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Prostate-specific antigen in serum during the menstrual cycle

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We previously found that prostate-specific antigen (PSA) expression in the female breast is regulated by steroid hormones and their receptors. We have now examined whether the PSA concentration in serum changes during the menstrual cycle of healthy women. Among 14 women studied, 3 had serum PSA ≥4 ng/L; their changes in PSA content during the menstrual cycle were studied in 7 informative cycles. We found that PSA concentrations in serum are highest during the mid- to late follicular phase, drop continuously with a half-life of 3–5 days between the late follicular phase and midcycle, and reach a minimum during the mid- to late luteal phase. PSA changes do not correlate with changes in lutropin (LH), follitropin (FSH), or estradiol concentrations. However, PSA peaks seem to follow the progesterone concentration peaks, with a delay of 10–12 days. Sera of some volunteers were tested for their ability to upregulate PSA protein and PSA mRNA in a tissue culture system based on the T-47D breast carcinoma cell line. Only sera obtained during the mid- to late luteal phase were able to upregulate the PSA mRNA and protein. In stimulation experiments in vitro, progesterone, but not LH, FSH, estradiol, human chorionic gonadotropin, prolactin, or growth hormone, was able to upregulate PSA mRNA and protein in the T-47D cell line. These data suggest that PSA is produced in a cyclical manner during the menstrual cycle.

There is a dynamic relationship between pituitary gonadotropin and ovarian steroid secretion during the menstrual cycle. The early follicular phase is characterized by relatively high plasma concentrations of follitropin (FSH)[4] and by low concentrations of lutropin (LH), estradiol, progesterone, and inhibin. During the middle of the follicular phase, serum estradiol increases rapidly and suppresses secretion of FSH by a selective action on the pituitary. Plasma inhibin also increases during this time and may also play a role in suppressing FSH release *[1–4]*. In the late follicular phase, the increase in estradiol is important in the development of the midcycle LH surge, and exerts a positive feedback effect and enhances LH responsiveness to gonadoliberin (GnRH). Plasma progesterone is also increasing and can also augment LH response to GnRH. Thus, the combined effects of estradiol and progesterone result in markedly enhanced LH release that produces the midcycle LH surge. Concomitant with the abrupt increase in LH, serum estradiol falls precipitously and progesterone secretion increases. The increase in progesterone concentration appears to be the major factor that reduces GnRH secretion in the luteal phase. During this period, other peptide hormones and growth factors are also secreted *[4]*. Progesterone is the major endocrine product of the corpus luteum of the menstrual cycle.

Prostate-specific antigen (PSA) is a 33-kDa serine protease produced at high concentrations by prostatic epithelial cells and secreted into the seminal plasma. PSA production in the prostate is regulated by androgens through the action of the androgen receptor *[5]*. Recently, we have demonstrated that PSA is not a prostatic tissue-specific protein but is also expressed in the female breast and some other tissues in both males and females *[6]*. Normal, benign, and malignant breast tissue produces PSA *[7]*. Some, but not all, breast tumors produce PSA; our studies suggest that PSA is a favorable prognostic indicator in breast cancer *[8]*. Previously we found a close association between PSA presence in breast tumors and

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[4] Nonstandard abbreviations: FSH, follitropin (follicle-stimulating hormone); LH, lutropin (luteinizing hormone); GnRH, gonadoliberin; PSA, prostate-specific antigen; and hCG, human chorionic gonadotropin.

the presence of both estrogen and progesterone receptors; this association was stronger between PSA and progesterone receptors *[9]*. We have also found PSA in amniotic fluid at increasing concentrations between gestational weeks 11–22 *[10]*. PSA concentrations were also greater in serum of pregnant women than in nonpregnant controls *[10]*. We speculated that PSA is upregulated by placental steroids during pregnancy.

To investigate the mechanism of PSA gene regulation in the breast, we have developed a tissue culture system that reproduces in vitro the phenomenon of PSA production by breast cells. The steroid hormone receptor-positive breast carcinoma cell line T-47D does not produce detectable PSA when cultured in media lacking steroid hormones. Upon stimulation by steroid hormones, however, this cell line produces PSA in a dose-dependent manner. Strong positive regulators of PSA mRNA and protein expression in this system are androgens and progestins; estrogens not only do not induce upregulation but also partially block the effects of androgens and progestins *[11, 12]*.

