Prostate-Specific Antigen in Milk of Lactating Women

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Prostate-specific antigen (PSA) is believed to be a highly specific marker for normal and cancerous prostatic tissue. We recently found that 30–40% of breast tumors produce PSA. Other data from our group suggest that normal breast can also produce PSA under conditions of stimulation by steroid hormones. In addition, we detected PSA in amniotic fluid. Here we report the presence of PSA in breast milk of lactating women. PSA concentrations in breast milk were quite variable, ranging from <0.01 μg/L in 4 of 38 milks to 350 μg/L; the median was 0.47 μg/L. PSA concentration in breast milk was not correlated with mother's age or the sex of the newborn. It did tend to decrease with increasing time postdelivery, but was still detectable 2 weeks postdelivery. PSA in milk was equally measurable by a highly sensitive PSA assay based on time-resolved fluorometry and by the IMx® automated PSA method. As confirmed by Western blot analysis, PSA in milk was present predominantly in its 33-kDa form; the PSA–α_1-antichymotrypsin complex (100 kDa) was also present but its concentration was <25% of total PSA. We conclude that the female breast can produce PSA and that PSA is secreted into the milk during lactation; however, the biological role of PSA in milk is unknown. These and other data presented by our group suggest that PSA, a serine protease, may play a role in control of growth in mammary and other tissues through regulation of growth factors, cytokines, and growth-factor-binding proteins.

Indexing Terms: *breast/breast cancer/breast milk/growth factors/serine proteases*

Prostate-specific antigen (PSA) is a 33-kDa serine protease present at high concentrations in sperm.[2] Originally thought to be secreted exclusively by the epithelial cells of the prostate gland (*1, 2*), more recently this protein has been demonstrated in the periurethral and perianal glands (*3–5*) and very rarely in tumors of the salivary glands (*6*). We have found that PSA is present in 30% of female breast tumors and that its production in these tumors is associated with the presence of steroid hormone receptors (*7, 8*). We have also observed a significant advantage in both disease-free and overall survival of breast cancer patients whose tumors are PSA-positive (unpublished data). In addition, we have reproduced the phenomenon of PSA production by breast tumors by using breast cancer cell lines stimulated by various steroid hormones (*9*).

We speculate that the normal female breast may have the ability to produce PSA under conditions of stimulation by steroid hormones. To examine this possibility, we have analyzed milk from lactating women at various times postdelivery. If steroid hormones produced by the placenta could stimulate the normal breast to produce PSA, the mechanism might be similar to PSA production by breast cancer cell lines stimulated in vitro with steroid hormones (*9*). As reported here, our data suggest that PSA is produced in relatively high amounts by the normal breast of lactating women and that PSA is secreted into the milk. We do not yet know if PSA in milk has any physiological significance.

Materials and Methods

Samples

Breast milk, collected aseptically from women postdelivery, was stored at −20°C until analysis (within 3 weeks). For PSA assay, the breast milk was thawed and centrifuged at 12 000*g* for 10 min. After removing the top lipid layer, we analyzed the clear supernate.

Procedures

Immunoassays. PSA in milk was analyzed by a highly sensitive and specific immunofluorometric procedure described in detail elsewhere (*10*). Briefly, the PSA assay uses a mouse monoclonal anti-PSA capture antibody coated to polystyrene microtiter wells, a biotinylated polyclonal rabbit detection antibody, and alkaline phosphatase (ALP)-labeled streptavidin. In the assay, 50 μL of sample is first incubated with the coating antibody in the presence of 50 μL of assay buffer. After incubation for 3 h and six washes, the biotinylated polyclonal anti-PSA antibody is added and incubated for 1 h. After washing (six more times), ALP–streptavidin conjugate is added, incubated for 15 min, and washed again (six times). The ALP activity is measured by adding the substrate 5-fluorosalicyl phosphate, incubating for 10 min, and then adding a Tb^{3+}-EDTA solution to form a ternary fluorescent complex between the released 5-fluorosalicylate, Tb^{3+}, and EDTA. The fluorescence is measured in the time-resolved fluorometric mode. A similar assay, which measures specifically the PSA–α_1-antichymotrypsin complex, was also used as described in detail elsewhere (*10*). PSA was also measured in milk with the IMx® automated PSA method from Abbott Labs., Abbott Park, IL (*11*).

