF) into small peptides. Although the majority of the PSA in SF is enzymatically active, about 20–30% is inactive, primarily due to clipping between residues 145–146 (lysine-lysine) (14). The nicked PSA remains connected by the internal disulfide bonds, but does not complex to any protease inhibitors.

The predominant form of immunoreactive PSA in the male serum is the one complexed to ACT (13,14). Our results confirm that the minor PSA species is, indeed, F-PSA in normal male serum and serum of post-radical prostatectomy prostate cancer patients (Figure 3). The F-PSA in serum has not been fully characterized. The uncomplexed and enzymatically inactive PSA could be either the internally clipped PSA or the 244 amino acid proform (zymogen), or even KLK2, a kallikrein highly homologous to PSA. Although PSA may possibly be autocatalytic, the cleavage sites observed are highly suggestive of a trypsin-like enzyme. A speculation has been made that this trypsin-like activity and, hence, the inactivation of PSA by nicking may be attributable to KLK2 (19). However, it seems that this inactivation occurs before PSA is released into the circulation, because the huge excess of protease inhibitors in the blood would have likely complexed with the otherwise nonclipped enzymatically active PSA.

The molecular characterization of immunoreactive PSA in cytosolic breast tumor extracts and

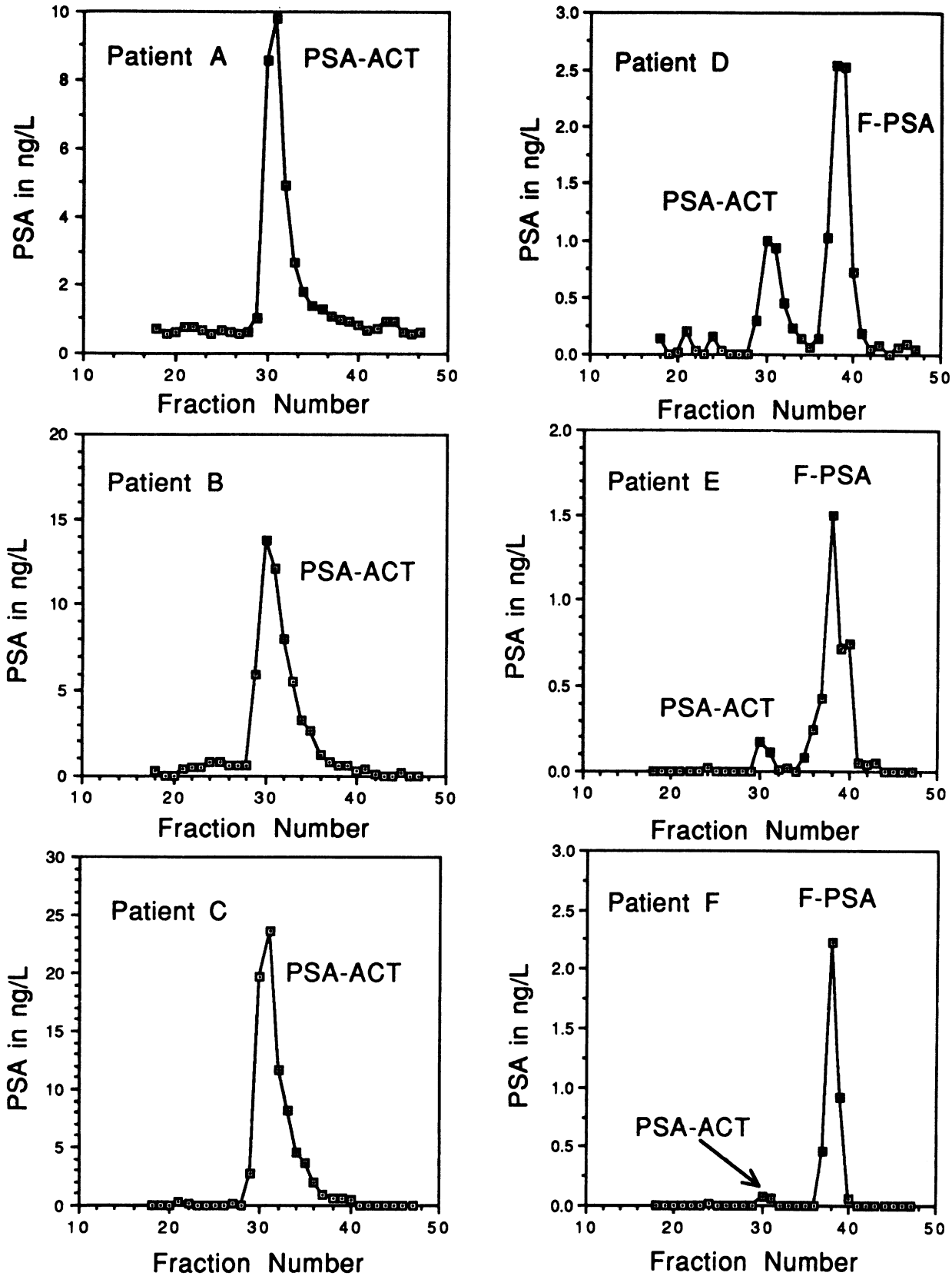

Figure 1 — Separation of total serum PSA by high-performance liquid chromatography and assay of the fractions by a highly sensitive time-resolved immunofluorometric methodology. Patients are described in Table 1. The PSA-α_1-antichymotrypsin complex (PSA-ACT) elutes at fraction 30 ± 1 (molecular weight of ~100 KDa). Free PSA (F-PSA) elutes at fraction 39 ± 1 (molecular weight of ~33 KDa). PSA is circulating as a complexed form (PSA-ACT) in the serum of healthy women, and the major molecular form in the serum of breast cancer women is F-PSA.

