

Oral Contraceptive-induced Expression of Prostate-specific Antigen in the Female Breast*

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Prostate-specific antigen (PSA) is widely used as a tumor marker of prostatic adenocarcinoma. We recently found that 30% of breast tumors produce PSA and that PSA is a favorable prognostic marker in female breast cancer. We measured immunoreactive PSA in cytosolic extracts of normal breast tissue from eight women receiving no medication and one woman who was receiving the progestin-containing oral contraceptive Brevicon. None of the eight cytosolic extracts of normal breast tissue contained appreciable amounts of immunoreactive PSA. However, left and right breast tissues from the woman receiving Brevicon contained high levels of PSA. This immunoreactive species was shown to have a molecular weight identical to that of seminal PSA. Furthermore, reverse transcription of RNA and polymerase chain reaction amplification produced a 571-base pair cDNA that hybridized to a labeled cDNA PSA probe. Upon sequencing, the cDNA polymerase chain reaction product was found to have 100% homology with cDNA from prostatic tissue. PSA production by breast carcinoma cell lines was achieved after *in vitro* stimulation with norethindrone and ethinylestradiol. Our data suggest that PSA can no longer be regarded as a specific prostatic protein because it is produced by breast tumors with good prognosis and by normal breast tissue after steroid hormone stimulation.

Oral contraceptives are among the most widely used drugs, but the genes that are regulated by these agents are currently unknown. No genes have as yet been found which are specifically regulated by oral contraceptives in the female breast. We have recently found that about 30% of female breast tumors overexpress prostate-specific antigen (PSA)¹ (1), a protein that was thought to be produced exclusively by the epithelial cells of the prostate gland (2, 3). PSA overexpression in breast cancer is associated with the presence of steroid hormone receptors (4). The mechanism of such overexpression in breast cancer is currently obscure. It has been hypothesized that either ovarian or adrenal steroids or tumor-derived molecules derepress the

PSA gene through the steroid hormone receptors or that the PSA gene is constitutively overexpressed because of poststeroid hormone-receptor complex defects, loss of physiological repressors, or because of defects in the hormone response elements. We have observed a significant advantage in both overall and disease-free survival of breast cancer patients who are PSA-positive.² As PSA was found to be associated with more benign breast tumors, we speculated that it could also be expressed by normal breasts either under physiological circumstances or after steroid hormone stimulation. In this paper we demonstrate that the breasts of one woman who was receiving a progestin-based oral contraceptive contained high levels of PSA. This protein was absent from the breasts of eight other women who were not receiving any medication. These data strongly suggest that PSA is not a prostate-specific protein and that it can be produced by the female breast either in cases of malignancy or after stimulation by steroidal compounds. The biological role of PSA in either normal or malignant breast is currently under investigation.

MATERIALS AND METHODS

Patients—We have studied nine women who underwent breast reduction surgery for cosmetic reasons. Normal breast tissue was immediately frozen on dry ice after resection and was stored at -70°C until extraction. Eight of the nine women were not receiving any medication. One woman was receiving Brevicon, a highly prescribed oral contraceptive containing 1 mg of norethindrone (a progestin) and 0.035 mg of ethinylestradiol/tablet. Breast tissue was obtained from both the left and right breasts for each patient.

Preparation of Cytosolic Extracts—Breast tissue cytosolic extracts were prepared as described previously (1).

Measurements—PSA in the cytosolic breast extracts was measured with a highly sensitive and specific immunofluorometric technique described in detail elsewhere (6). PSA was also measured in the cytosolic breast extracts with commercially available kits: the Hybritech Tandem-E PSA kit (Hybritech Inc., San Diego) and the IMx automated PSA method (Abbott Laboratories, Chicago). PSA in the tissue was expressed as ng/mg of total protein.

High Performance Liquid Chromatography (HPLC)—HPLC was performed as described previously (6).

Western Blot Analysis—All necessary reagents and equipment for Western blot analysis were purchased from Novex (San Diego). The manufacturer's protocols were followed throughout. Samples for Western blot analysis were electrophoresed under reducing conditions on 8–16% gradient polyacrylamide minigels, and proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham Corp.). Membranes were blocked for 1 h and processed further as recommended by the manufacturer of the ECL-Western blot detection kit (Amersham Corp.). Briefly, the membrane was probed with a polyclonal anti-PSA antibody (from Medix Biotech, Foster City, CA, 1 mg/ml, diluted 2,000-fold in the blocking buffer), washed, and reacted with a horseradish peroxidase-conjugated goat anti-rabbit antibody. The enzyme activity

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¹ The abbreviations used are: PSA, prostate-specific antigen; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; bp, base pair(s); ACT, α_1 -antichymotrypsin.