Regulation of the PSA gene by steroid hormones in the breast led us to speculate that this protein may be differentially expressed during the menstrual cycle. To investigate this possibility, we studied PSA concentrations in the serum of women during the menstrual cycle and correlated these with the concentrations of steroid and peptide hormones. In addition, we examined the ability of female serum during the menstrual cycle to induce PSA mRNA and protein expression in the tissue culture system. Our data support the view that the PSA protein is differentially expressed during the menstrual cycle, with the greatest concentrations occurring during the mid- to late follicular phase and the lowest being found during the mid- to late luteal phase. The peak in serum PSA concentration follows the peak in serum progesterone concentration but with a delay period of ~10–12 days. These data support the view that the PSA gene is regulated by corpus luteum steroids during the menstrual cycle.

Materials and Methods

SUBJECT SELECTION

Fourteen healthy women of reproductive age with regular ovulatory menstrual cycles, as determined by history and either basal body temperature charting or luteal-phase progesterone measurements, were studied throughout one or two menstrual cycles. Blood was collected every 3–4 days. Criteria for inclusion in this study were: age between 20 and 46 years, regular menstrual cycle, and taking no birth control pills or other medications. All women had normal screening medical histories; one reported a past history of breast fibroadenoma that was not treated. Six of the volunteers were nulliparous (no pregnancy), and eight had one to five uneventful pregnancies. Two volunteers were black, 2 Asian, and 10 white. Menstrual cycle regularity and ages of the women were 26–39 days and 29–46 years, respectively.

PROCEDURES

Tissue culture stimulation experiments. The T-47D breast carcinoma cell line was obtained from the American Type Culture Collection. This cell line is positive for estrogen, progesterone, androgen, and glucocorticoid receptors *[13, 14]*. T-47D cells were cultured in RPMI media (Gibco BRL) supplemented with glutamine (200 mmol/L), bovine insulin (10 mg/L), fetal bovine serum (100 g/L), antibiotics (penicillin, streptomycin), and antimycotics (amphotericin B). The cells were cultured to near confluency in plastic culture flasks and then transferred to phenol red-free media containing charcoal-stripped fetal bovine serum, 100 g/L, with antibiotics/antimycotics. Phenol red-free media were used, given findings that phenol red has weak estrogenic activity *[15]*; charcoal-stripped fetal bovine serum is devoid of any steroid hormones.

The T-47D cells were aliquoted into 24-well tissue culture plates (Corning no. 25820) and cultured to confluency with a change in media at 3 days. Stimulations were carried out with confluent cells in 1 mL of phenol red-free media containing charcoal-stripped fetal calf serum (100 g/L) and antibiotics/antimycotics. Stimulation was initiated by adding 1 mL of filter-sterilized serum sample and incubating for 24 h. Tissue culture supernatant (~150 μL) was removed for PSA protein analysis after 24 h. Slight modifications of this protocol were introduced as necessary. Appropriate multiple negative controls (no serum added) were included in each experiment. Positive controls consisted of T-47D cells stimulated with the synthetic progestin Norgestrel, which was found previously *[11, 12]* to induce PSA mRNA and PSA protein production by T-47D cells. Additional experiments were performed with progesterone at 0.11 μmol/L–10 pmol/L, prolactin at 5 μg/L–5 ng/L, growth hormone at 10 μg/L–10 ng/L, LH at 10 μg/L–10 ng/L, FSH at 10 μg/L–10 ng/L, and human chorionic gonadotropin (hCG) at 5–5000 IU/L (all concentrations are the final concentrations in the microtiter wells, and all the human hormones were purchased from Sigma Chemical Co.). The mRNA from the T-47D cells was extracted for PSA mRNA amplification as described below.

Measurement of PSA protein. PSA protein was measured with a highly sensitive time-resolved immunofluorometric procedure described in detail elsewhere *[16]*. This ultrasensitive assay can measure PSA at 1–2 ng/L (and up to 10 000 ng/L) with a precision of <10%. Further within-run precision data with a female serum pool confirmed a CV of 8.5% at a PSA concentration of 4.9 ng/L (n = 10). PSA measurements in all assays were performed in duplicate or triplicate. PSA protein in all sera and tissue culture supernatants was measured undiluted in 100-μL aliquots per assay.