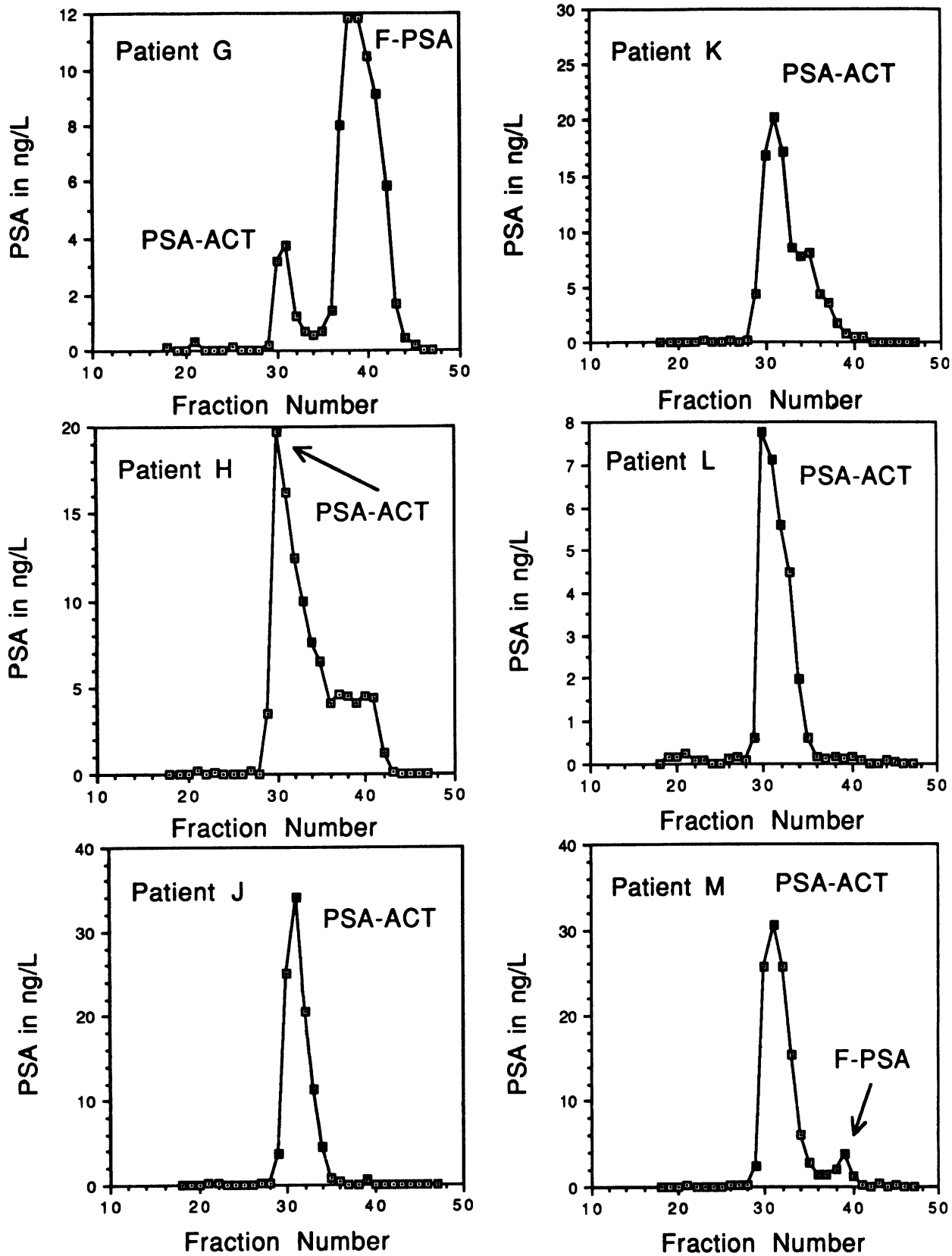

Figure 2 — Separation of total serum PSA by high-performance liquid chromatography and assay of the fractions with a highly sensitive time-resolved immunofluorometric methodology. Patients are described in Table 1. The data represent serum fractions of postoperative female patients with primary breast cancer. PSA-ACT elutes at fraction 30 ± 1 (molecular weight of ~100 KDa). Free PSA (F-PSA) elutes at fraction 39 ± 1 (molecular weight of ~33 KDa). PSA is circulating primarily as a complexed form (PSA-ACT) in 6 of 7 postsurgical sera of women with breast cancer (1 serum with 100% PSA-ACT is not shown). The minor molecular form of PSA in some postoperative sera is F-PSA. In patient G (for which no current clinical status of the disease was available), the primary molecular form is F-PSA; we suspect that this case represents breast cancer relapse.