Milk samples with PSA concentrations >10 μg/L

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[2] Nonstandard abbreviations: PSA, prostate-specific antigen; ALP, alkaline phosphatase; and IGFBP, insulin-like growth factor binding protein.

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were diluted in PSA-negative female serum and reanalyzed. Further dilution experiments with female serum as diluent were performed to exclude the possibility of matrix artifacts with the immunofluorometric procedure or the IMx assay.

HPLC. For HPLC we used a Shimadzu system (Shimadzu Corp., Kyoto, Japan), isocratically, with a mobile phase of 0.1 mol/L Na_2SO_4–0.1 mol/L NaH_2PO_4, pH 6.80, at a flow rate of 0.5 mL/min. The column was a 600 × 75 mm Bio-Sil SEC-250 (Bio-Rad Labs, Richmond, CA), calibrated with a molecular mass standard solution from Bio-Rad. Fractions (0.5 mL) were collected after injection of the sample and analyzed for PSA and for the PSA–α_1-antichymotrypsin complex (*10*).

Western blot analysis. All necessary reagents and equipment for Western blot analysis were purchased from Novex, San Diego, CA; the manufacturer's protocols were followed throughout. Samples for Western blot analysis were electrophoresed under reducing conditions on 8–16% gradient polyacrylamide minigels, and the proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham International, Arlington Heights, IL). Membranes were blocked for 1 h and further processed as recommended by the manufacturer of the ECL–Western blot detection kit (Amersham). Briefly, the membrane was probed with a polyclonal anti-PSA antibody (Medix Biotech, Foster City, CA; 1 g/L, diluted 2000-fold in the blocking buffer), washed, and reacted with a horseradish peroxidase-conjugated goat anti-rabbit antibody. The enzyme activity on the membrane was marked by chemiluminescence recorded on x-ray film. Biotinylated molecular mass markers were visualized by reacting with a streptavidin–horseradish peroxidase conjugate added simultaneously with the goat anti-rabbit antibody.

Results

We initially analyzed 38 different milk samples from 38 lactating women; the distribution of PSA results was as follows:

PSA concn, μg/L	No. of samples
<0.010	4
0.010–0.049	6
0.050–0.099	4
0.10–0.49	5
0.50–0.99	4
1.00–3.99	5
4.00–9.99	5
>10	5

All but four milk samples contained detectable amounts of PSA by the method used (detection limit 0.01 μg/L) (*10*); the median concentration was 0.47 μg/L. Five milk samples had extremely high PSA concentrations: 10.6, 15.0, 15.6, 72, and 350 μg/L.

The distribution of PSA concentrations in 16 samples for which we had information on time of collection postdelivery, sex of newborn, and mother's age is shown in Fig. 1. We found no statistically significant linear correlation between PSA concentration and either mother's age or time of milk collection postdelivery, even after logarithmic transformation of PSA concentrations. However, we did see a trend between the logarithm of PSA concentration and time of milk collection postdelivery in days: The slope was −0.21 (P = 0.11), and the correlation coefficient was 0.42 (P = 0.10). For a log-log plot (logarithm of PSA concentration vs the logarithm of time of milk collection), the slope was −1.93 (P = 0.059) and the correlation coefficient increased to 0.48 (P = 0.061). These data suggest that PSA concentration in milk declines with time postdelivery. This trend, shown in Fig. 1A, is supported by the observation that the highest PSA concentrations in milk were associated with milk collected between 3 and 4 days postdelivery (the usual time of initiation of milk production in lactating women).

The distribution of PSA concentrations in milk according to the sex of the newborn is shown in Fig. 1B. The mean PSA concentrations of the two distributions were not significantly different (unpaired t-test, P = 0.31).