Measurement of hormones in serum. All serum samples collected during the course of the menstrual cycle were

analyzed for concentrations of the hormones FSH, LH, estradiol, and progesterone. FSH and LH were assayed with the Access® Immunoassay Analyzer (Sanofi Diagnostics Pasteur). The lowest detectable concentration of FSH and LH distinguishable from zero with 95% confidence in these assays is 0.2 IU/L, and both assays exhibit total imprecision <10% across the assay range. Estradiol was measured by a solid-phase, chemiluminescence enzyme immunoassay system (Immulite®; Diagnostic Products Corp.), which had a detection limit of 0.044 nmol/L and a CV <10%. Progesterone was measured with the Ciba-Corning ACS® progesterone chemiluminescence immunoassay, with a minimum detectable concentration of 0.35 nmol/L and CVs <10% within the measuring range.

Extraction of total RNA. Total RNA from T-47D cells was extracted with the commercial reagent, TRIZOL™ (Gibco-BRL), according to the manufacturer's recommendations. The quality and quantity of the extracted RNA were checked by spectrophotometric measurements at 260 and 280 nm.

Reverse transcription. To reverse-transcribe 1 μg of total RNA, we used oligo dT primers and Superscript II™ reverse transcriptase (Gibco BRL). Briefly, the RNA and 500 ng of oligo dT primers were first denatured for 10 min at 70 °C, chilled on ice for 1 min, and then incubated for 1 h at 42 °C in a 20-μL reaction mixture containing 1× PCR buffer (Boehringer Mannheim; Tris 10 mmol/L, pH 8.3, plus KCl 50 mmol/L), 2.5 mmol/L $MgCl_2$, 1 mmol/L deoxynucleoside triphosphates, 10 mmol/L dithiothrietol, and 200 units of Superscript II reverse transcriptase. The reaction was terminated by heating for 15 min at 70 °C. Template RNA was digested by incubation with RNase H for 20 min at 37 °C.

PCR procedure. The two oligonucleotides we used to amplify the cDNA of PSA by PCR were originally proposed by Deguchi et al. *[17]* and have the following sequences:

PSA-1: 5′-TGC-GCA-AGT-TCA-CCC-TCA-3′
PSA-2: 5′-CCC-TCT-CCT-TAC-TTC-ATC-C-3′

These primers amplify a 754-bp fragment of PSA cDNA. Actin primers, which amplify a 372-bp fragment of actin cDNA, were used as controls; their sequences have been described previously *[18]*.

In the PCR, 5 μL of cDNA was added to 45 μL of PCR mix containing 1× PCR buffer, 1.5 mmol/L $MgCl_2$, 500 nmol of the PCR primers, 200 μmol/L of deoxynucleostide triphosphates, and 2 units of Taq DNA polymerase (Boehringer Mannheim). PCR was performed on a Perkin-Elmer 2400 thermal cycler for 30 cycles according to the following program: 94 °C for 30 s (5 min for the first cycle); 56 °C for 30 s; and 72 °C for 30 s (7 min for the last extension). Actin cDNA was amplified from 1 μL of the cDNA preparation under the same conditions used for PSA cDNA. From each PCR reaction 20 μL was electrophoresed on agarose gels (20 g/L) and visualized by ethidium bromide staining.

In other experiments, we incorporated digoxigenin-11-dUTP (Boehringer Mannheim), 0.7 μmol/L, into the PCR reaction mixture and detected the PCR product after Southern transfer to nylon membranes by probing with anti-digoxigenin antibodies conjugated to alkaline phosphatase; activity of the enzyme was detected with chemiluminescence. This method is ~500-fold more sensitive than ethidium bromide staining in detecting the PCR products.

Results

We collected ~8–10 serum samples from each of the 14 volunteers during the menstrual cycle. Analysis for PSA determined that in 11 patients PSA was <4 ng/L in all samples collected. Because the detection limit of the assay used is 1–2 ng/L, we did not use these data for further analysis. Among the three volunteers whose serum PSA content was ≥4 ng/L, we collected additional sera from two. These new sera spanned two consecutive menstrual cycles and were collected at least 2 months after the initial collection; thus, in total, we studied 7 informative menstrual cycles from three different patients. The days of the menstrual cycle in each case were verified by analysis of progesterone, estradiol, LH, and FSH.

In Fig. 1, we present the serum PSA changes during the menstrual cycle of one patient (3 cycles) and in Fig. 2 the data for the other two patients (3 cycles and 1 cycle). The progesterone values are also presented; however, when we plotted the PSA data along with the LH, FSH, or estradiol data, no recognizable pattern or relationship was seen (data not shown). Figs. 1 and 2 show a consistent pattern, in which PSA peaks during the mid- to late follicular phase and reaches a minimum during the mid- to late luteal phase. The difference between PSA and progesterone peaks is ~10–12 days.