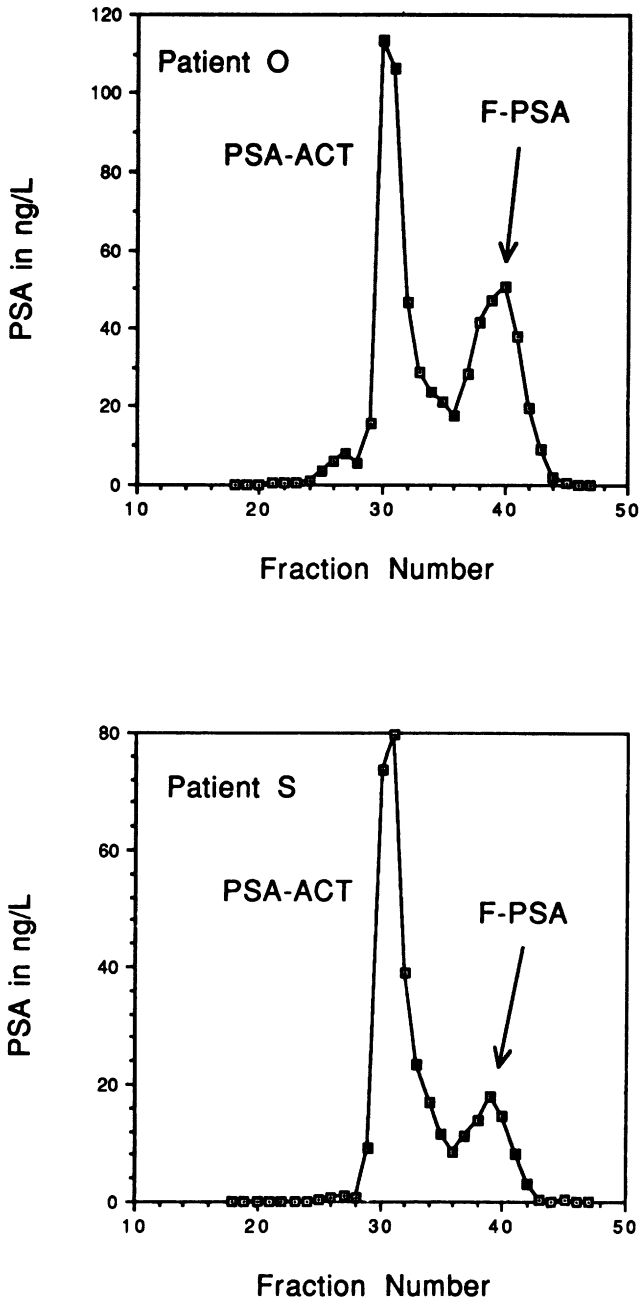

Figure 3 — Separation of male serum total PSA by high-performance liquid chromatography and assay of the fractions with a highly sensitive time-resolved immunofluorometric methodology. Patients are described in Table 1. The data represent serum fractions from a healthy male (upper panel) and a prostate cancer patient post-radical prostatectomy (lower panel). The PSA-α_1-antichymotrypsin complex (PSA-ACT) elutes at fraction 30 ± 1 (molecular weight of ~100 KDa), and the free PSA (F-PSA) elutes at fraction 39 ± 1 (molecular weight of ~33 KDa). For all sera from male patients, the predominant molecular form in circulation is PSA-ACT.

healthy breast tissue has shown that the predominant molecular form is the F-PSA (4,7). However, the presence of an enzymatic activity or the determination of its physicochemical and biomolecular properties have not been examined in the breast as yet, mostly due to the production and presence of minute amounts in comparison to those of the prostate gland. We have previously demonstrated that fewer than 5% of women have serum PSA concentrations of ≥ 50 ng/L (15). A recent study involving the measurement of PSA with an optimized ultrasensitive assay (biological detection limit of 1 ng/L) (18) from sera of 212 healthy women, revealed that 32% of the women had PSA values of ≤1 ng/L and the median was 2 ng/L. We have previously reported, in a study examining female serum total PSA levels, that there is no association of breast tumor PSA levels with serum PSA, either pre- or postoperatively and, also, no substantial difference of serum PSA levels between healthy women and women with breast cancer (17). The results of the present study indicate that the predominant and, quite possibly, the only molecular form of circulating PSA existing in the serum of healthy women is PSA complexed with ACT (Figure 1). Moreover, the predominant molecular form of PSA in the presurgical serum of women with breast cancer is the F-PSA; presumably