² H. Yu, M. Giai, E. P. Diamandis, D. J. A. Sutherland, M. A. Levesque, R. Roagna, R. Ponzzone, P. Sismondi, and D. Katsaros, submitted for publication.

on the membrane and was revealed by chemiluminescence, captured on x-ray film. Biotinylated molecular weight markers were visualized by reacting with a streptavidin-horseradish peroxidase conjugate added simultaneously with the goat anti-rabbit antibody.

Tissue Culture—The T-47D and MCF-7 breast carcinoma cell lines and the LNCaP prostate carcinoma cell line were obtained from the American Type Culture Collection (Rockville, MD). These cell lines were cultured as described previously (7). Stimulation experiments with steroids, obtained from Sigma, were performed as described previously (7). PSA was measured in the supernatants of the tissue cultures with the immunofluorometric procedure (6).

RNA Extraction—Total RNA extraction was performed using the RNazol B method (Tel-Test Inc., Friendswood, TX) following the manufacturer's instructions.

cDNA Synthesis—One microgram of total RNA was used for the synthesis of the first strand of cDNA using the SuperScript II reverse transcriptase (Life Technologies, Inc.). Briefly, RNA, oligo(dT)₁₂₋₁₈ primers (100 ng), and random hexamer primers (100 ng) were first denatured for 5 min at 70 °C, chilled on ice for 1 min, and then incubated for 1 h at 37 °C in 20 μ l of a reaction mixture containing 1 \times first stand buffer, 250 μ M deoxynucleoside triphosphate mix (Boehringer Mannheim), 10 mM dithiothreitol, and 200 units of SuperScript II reverse transcriptase.

Oligonucleotide Primers—Oligonucleotides were synthesized by the Jefferson Cancer Institute Nucleic Acid Facility (Philadelphia) and used without further purification. The PSA oligonucleotide sequences used as primers were: PR5, 5'-AGCCCCAAGCTTACCACCT-3'; PR6, 5'-CAGACTGCCCTGCCACGA-3'; PR2 and PR3 sequences have been published previously (8). Previously published actin PCR primer sequences were used to rule out degraded RNA (9). Amplification yielded a 571-base pair (bp) PSA cDNA fragment with PR5 (from exon 1) and PR2 (from exon 4) oligonucleotide primers and a 154-bp cDNA fragment with actin oligonucleotide primers.

PCR Procedure and Southern Blot Analysis—One microliter of cDNA was added to 39 μ l of PCR mix containing 1 \times PCR buffer (Boehringer Mannheim), a 18 μ M concentration of each primer, 250 μ M deoxynucleoside triphosphate mix, and 1.25 units of *Taq* DNA polymerase (Boehringer Mannheim). PCR was performed with primers PR5 and PR2 for 30 cycles according to the following program: 94 °C for 1 min (2 min for the first cycle), 56 °C for 1 min, and 72 °C for 2 min (5 min for the last extension). Actin fragments were amplified from 1 μ l of the cDNA preparation under the same conditions used for PSA PCR. Twenty microliters of each PCR reaction was electrophoresed in 2% agarose gel and visualized by ethidium bromide staining.

After electrophoresis the PSA cDNA PCR products were transferred onto Hybond nylon membranes (Amersham) and hybridized with a PSA cDNA probe (kindly provided by Dr. Jose Moreno). Southern blotting, probe radiolabeling, hybridization, and autoradiography were performed by standard techniques (10). A second PCR amplification was performed on the remaining PCR reaction to provide sufficient material for subsequent analysis. Finally, the fragments were purified by ion exchange columns (Qiagen, Dusseldorf, Germany) according to the instructions of the manufacturer.

Sequencing—An automated 373A DNA sequencer (Applied Biosystems) and dye terminator kits from the same manufacturer were used for direct sequencing of the PSA cDNA fragments by the dideoxynucleotide chain termination method using fluorescent labels. The coding and noncoding strands of each fragment were sequenced with primers generating overlapping sequence data. Sequences were reassembled and analyzed using the SAP program (11).