We further studied PSA concentrations in milk from two women for whom we had serial postdelivery milk

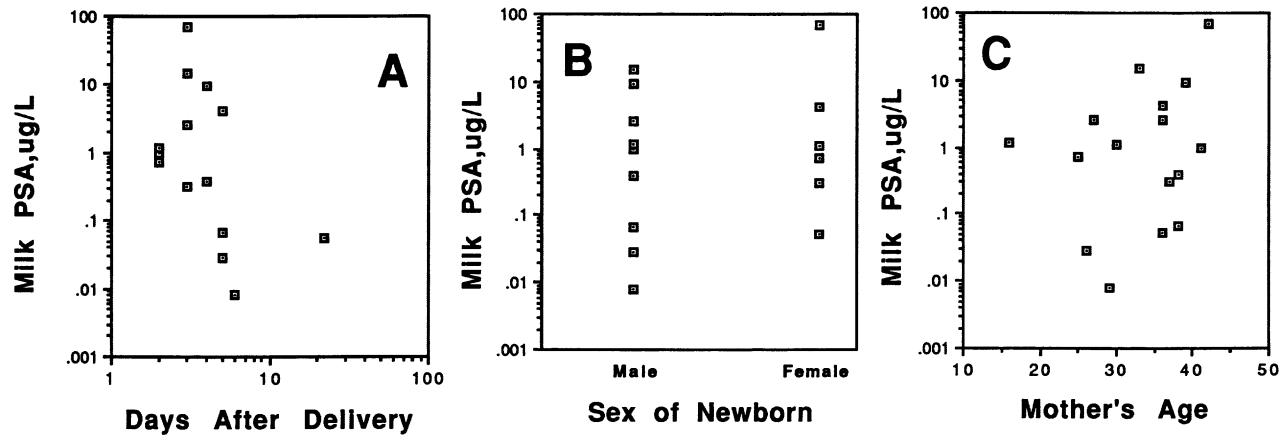

Fig. 1. PSA concentrations in 16 milk samples obtained at various times postdelivery (*A*) and plotted according to the sex of the newborn (*B*) or maternal age (*C*).

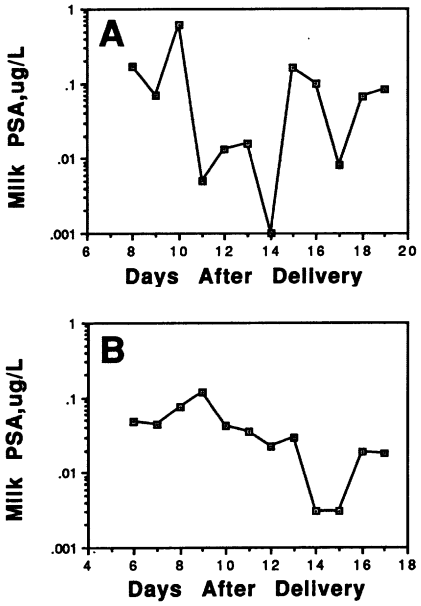

Fig. 2. Changes of PSA concentrations in milk according to days postdelivery in two different women (*A* and *B*).

samples. PSA was still detectable 17–18 days postdelivery but seemed to decline with time (Fig. 2).

To exclude matrix effects from the milk in the performance of the PSA assay, we diluted from 2- to 160-fold one milk sample having a high PSA concentration with PSA-negative female serum. The relationship between PSA concentration and dilution was linear in the PSA concentration range between 0.30 and 7 μg/L (Fig. 3). PSA concentrations >10 μg/L fall outside the range of our PSA assay (*10*). We further compared PSA values in milk obtained with our assay (y) (*10*) and with the IMx method (x), which is widely used for monitoring PSA in prostate cancer patients (*11*). For the 18 milk samples tested, the regression equation was $y = 1.01x - 0.53$, (r^2 = 0.988). The range of PSA concentrations in these samples was 0.1–70 μg/L.

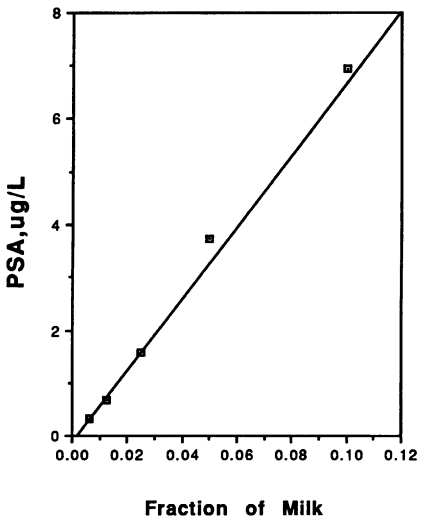

Fig. 3. Linearity of dilution of a milk sample with high PSA concentration (~72 μg/L) diluted with a PSA-free female serum sample and reanalyzed with our ultrasensitive PSA assay (*10*).