the internally clipped and nonenzymatically active form of PSA. The results indicate that the female serum presents differences with respect to the presence of PSA molecular form variants between healthy and breast cancer-afflicted subjects. Determination of the PSA molecular forms in 7 postoperative sera from women with breast cancer indicated, with one exception that we speculate to be a relapsed case, that the major PSA molecular form is the PSA-ACT complex. It seems that the degree of posttranslational modification with reference to PSA clipping could be a distinquishable feature for the diagnosis and monitoring of breast cancer.

PSA, a kallikrein-like serine protease with chymotrypsin-like enzymatic activity, involved directly in the liquefaction of the seminal coagulum, may have new biological functions in nonprostatic normal tissues and metastatic sites (reviewed elsewhere) (20).

Our extensive, recently published data on nonprostatic PSA and the data presented here allow us to propose a simple diagram covering PSA production by breast epithelial cells (Figure 4). We suggest that normal breast epithelial cells secrete enzymatically active PSA that binds to α_1-antichymotrypsin when it enters the general circulation. Breast cancer cells seem to produce enzymatically inactive PSA that does not bind to ACT and circulates as a free 33 KDa protein. We still do not know if free PSA represents internally clipped PSA, pro PSA, KLK-2, or even mutant PSA produced by the tumor. Alternatively, the tumor may produce an endopeptidase that cleaves enzymatically active PSA. The consequences of the loss of enzymatically active PSA from the breast are not known, nor it is known if this loss occurs before or after the malignant transformation.

To summarize, our report examines the molecular forms of PSA in the serum of healthy women and women with breast cancer. The results indicate that the molecular forms of PSA differ in females with or without breast cancer. The clinical value of PSA mo-