We further examined whether serum obtained during the menstrual cycle could stimulate PSA production in the breast carcinoma cell line T-47D by measuring the PSA mRNA and the PSA protein. We found that nonstimulated T-47D cells do not produce PSA protein and do not express PSA mRNA. Fig. 3 shows the results of stimulating the cells with serum obtained during the menstrual cycle. The ability of the serum to induce PSA production parallels the concentrations of progesterone present, the greatest stimulation being achieved with the serum containing the most progesterone (day 24 of the menstrual cycle).

PSA mRNA can be detected in trace amounts in T-47D cells if the serum used for stimulation is collected during the follicular phase of the cycle. When the serum is collected during the luteal phase, PSA mRNA expression is dramatically increased (Fig. 4). These changes in PSA mRNA concentrations parallel the changes in progester-

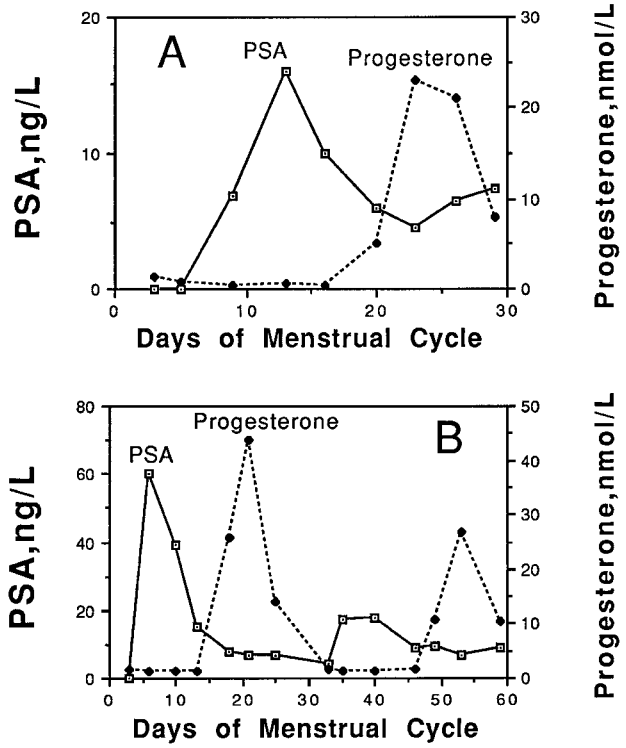

Fig. 1. Serum PSA (□) and progesterone (♦) during menstrual cycles of one volunteer: (*A*) in one menstrual cycle; (*B*) in two additional consecutive menstrual cycles collected at least 2 months after the sera of panel *A*.

Notice the peak of PSA, which follows the progesterone peak with a delay of 10–12 days.

one concentrations. PSA mRNA is undetectable in T-47D cells that are not stimulated by serum (data not shown).

We further stimulated T-47D cells with the glycoprotein hormones LH, FSH, and hCG and with prolactin and growth hormone. Despite the wide range of concentrations used, none induced PSA production. Progesterone was able to induce T-47D cells for PSA protein production and PSA mRNA expression at concentrations of 0.1-100 nmol/L *[12]*.

Discussion

Extensive evidence now indicates that PSA is produced by normal, hyperplastic, and cancerous breast tissue *[6–8]* and possibly by other female tissues as well *[19]*. PSA expression in the female breast is under the control of steroid hormones and their receptors *[9, 11]*. Recently, using a breast carcinoma cell line in a tissue culture system *[11, 12]*, we demonstrated that the PSA gene is upregulated by androgens and progestins but not estrogens.

The PSA concentration in female serum is very low and usually not measurable by commercial PSA assays. Using highly sensitive PSA procedures we developed *[16]*, we demonstrated that many female sera have measurable PSA concentrations *[20, 21]*. We do not know why some women have measurable serum PSA and some do not,