Dilutions ranging from 8- to 160-fold showed excellent linearity ($y = -0.143 + 72.1x$, $r^2 = 0.997$), confirming that the milk matrix does not affect the performance of the immunological assay.

We further characterized the molecular forms of PSA in breast milk by using size-exclusion HPLC (Fig. 4). Two milk samples with PSA concentrations of ~72 and 350 μg/L were injected into the HPLC column (50-μL samples) and the collected fractions were analyzed by a method that measures both free PSA and PSA bound to α_1-antichymotrypsin or by a method that measures only the complexed PSA, as described elsewhere (*10*). The major immunoreactive fraction in both milks was free PSA (molecular mass, 33 kDa; Fig. 4). The complexed PSA, with a molecular mass of ~100 kDa, is specifically recognized by the ACT-PSA assay and constituted ~25% of PSA in one milk sample and <10% in the other. We previously demonstrated that the PSA in breast tumors is present in its free 33-kDa form (*6*). In male serum the PSA–α_1-antichymotrypsin complex constitutes the majority of total immunoreactive PSA (*10*).

We also characterized the presence of PSA in breast milk with Western blot analysis. A PSA band at 33 kDa was seen in three milks that were highly positive for PSA but was absent in three milks with very low PSA concentrations. PSA was also detected in tissue culture supernates from the LNCaP prostatic carcinoma cell line (positive control). In all milks tested and in an amniotic fluid sample, which was included for comparison, another immunoreactive band was detected at 25–28 kDa. However, this band was not measurable by the immunofluorometric procedure (*10*) and was not detected by nonimmune rabbit serum. The identity of this band is currently under investigation.

Discussion

The physiological role of PSA, a serine protease thought to be produced exclusively by prostatic epithelial cells, has been linked to the process of semen liquefaction after ejaculation (*12*). Because PSA was considered a specific biochemical marker of prostatic cells, it was proposed as an ideal tumor antigen useful for prostate cancer diagnosis, screening, monitoring and detection of micrometastasis (*1, 2, 13*); PSA presence in tumors of nonprostatic origin was reported as an extremely rare event (*6*). However, we have recently discovered that PSA is present in about 30–40% of female breast tumors (*7, 8*). The presence of PSA is associated with breast tumors that are steroid hormone receptor-positive and with patients with early-stage disease and age <55 years (*7, 8*). Preliminary clinical data (unpublished) suggest that PSA is a favorable prognostic marker in breast cancer.

We have recently studied breast carcinoma cell lines in culture and found that these do not produce PSA. However, cell lines that are steroid hormone receptor-positive could be stimulated by glucocorticoids, androgens, and progestins, but not estrogens, to produce PSA (*9*). We have thus assumed that steroid hormone receptor-positive breast cells have the capacity to express PSA, provided they are stimulated by the corresponding steroids. We do not as yet know if the subset of breast

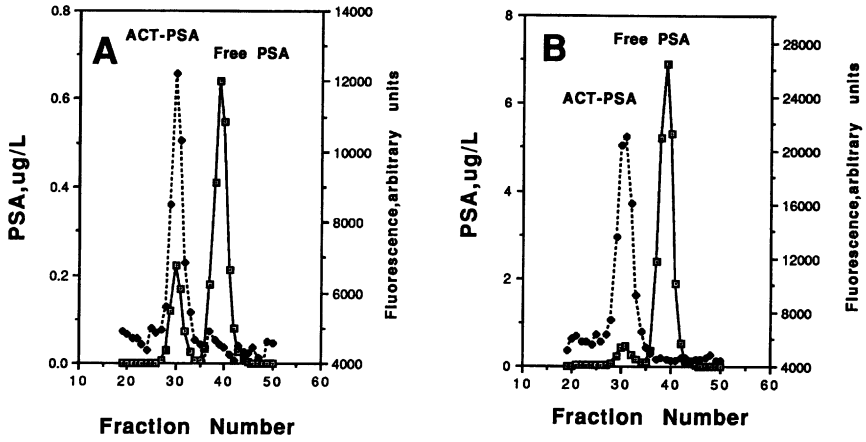

Fig. 4. Gel-filtration HPLC fractions (0.5 mL) analyzed for free PSA and for α_1-antichymotrypsin-bound PSA (ACT-PSA) by an assay that measures both (——) and by an assay that measures only ACT-PSA (- - - -) after injection of 50-μL milk samples with PSA concentrations of 72 μg/L (*A*) and 350 μg/L (*B*).