RESULTS

We have prepared cytosolic extracts from 18 normal breast tissues removed from nine women (left and right breasts) during cosmetic breast reduction surgery. PSA immunoreactivity was measured in these extracts using a highly specific and sensitive immunofluorometric technique (6) and by two widely used commercial PSA assays. Breast extracts from eight of the nine women were found to contain <0.03 ng of PSA/mg of total protein and were considered negative for PSA. Two breast extracts from the same woman (left and right breasts) had a relatively high concentrations of PSA (0.11 and 1.53 ng/mg). None of the eight PSA-negative women was receiving oral contraceptives or other medications. The woman with PSA-positive breasts was receiving only one medication, Brevicon.

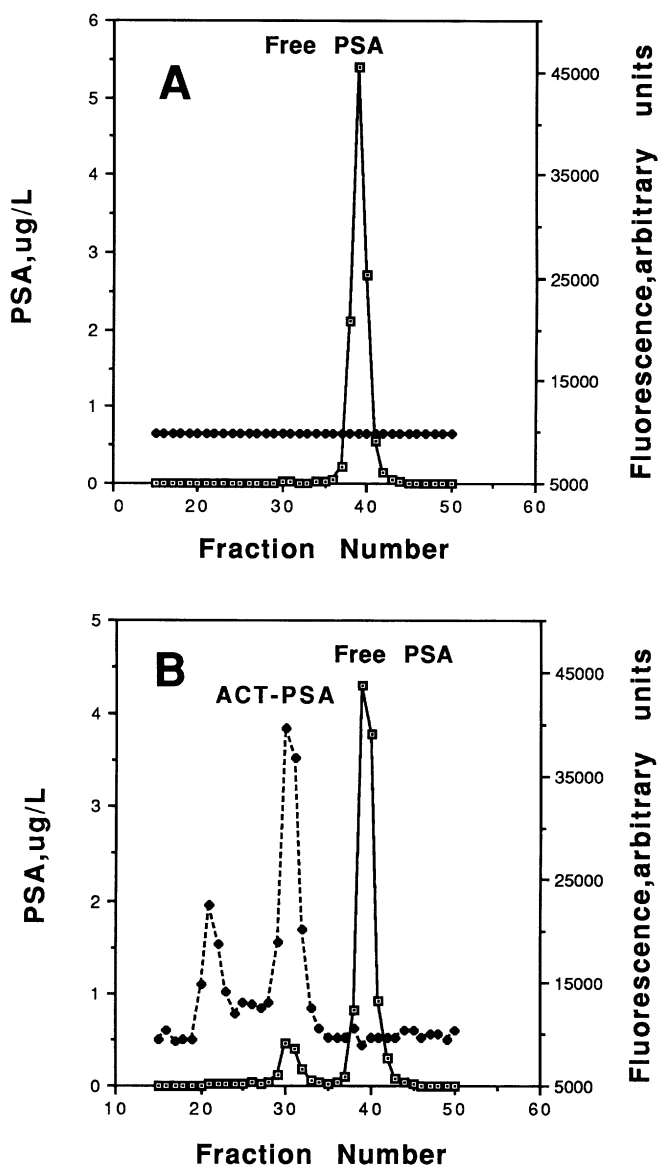


FIG. 1. HPLC with a gel filtration column. Details of the method are given in Ref. 6. Each HPLC fraction (0.5 ml) was analyzed with an assay that measures free and α_1 -antichymotrypsin-bound PSA (ACT-PSA) (\square) or an assay that measures only ACT-PSA (\blacklozenge). The response of the latter assay is in arbitrary fluorescence units since no ACT-PSA standard exists. *Panel A*, injection of purified seminal PSA, which elutes at fraction 39 corresponding to a molecular mass of 33 kDa. No ACT-PSA is detected. *Panel B*, injection of a breast extract from the woman receiving the oral contraceptive Brevicon. The PSA assay detects two peaks: one at fraction 39 (free PSA, major peak) and one at fraction 30 (100-kDa minor peak). The latter peak is ACT-PSA as confirmed by the ACT-PSA assay. The identity of the minor peak at fraction 21 (650 kDa) is unknown. These data confirm that more than 80% of the breast tissue PSA is in the free, 33-kDa form. 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 49 (1.4 kDa).