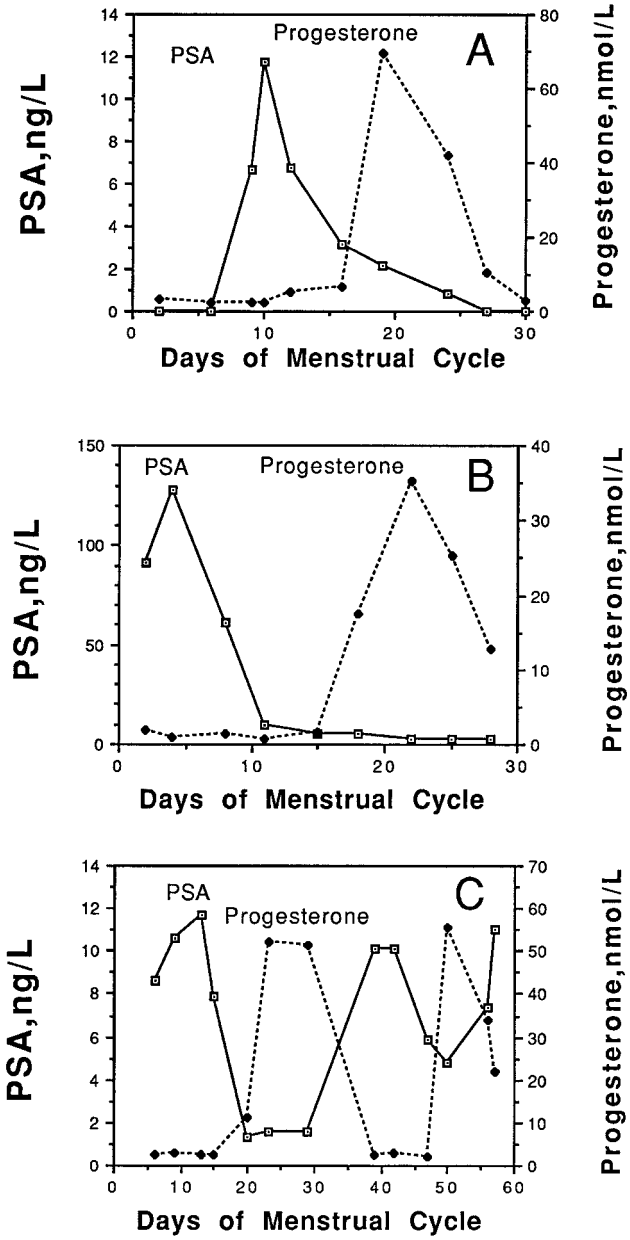

Fig. 2. Serum PSA (□) and progesterone (♦) during menstrual cycles of two additional volunteers: (*A*) in one menstrual cycle of the second volunteer; (*B*) in one menstrual cycle of the third volunteer; (*C*) in two additional consecutive menstrual cycles of the second volunteer, collected at least 2 months after the sera of panel *A*.

but perhaps it is related to an ability of PSA to diffuse from the breast tissue into the general circulation. We have further demonstrated that much higher PSA concentrations can be found in female breast secretions. For comparison purposes, we here report the approximate concentrations (ng/L) of PSA in various human fluids: seminal plasma, 10^9; male serum, 1000–2000; normal breast discharge fluid, 5×10^6; milk of lactating women, 10^5; female serum, 2–4. That is, the difference between PSA concentrations in the prostatic or breast secretion

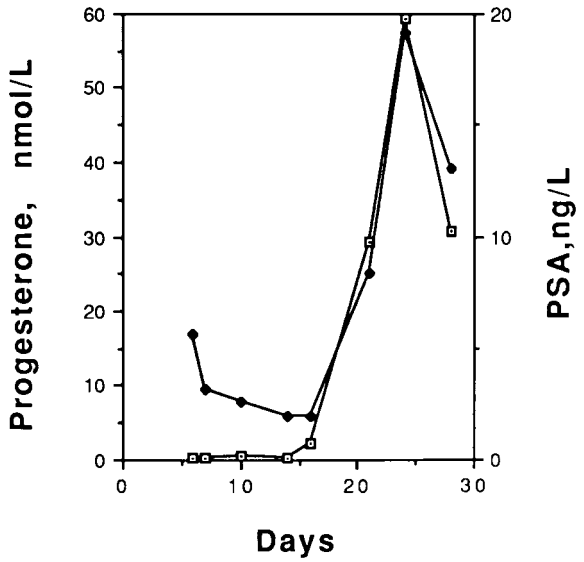

Fig. 3. Serum progesterone concentrations during the menstrual cycle of a normally cycling volunteer (□), and the PSA concentration in the tissue culture supernatants stimulated by those sera in the breast carcinoma cell line T-47D (◆).

The sera containing high concentrations of progesterone induced PSA production in cell line T-47D. See Fig. 4 for data on PSA mRNA expression.