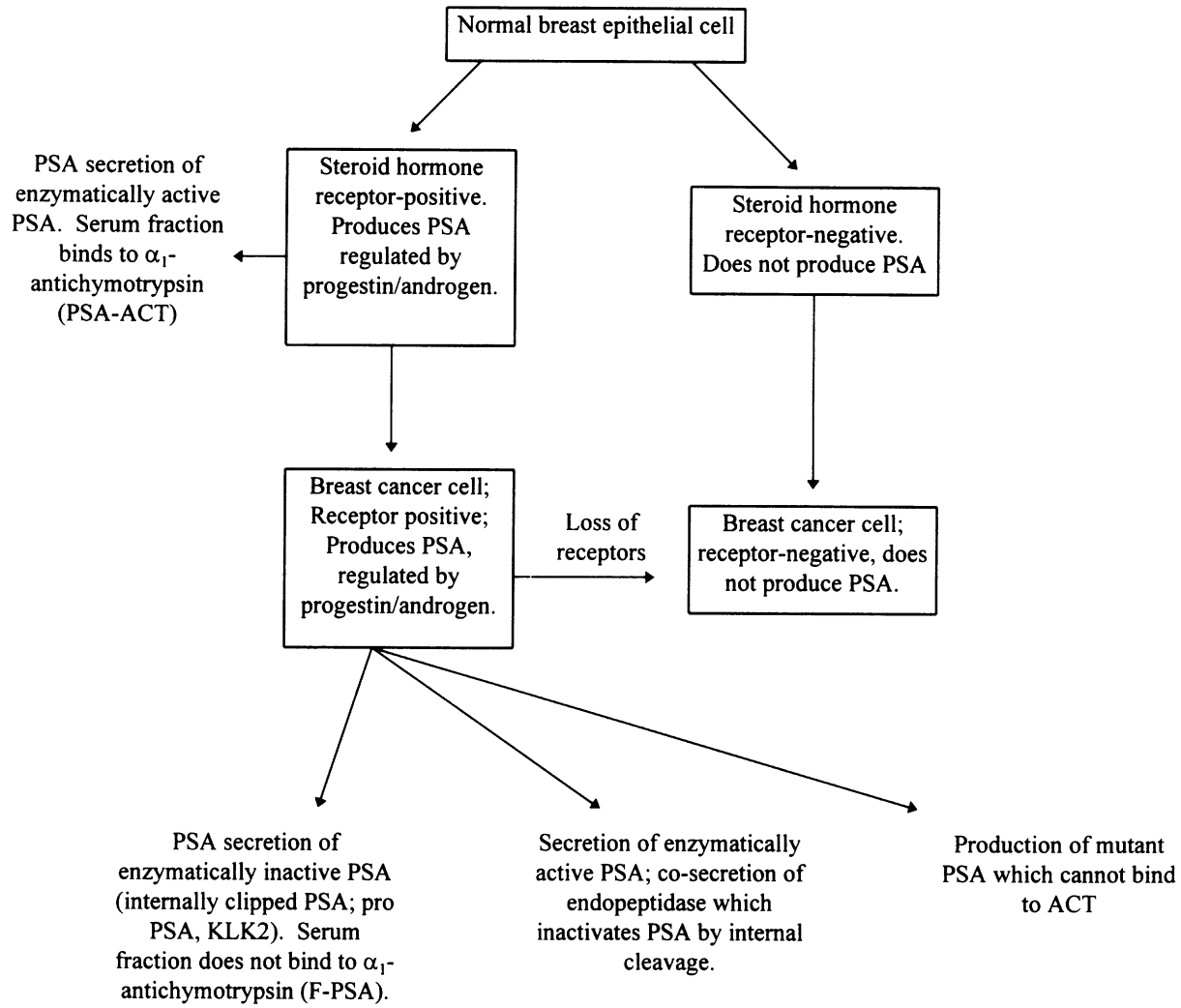

Figure 4 — PSA is produced by steroid hormone receptor-positive breast epithelial cells under regulation by progestins/androgens. Normal epithelial cells produce and secrete enzymatically active PSA that binds to α_1-antichymotrypsin when it enters the general circulation. Breast tumors seem to produce and secrete enzymatically inactive PSA (either internally clipped PSA, pro PSA, or KLK-2) that cannot bind to α_1-antichymotrypsin when it enters the general circulation.

lecular forms was also examined by other investigators for males (21). Determination of the proportions of F-PSA and PSA-ACT may assist in the discrimination of prostate cancer and benign prostatic hyperplasia (BPH). The observation reported in this article concerning females with and without breast cancer, as well as during treatment, needs confirmation with a larger study. The prospect of measuring PSA molecular forms in female serum may prove clinically useful for the diagnosis and management of breast cancer.

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