The PSA-positive and -negative results in the breast extracts by the immunofluorometric procedure were verified by using two widely used commercial PSA methods: the IMx from Abbott Laboratories and the Tandem-E kit from Hybritech Inc. Additionally, the highly positive breast extract was serially diluted in female serum from 2- to 32-fold and analyzed by immunofluorometry and the IMx assay. Excellent agreement between results was obtained. The serum from the patient on oral contraceptives and sera from another 10 women on oral contraceptives were analyzed for PSA and found to contain

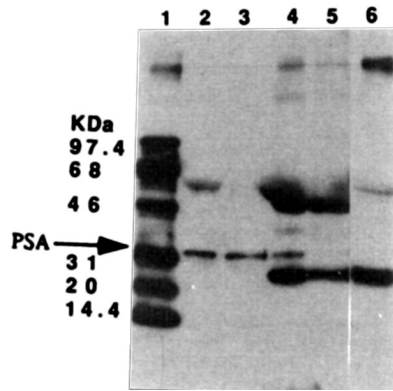


FIG. 2. **Western blot analysis.** Samples were electrophoresed on 8–16% gradient polyacrylamide minigels under reducing conditions, electrotransferred to nitrocellulose membranes, and probed with a rabbit polyclonal anti-PSA antibody. Detection was achieved by using a horseradish peroxidase-conjugated goat anti-rabbit antibody and chemiluminescence. *Lane 1*, molecular mass markers. *Lane 2*, purified seminal PSA dissolved in bovine serum albumin; the PSA band appears at 33 kDa (just above the marker at 31 kDa). *Lane 3*, supernatant from a prostatic carcinoma cell line (LNCaP) producing PSA. *Lane 4*, PSA-positive normal breast extract from the woman receiving Brevicon, containing a band at 33 kDa. *Lane 5*, another normal breast extract tested negative for PSA by the immunofluorometric procedure. *Lane 6*, an amniotic fluid tested for comparison; the identity of the band at 28 kDa, present in PSA-positive and PSA-negative normal breast extracts and in amniotic fluids, is unknown (16).

<0.02 $\mu\text{g/l}$ PSA.

The highly positive breast extract was subjected to HPLC (Fig. 1), and fractions were analyzed by two immunofluorometric procedures that measure either total PSA (free PSA plus PSA bound to α_1 -antichymotrypsin) or specifically the PSA- α_1 -antichymotrypsin (ACT) complex (6). More than 80% of the total PSA in normal breast was in the free, 33-kDa form; a small proportion was present as ACT-PSA complex (100 kDa). Another minor species, containing PSA and ACT, was also detected (660 kDa), but its identity is unknown. The presence of PSA in the highly positive breast extract was further confirmed by Western blot analysis (Fig. 2). The 33-kDa form of PSA, shown here to be present in normal breasts stimulated by oral contraceptives, is similar to the form of PSA found in breast tumors (1). In male serum, the majority of PSA is present as ACT-PSA complex with a molecular mass of 100 kDa (data not shown) (6).

To study the oral contraceptive-induced PSA production further, we cultured T-47D and MCF-7 breast carcinoma cell lines in the absence of any steroid hormones or in the presence of norethindrone or ethinylestradiol at various concentrations (Fig. 3). No PSA was detected in the tissue culture supernatants in the absence of steroid hormones after 11 days of confluent cultures. Ethinylestradiol stimulated low levels of PSA production at concentrations $\geq 10^{-8}$ M. Norethindrone was effective in mediating intense PSA gene expression at concentrations as low as 10^{-10} M. Other progestins were also effective in mediating PSA gene expression (data not shown). The identity of PSA in the tissue culture supernatants was characterized further by HPLC and Western blot analysis (data not shown).