The response of the ACT-PSA assay is shown in arbitrary fluorescence units since no standard for the complex exists. The PSA assay detects two peaks, one at fraction 39 (free PSA, 33 kDa, major peak) and one at fraction 31 (100 kDa, minor peak); the latter peak is ACT-PSA, as confirmed by the ACT-PSA assay. The HPLC column was calibrated with molecular mass standards eluting at fraction 21 (660 kDa), 28 (160 kDa), 37 (44 kDa), 42 (17 kDa), and 19 (1.4 kDa).

tumors that produce PSA do so because of stimulation by adrenal, ovarian, or tumor-derived stimulants or because of loss of physiological repressors or other defects. However, we speculate that normal breast cells, which are steroid hormone receptor-positive, could produce PSA under conditions of stimulation by steroid hormones. We recently found a patient who was receiving progestin-containing oral contraceptives and from whom we had normal breast tissue available for experimentation. This stimulated breast tissue contained very high concentrations of PSA; breast tissue from eight women not receiving any medication were found to be PSA-negative (unpublished data).

In examining whether the milk of lactating women contains PSA, we hypothesized that the massive amounts of steroid hormones produced by the placenta during pregnancy could stimulate PSA production in the breast of pregnant women and that PSA could be secreted into the milk. We found that the vast majority of milk samples examined contained PSA (see *Results*), although PSA concentrations seemed to decline with time postdelivery (Figs. 1A and 2), perhaps because of the decrease in stimulating steroids after removal of the placenta. Most of the PSA in milk was present in its free 33-kDa form; a minor fraction was bound to α_1-antichymotrypsin. Free PSA was also predominantly present in breast tumors (*7*), normal breast tissue, and amniotic fluid (unpublished data). In contrast, the majority of PSA in male serum is bound to α_1-antichymotrypsin (*10*). We assume that the free PSA fraction is enzymatically active but we have not yet tested this hypothesis.

The PSA concentrations in the milk samples tested varied considerably, from <0.01 μg/L to 350 μg/L. The reason for this variation is unknown, but we speculate that the concentrations of steroid hormone receptors in the breast are involved, given our previous findings that PSA production in breast tumor tissue or cell lines is mediated by the steroid hormone receptors (*7–9*).

The 25–28-kDa band was seen in Western blots of all milks examined and in amniotic fluid but was not measured by the "sandwich-type" assay for PSA (*10*). We have not as yet characterized this band; it may represent a PSA fragment or a PSA-like cross-reacting molecule recognized only by the polyclonal anti-PSA antibody.

The physiological role of PSA production by the breast epithelial cells during pregnancy is currently unknown. However, recent data on prostatic tissues offer clues that PSA may be involved in growth regulation of mammary and other tissues. The sequence of the PSA shows extensive homology with γ-nerve growth factor (56%), epidermal growth factor binding protein (53%), and α-nerve growth factor (51%) (*14*). PSA can hydrolyze the A and B chains of insulin and recombinant interleukin-2 (*14*), and can enzymatically digest insulin-like growth factor binding protein III (IGFBP-3), the major binding protein of insulin-like growth factor I. This latter activity is thought to regulate the concentration of insulin-like growth factor through release from IGFBP-3 (*15*). Additional data support the hypothesis that PSA is a regulator of IGFBP-2 and IGFBP-3 in patients with prostate cancer (*16*). Killian et al. (*17*) have recently found that PSA has mitogenic activity, due to activation by PSA of latent transforming growth factor-β and modulation of cell adhesion. Others have shown that PSA binds and inactivates protein C inhibitor (*18*).

Our findings of PSA in primary and metastatic (*19*) breast tumors, stimulated normal breast tissue, amniotic fluid, and breast milk and the data presented by others suggest that PSA can no longer be regarded as a specific prostatic marker and as a physiological molecule associated only with semen liquefaction. Instead, PSA should be regarded as a molecule that could be produced by cells bearing steroid hormone receptors when exposed to conditions of steroid hormone stimulation. PSA may also be a new candidate growth factor or growth factor regulator. Its biological role in breast milk needs further investigation.

Finally, in parallel with other known tumor markers (e.g., α-fetoprotein), PSA, now found in primary and metastatic tumors and during pregnancy may represent another oncofetal tumor marker.

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