K1 deficiency occurs in elderly subjects (4–7) days after bone fracture. It is known that vitamin K1 deficiency occurs in elderly subjects (6). However, in our study, because all the subjects were males and their ages were not relatively old, together with the fact that the age range of the controls was similar to that of the patients, it is unlikely that age had an effect on our observations. In control subjects, vitamin K1 concentrations in serum can be calculated from the concentrations of apolipoproteins A1 and B (3). However, in patients after bone fracture, the changes in serum vitamin K1 concentrations are not paralleled with changes in their carrier systems, the lipoproteins. There is no evidence that after bone fracture apolipoprotein A1 and B concentrations in serum are altered. It appears that serum vitamin K1 is utilized independent of its lipoprotein carriers in serum. These observations support the concept that vitamin K1 is sequestered from lipoproteins in the circulation for use, perhaps at the fracture site (4). The current results indicate that the mode of sequestration of vitamin K1 is independent of the metabolism of lipoproteins, an observation that has not previously been reported. In patients without bone fractures, the vitamin K1 concentration can be predicted from the concentrations of apolipoproteins A1 and B, whereas it cannot be predicted by the same equation in patients with bone fractures. The mechanism of vitamin K1 delivery to the fracture site remains to be elucidated, but it could conceivably operate via a putative receptor in which the vitamin K1 is selectively taken up, analogous to the interaction of HDL with cells without the loss of its apolipoprotein components. This study further indicates a need to validate the use of equations containing the serum concentrations of apolipoproteins A1 and B to calculate vitamin K1 concentrations in serum (3) in patient groups where changes in vitamin K1 metabolism are in question.

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Prostate-specific antigen (PSA) is a member of the human kallikrein family of serine proteases that for a long time was thought to be produced exclusively by the epithelial cells of the prostate gland (1). Because of its tissue specificity, PSA has been widely used as a marker for diagnosing and monitoring prostate cancer (2). However, recent studies have demonstrated the widespread distribution of PSA in a variety of tumor types, healthy tissues, and biological fluids [reviewed in Ref. (3)]. Although the physiological role and the biological significance of extraprostatic PSA currently are unknown, it has been suggested that this serine protease should be regarded as a growth factor regulator produced by cells bearing steroid hormone receptors (4, 5). Several studies have biochemi-
cally and molecularly demonstrated that female breast produces and secretes PSA through steroid hormone regulation (in particular by androgens and progestins but not estrogens) (6, 7). Moreover, PSA has been suggested as a marker of good prognosis for women with breast cancer (8), even if there is contradictory evidence regarding this argument (9, 10).

Over recent years, analysis of nipple aspirate fluids (NAFs) from nonlactating women has attracted considerable interest as a rapid and noninvasive method to assess the environment and metabolic activity within the mammary gland (11). Light microscopic studies have revealed the presence of abnormal apocrine epithelial cells in fluids from women with breast diseases, allowing the identification of women who may be at increased risk of developing breast cancer (12). Similarly, biochemical analysis of breast NAFs has demonstrated the presence of a variety of substances, including hormones, enzymes, electrolytes, proteins, and toxic compounds, that may be involved in the malignant transformation (13–15). Recent studies have demonstrated the presence of measurable amounts of total PSA in NAF, which are inversely correlated with breast cancer risk and associated with and/or influenced by progesterone action (16–19).

In the frame of a study on breast NAFs, we recently described the biochemical profile and the ultrastructural features of the cellular components of a subtype of NAF—previously named type II NAF (15)—demonstrating that it is characterized by the presence of some peculiar milk proteins and by biosynthetically active apocrine cells. Interestingly, this NAF subtype shows a higher content of PSA in extracellular fluid with respect to milk (Malatesta et al., submitted for publication). In the present study, we evaluated the distribution of the molecular forms of PSA present in NAF and we localized ultrastructurally PSA molecules to investigate the capability of breast apocrine cells found in NAF to produce and/or secrete this serine protease.