(i.e., seminal plasma or breast discharge fluid) and the corresponding serum was ~10^6-fold. The physiological role of PSA in seminal plasma seems to have been established *[22]*. The role of PSA in the breast and its secretions is still obscure. We found that breast cancer patients with PSA-producing tumors may have better prognosis than do patients with tumors that do not express PSA *[8]*.

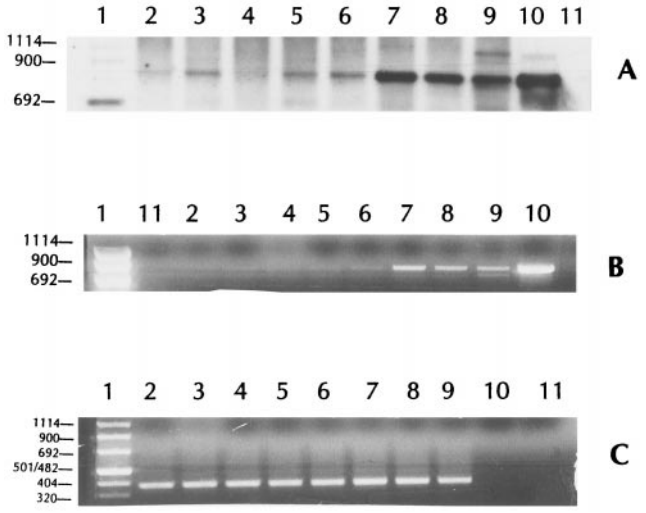

Fig. 4. Reverse transcription PCR of PSA mRNA (*A*, *B*) and actin (*C*) obtained from T-47D cells stimulated with serum of one cycling volunteer.

Lanes 2–9: Results for serum drawn at days 5, 7, 10, 14, 16, 21, 24, and 28 of the menstrual cycle. *Lane 10* represents a positive PCR control (plasmid containing full-length cDNA for PSA); *lane 11* is the negative PCR control (no target). Notice the intense PCR bands produced when serum from the luteal phase of the cycle is used to stimulate T-47D cells. The PCR product was detected with chemiluminescence (*A*) or with ethidium bromide staining of agarose gels (*B*, *C*).

Because PSA expression is under the control of steroid hormones and their receptors, we speculated that its concentration may change during the menstrual cycle. Now we have demonstrated this, by assessing the concentrations of serum PSA during the menstrual cycle of healthy women and by examining the ability of serum from these women to stimulate PSA production and PSA mRNA expression in a tissue culture system. We found that serum PSA concentrations change widely during the menstrual cycle and follow a specific pattern. This pattern was similar in the three patients studied and was reproducible on repeated cycles from the same patients (Figs. 1 and 2). The increase in PSA concentration follows the progesterone concentration increase but with a 10- to 12-day lag period. On the basis of many recent indirect findings, we suggest that the target tissue producing PSA is the female breast, but the contribution of other steroid hormone-responsive tissues, e.g., the endometrium, cannot be excluded *[19]*. Recently, Clements et al. measured PSA mRNA in endometrial tissue of normal cycling women and found the highest amounts during the follicular phase and the lowest during the late luteal phase *[23]*, in general accordance with the data reported here. They saw no association between the changes of PSA and changes in the concentrations of LH, FSH, or estradiol. Our tissue culture system with T-47D breast carcinoma cells confirmed the following: (*a*) that progesterone can upregulate PSA mRNA and protein production; (*b*) that estradiol, LH, FSH, prolactin, growth hormone, and hCG do not mediate PSA production in the T-47D cell line; (*c*) that sera collected during the luteal phase of the cycle, but not sera collected during the follicular phase or mid-cycle, have the ability to upregulate PSA protein production and increase PSA mRNA expression in the breast carcinoma cell line T-47D.

The data presented allow us to speculate that corpus luteum steroids stimulate target tissues capable of producing PSA (one of which is the breast) for PSA production and release into the mammary ducts. A fraction of this PSA diffuses into the general circulation and can be measured in the serum. Peak concentrations of PSA appear 10–12 days after the progesterone peak. Once the corpus luteum regresses, PSA concentration decreases, with an apparent half-life of ~3–5 days. Given these findings, we suggest that PSA is a protein regulated by corpus luteum steroids. Because this protein has serine protease activity, it will be interesting to find its biological role in the breast and its secretions and its possible physiological substrates *[6]*.

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