Two RNA samples isolated from normal human breast tissue obtained at reduction mammoplasty were analyzed for PSA gene expression by PCR. Southern blot hybridization of the PCR products with a PSA cDNA probe detected a PSA band of the expected size (571 bp) in the sample obtained from the woman who was receiving oral contraceptives. The other sample, obtained from a woman not receiving any medication, was

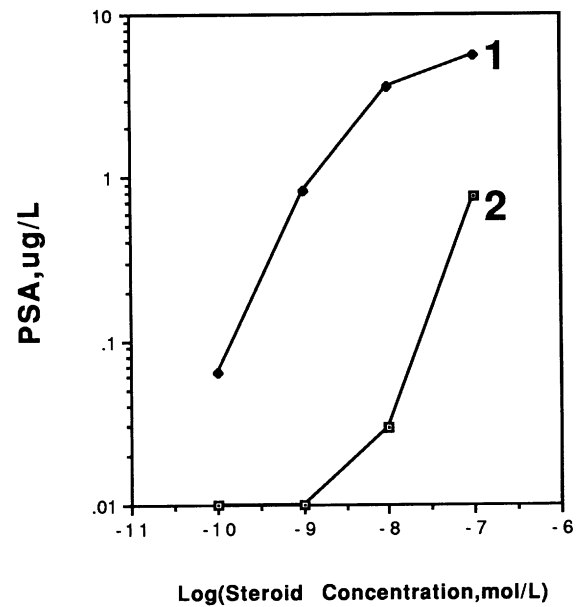


FIG. 3. **Production of PSA by the breast carcinoma cell line MCF-7.** Cells were grown to confluence and then stimulated with varying concentrations of either norethindrone (1) or ethinylestradiol (2) at the final concentrations indicated, in the absence of fetal calf serum from the culture medium. PSA was measured in the culture supernatant 10 days poststimulation. No PSA was detected in cell cultures grown identically but either nonstimulated or stimulated with the solvent alone (ethyl alcohol). Norethindrone stimulates PSA production at concentrations as low as 10^{-10} M.

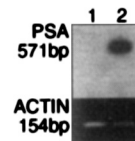


FIG. 4. *Top panel*, reverse transcription-PCR of RNA isolated from normal breast tissue and detected by Southern blotting and hybridization of the filter with a radiolabeled PSA cDNA probe. A PSA hybridization band of 571 bp is detected in *lane 2*, but the band is absent in *lane 1*. *Bottom panel*, ethidium bromide-stained agarose gel of β -actin reverse transcription-PCR products. *Lane 1* represents RNA isolated from breast tissue negative for PSA protein immunoreactivity. *Lane 2* represents RNA isolated from the breast tissue of the woman receiving Brevicon. This breast tissue was positive for PSA protein immunoreactivity.

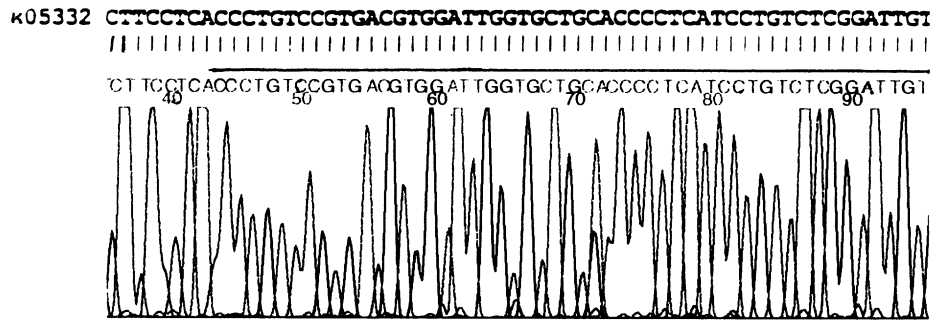
PSA-negative (Fig. 4). To determine whether the PSA mRNA detected in breast tissue is identical to the mRNA present in prostate tissue, we performed DNA sequence analysis on the PSA cDNA fragment isolated from breast tissue and compared it with the published PSA cDNA sequence. Partial results are shown in Fig. 5. DNA sequence analysis of the breast tissue PSA cDNA fragment showed 100% identity with the PSA cDNA sequence data from normal prostate tissue recently obtained by Monne and Croce³ (GenBank accession number U17040). No mutations were identified in the 571-bp fragment that was sequenced.

DISCUSSION

PSA is a serine protease found at very high concentrations in sperm. The molecular mass of the glycosylated and nonglycosylated PSA is 28.430 and 26.079 kDa, respectively (12). However, with polyacrylamide gel electrophoresis, PSA runs as a 32–33-kDa protein. It has been suggested that PSA is involved in semen liquefaction postejaculation. PSA is considered a

³ M. Monne and C. M. Croce, manuscript in preparation.