After informed consent, NAFs were collected by manual compression of the four periareolar breast quadrants from 75 nonlactating women, including 50 women (ages, 21–33 years) with benign breast diseases (diagnosed on the basis of clinical, echographic, and/or mammographic and cytological approaches) and 12 (ages, 35–42 years) with infiltrating ductal carcinoma (diagnosed by surgical biopsy). The remaining 13 subjects (ages, 24–36 years) were healthy control women. Women reporting pregnancy, lactation, or clinical symptoms referable to the breast within 3 years prior of this study as well as those who had been medically, hormonally, or surgically treated for breast disease during the previous 6 months were excluded from this study.

For biochemical studies, NAF samples (1–500 μL) were centrifuged at 19 000g for 15 min at 4 °C; after removal of the top lipid layer, the clear supernate was analyzed for free and total PSA content (expressed as μg/L) by a commercially available kit based on a microparticle enzyme immunoassay with a mouse anti-human monoclonal antibody (AxSYM®; Abbott Laboratories). The PSA determination procedure, performed according to the manufacturer’s recommendations, has been described in detail elsewhere (20). 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. To exclude the possibility of “matrix” artifacts caused by interfering substances present in NAF (such as lipids, proteins, and hormones), the samples were serially diluted in PSA-negative healthy female serum and reanalyzed for the response linearity (range of dilution, 0- to 90-fold). The analytical recovery of at least two concentrations of purified human PSA (Sigma) added to the NAF supernatants was tested, and the recovered amount of PSA was calculated by subtracting the concentration initially present from the measured concentration after the addition (21).

NAF protein components were separated on a 600 × 9 mm column of Sephacryl S-200 (Pharmacia) and eluted according to the previously described method (22). Reagents and equipment for Western blotting were purchased from Bio-Rad; our protocols were followed throughout, using an anti-human PSA monoclonal antibody (Dako) (23). For thermal inactivation studies, aliquots of NAF were incubated in a thermostable water bath for different lengths of time at 55 °C or at different temperatures (45, 55, 65, 75, and 85 °C) for 30 min (22). Five repeated freeze-thaw cycles were also performed using NAF aliquots stored in cryotubes (Nunc). The results obtained from two independent experiments and at least in triplicate (mean ± SE) were statistically analyzed using the StatView, Ver. 4 package (Abacus Concepts) on a Macintosh Power PC (Apple).

To obtain a detectable amount of immunolabeling, electron microscopic analysis was carried out on NAF samples previously demonstrated to contain large amounts of PSA. Immediately after collection, NAFs were mixed 1:1 by volume with the fixative solution (0.1 mol/L Sørensen phosphate-buffered 80 mL/L paraformaldehyde and 10 mL/L glutaraldehyde, pH 7.4). Fixation was performed for 1 h at 4 °C after which the cell suspensions were centrifuged at 340g at 4 °C for 10 min. The supernatants were removed, and the cell pellets were washed in Sørensen buffer and embedded in 1.5% agar-agar. After the specimens were washed in phosphate-buffered saline, free aldehydes were blocked in 0.5 mol/L NH₄Cl in phosphate-buffered saline at 4 °C for 45 min. After another wash in phosphate-buffered saline, the specimens were dehydrated through graded concentrations of ethanol and embedded in LRWhite resin. Resin polymerization was carried out with ultraviolet light to avoid thermal PSA denaturation (22). Ultrathin sections were placed on nickel grids coated with a Formvar layer and then processed for immunocytochemistry using a rabbit anti-human PSA antibody (Biomedica) according to our previously reported protocol (24). Control grids were incubated under the same conditions as the experimental samples except that the primary antibody was omitted (22). Immunolabeled sections were observed in a Zeiss EM 902 electron microscope operating at 80 kV.
The present work was carried out in accordance with the ethical standards of Helsinki Declaration of 1975, as revised in 1983.

The mean NAF total PSA concentration in the women examined (n = 72) was 37.78 ± 8.11 μg/L, whereas the mean NAF free PSA concentration was 10.39 ± 2.23 μg/L (P < 0.001). The linearity and interference studies revealed a good correlation between PSA concentration and dilution (n = 8; r² = 0.98), demonstrating that “breast NAF matrix” did not affect the performance of PSA assay.

The analytical recovery of purified PSA added to NAF samples was 98% ± 5%. Assay reproducibility (CV) was determined by assaying NAF samples in replicates of three in at least two independent analyses; the within-run CV was 2.4%, and the between-run CV was 3.9%.