FIG. 5. Representative chromatogram of a 60-nucleotide region of the PSA cDNA sequence from normal breast tissue. Total RNA was isolated from a breast tissue specimen obtained at reduction mammoplasty from a woman receiving Brevicon. RNA was reversed transcribed to cDNA, amplified by PCR, purified, and directly sequenced. We obtained a 100% identity between the PSA cDNA sequence from breast tissue (bottom) and the PSA cDNA published sequence (top).



highly specific biochemical marker of the prostate gland and is currently used for prostate cancer diagnosis, population screening, and postsurgical monitoring of patients with prostate cancer (2, 3). Recently, we have demonstrated that PSA is produced by 30% of breast tumors and provided evidence that this marker may be a new favorable prognostic indicator of the disease (1). Patients whose tumors produce PSA live longer and relapse less frequently in comparison with patients with PSA-negative tumors.²

In this paper we have examined if PSA could also be produced by normal breasts either under physiological conditions or under conditions of stimulation by exogenously administered steroid hormones. Eight patients who received no medication had breast PSA levels below 0.03 ng/mg protein and were considered negative for PSA. One patient who was receiving a progestin-containing oral contraceptive was found to have breast PSA levels of 0.11 and 1.53 ng/mg total protein (left and right breast, respectively). This immunoreactive PSA molecule was measurable by the immunofluorometric assay (6) as well as by commercial PSA assays that are used widely for prostate cancer diagnosis. We have verified, using HPLC (Fig. 1), that the immunoreactive PSA species in breast is present in two molecular forms: as a 33-kDa protein, corresponding to the seminal form of prostatic PSA; and as a 100-kDa protein, corresponding to PSA complexed with α_1 -antichymotrypsin. The latter form, which is predominantly found in the serum of prostate cancer patients, was present in the breast extract at relatively low levels (<10% of total PSA); the major form was comprised of the 33-kDa protein (Fig. 1B). We have further characterized PSA in the positive breast extract using Western blot analysis. This data have shown that the PSA-positive breast extract, but not a PSA-negative breast extract, contained an immunoreactive band with a molecular mass of 33 kDa (Fig. 2). PSA was not elevated in the serum of patients receiving oral contraceptives. The additional band on Western blots, at 28 kDa, present in PSA-positive and PSA-negative breast extracts and in amniotic fluid, may represent a PSA isoform or a PSA fragment.

We have further molecularly characterized the presence of PSA in the normal breast at the mRNA level. For this analysis, we extracted total RNA from the PSA-positive and a PSA-negative breast tissue and amplified it, after reverse transcription, with specific primers derived from the known PSA gene sequence. We found that amplification occurred only with RNA from the PSA-positive breast tissue. The PCR product was hybridized with a specific cDNA probe for PSA and revealed the expected 571-bp fragment (Fig. 4). Furthermore, sequencing of the PCR product derived from breast tissue has shown that the sequence was identical to the sequence of the cDNA for PSA derived from prostatic tissue. No mutations were identified (Fig. 5).

The data presented support the notion that the PSA gene is

expressed in normal breast tissue under conditions of stimulation by steroid hormones. To reproduce the phenomenon *in vitro* we cultured T-47D and MCF-7 cells, two breast cancer cell lines that are both positive for steroid hormone receptors. Tissue culture supernatants from the nonstimulated cell lines contained no detectable PSA. However, upon stimulation with norethindrone or ethinylestradiol, the two components of the contraceptive used by the patient whose breast was positive for PSA, the cell lines produced PSA. Notably, norethindrone was active at concentrations as low as 10^{-10} M (Fig. 3).

The physiological relevance of our finding is currently unknown. However, it has recently been suggested (13, 14) that PSA, a serine protease, is a new potential growth factor regulator, enzymatically digesting insulin growth factor-binding protein-3 to release insulin growth factor-I or enzymatically activating latent human transforming growth factor- β and proteolytically modulating cell adhesion receptors (15). Our previous data on PSA gene expression in breast cancer that has good prognosis (1, 5) and the demonstration here that the PSA gene is regulated by oral contraceptives in normal breast suggest that this enzyme, until recently thought to be associated only with male prostatic tissue, may have important, previously unrecognized extraprostatic functions related to breast and other tissue growth and possibly breast cancer. This suggestion is further supported by our finding of the PSA presence in amniotic fluid during gestational weeks 13–21 (16) and in the milk of lactating women (5). The role of PSA in embryonal life and its involvement in growth factor regulation are currently under investigation.

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