The mean concentration of total PSA in healthy subjects and in women with benign breast diseases (n = 63) was 62.84 ± 10.28 μg/L, compared with the mean value of 0.18 ± 0.03 μg/L found in NAF from patients with breast cancer (n = 12; P < 0.001). In all samples examined, the percentage of free PSA was of ~28% ± 4%. A significant difference was also found between patients with benign breast diseases and patients with breast cancer [17.28 ± 2.83 μg/L in NAF from women with benign breast diseases (n = 50) vs 0.09 ± 0.01 μg/L in patients with breast cancer (n = 12; P < 0.001)].

The representative PSA immunogram of Sephacryl S-200 columns (n = 10) of selected NAF samples (from eight women with benign breast diseases; mean NAF total PSA content, 40.55 ± 5.25 μg/L) confirmed that the major immunoreactivities were in fractions 40–100, where the α1-antichymotrypsin (ACT)-PSA complex and free PSA were expected; in fact, the higher molecular mass PSA fraction (presumably ACT-bound) was of ~100 kDa and constituted ~70% of the total PSA found in breast NAFs. This finding was corroborated by Western blotting analysis, revealing PSA staining bands corresponding to the expected positions of ACT-bound (~100 kDa) and free (~33 kDa) human PSA (Fig. 1A).

The thermal inactivation profile showed a linear temperature-dependent thermolability (n = 10; r² = 0.97), with residual immunoreactivity of ~60% after 30 min at 55 °C and ~10% after 30 min at 75 °C. The freeze-thaw procedure did not significantly affect either the immunoreactivity or the PSA fraction concentration.

Ultrastructural observations of immunolabeled samples revealed that the epithelial cells obtained from NAF showed a cytoplasmic signal located mainly on free ribosomes—very numerous in these cells—and on small vacuoles containing homogeneous material (Fig. 1B). Some labeling was also observed along the cell surface. Cell nuclei and mitochondria appeared devoid of gold grains. Control samples showed only a negligible signal.

These results are in agreement with recent findings of PSA in NAF: previous studies have well documented the presence of total PSA in breast NAFs and its correlation with the progesterone content (16, 19), even if there are contradictory results/opinions about its utility as a marker for breast cancer risk (17–18). Although the physiological role of this serine kallikrein protease is unknown, our results suggest that breast apocrine epithelial cells (in both physiologic and benign breast disease conditions) may produce high amounts of PSA, which may be correlated to steroid hormone stimulation (6). Moreover, the high percentage of ACT-bound PSA (~70%) in our NAF samples is in accordance with the previously demonstrated high concentration of complexed ACT.
found in benign breast diseases (25). In addition, the presence of PSA (in particular in the uncomplexed form) in NAF is in accordance with the high concentration of enzymes, proteases, and some biochemical analytes that may be prone to metabolic alterations in ductal epithelial cells of women with breast diseases (11). In another study we described at the electron microscopic level the epithelial cells present in NAFs, giving evidence for highly metabolizing cells (Malatesta et al., submitted for publication). Accordingly, the intense anti-PSA labeling on ribosomes, sites of protein synthesis, strongly suggests that NAF cells are able to synthesize this protease; moreover, the presence of some labeling along the cell surface could indicate a secretory activity. The small vacuoles containing PSA could represent storage/complexation sites of this protease, although we cannot exclude that they can be removed by exocytosis. Therefore, breast epithelial cells present in NAFs could be a candidate source of PSA found in nipple aspirate extracellular fluid; similarly, in type I breast gross cysts, the apocrine cells occurring in the cystic fluid are responsible for PSA production and secretion (24).

The production and secretion of several proteases (including PSA) and biologically active compounds by female breast apocrine cells in association with a lack of physiologic control of the secretion/reabsorption mechanism (11) and/or with prolonged exposure to several biologically active compounds through the autocrine/paracrine mechanism (13) could, with age, make the highly metabolizing apocrine cells prone to premalignant transformation (26). However, the clinical significance of the PSA molecular forms and of its production/secretion by breast epithelial cells with respect to breast cancer risk should be evaluated cautiously (17,18).

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