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Association between steroid hormone receptors and PSA gene expression in breast cancer cell lines

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The prostate specific antigen (PSA) gene is a member of the human kallikrein gene family and is known that to be tightly regulated by androgens in the male prostate. The presence of PSA is strongly associated with presence of steroid hormone receptors. The aim of this research was to show differential expression and association between steroid hormone receptors and PSA gene expression in breast cancer cell lines. The cell lines investigated were steroid receptor-negative breast carcinoma cell lines BT-20 and HBT-100 and the steroid hormone receptor-positive breast carcinoma cell lines BT-474, ZR-75-1, MDA-MB-453 and MFM-233. Others include BG-1 (ovarian), MFE-296 (endometrium), HBT-161 (ovarian), HBT-173 (ovarian), A-427 (lung), SK-MES-1 (lung), NCI-H460 (lung), MIA PaCa (pancreas), and Colo320 (Colon) cell lines. All cell lines were cultured as confluent and then harvested. Stimulation test was carried out using steroid compound. The concentration of estrogen receptor (ER) and progesterone receptor (PR) in all tumor cell lines were measured with the abbot enzyme immunoassay kit. Among cell lines tested, only steroid receptor positive cells were able to produce PSA at the protein and the mRNA level in response to stimulatory effects of steroid hormones. Our data suggest that the expression of the PSA gene in different cancer cell lines may depends on the presence of steroid hormone receptors.

Key words: PSA, steroid hormone receptor, prostate cancer, breast cancer.

INTRODUCTION

Prostate specific antigen (PSA) is a serine protease produced at high levels by prostate epithelial cells and secreted into seminal plasma. PSA is the best tumor marker for diagnosis and prognosis of prostatic carcinoma (Rekasi et al., 2001). PSA gene localized on chromosome 19 and has 80% sequence similarity with human granular kallikrein gene (Riegman et al., 1989; Schedlich et al., 1987; Clements, 1989). PSA is generally believed to be express only by prostate epithelial cells (Papsidero et al., 1981). Expression of the PSA gene was demonstrated to be directly regulated by binding of androgen receptor (AR) (Katerina et al., 2002). Androgen plays essential roles in prostate proliferation and prostate cancer progression (Xiaomin and Chawnshang Chang, 2003). We have previously provided evidence that the PSA production was associated with the presence of steroid hormone receptors and this association was stronger between PSA and progesterone (Yu et al., 1994). A possible explanation to the fact that PSA expression by breast cancer cells confers prognostic advantage to breast cancer patients is that its presence may reflect the existence of a complete hormone receptor pathway. According to this hypothesis, extra-prostatic expression of PSA may be a consequence of the hormone alterations presumably involved in the development of carcinomas, without causing any direct effect on the spread of cancer. It has been reported that PSA is detectable in ovarian cancer cytosol, primary lung cancer tissues, endometrium, and other cancer tissues,

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Abbreviations: PSA, Prostate specific antigen; **ER**, estrogen receptor; **PR**, progesterone receptors; **AR**, androgen receptor; and **HRE**, hormone response element.

but the frequency is much lower compared to the frequency of detection in breast cancer (Levesque et al., 1995).

From these considerations, we were prompted to examine the possibility that steroid hormones and their cognate receptors could mediate production of PSA by non-prostatic tissues. In order to search the mechanism of PSA gene regulation in the non-prostatic tissues, such as breast, ovarian, lung and colon, we have developed a tissue culture system which reproduces *in vitro* the phenomenon of PSA production by these cells. Although BT-474 and ZR-75-1 were positive for steroid hormone receptors, only BT-474 breast carcinoma cell line was able to produce PSA in response to steroid hormones stimulation. The steroid hormone receptor-positive breast carcinoma cell line BT-474 does not produce detectable PSA when cultured in media devoid of steroid hormones. When stimulated by steroid hormones, these cell lines produce PSA in a dose-response manner. We used this system to study the mRNA expression of PSA with and without stimulation. In this report, we show that androgens, progestins, glucocorticoids up-regulate PSA gene expression in breast cancer cells. In addition, we provide indirect evidence that the PSA gene in some steroid hormone receptor positive cell line and/or steroid hormone receptor itself could be defective and needs to be investigated.

MARERIALS AND METHODS

Compounds

All steroidal and non-steroidal compounds used in this study were obtained from Sigma Chemical Co., Louis, MO., except for the following: ICI 102,780 and casodex (ICI 176,334) (Zeneca Pharm INC., Mississauga, ON, Canada); RU58,668, RU54,876, RU56,187, nilutamide (Anandron, RU23,908) and mifepristone (RU486, RU38,486) (Roussel-UCLAF, Romainville, France), vitamin D Analogs (Ro 23-7553, and Ro 24-5531) (Hoffman-La Roche Inc, Nutley, Newjersy, USA), EB-1089 (Leo Pharmaceutical products Ltd, Allerup, denmark), LG100153, LG100272 and Degnelin (Ligand Pharmaceuticals Inc, san Diego, CA). Hydroxyflutamide was a gift from Dr. Donna peehl, Stanford University. Oral contraceptive (LoEsrin, Ortho 7/7/7, and Demulin) were provided from Park Davis, Ortho and Searle, Canada. Stock Solutions (10^{-2} or 10^{-3} M) were prepared in absolute ethanol. More diluted solutions were also prepared in the same solvent.

Cell lines

The BT-474, ZR-75-1, MDA-MB-453 breast carcinoma cell lines, BG-1, HBT-75, HBT-161 ovarian carcinoma cell lines, and A-427, TBT-173, SK-MES-1, NCI-H460 lung tumors cell lines were obtained from the American type culture collection (ATCC, Rockville), MD. The MFE-296 endometrial cancer cells were obtained from Dr. R. Hackenberg, Klinikum der Philips-Universitat, Marburg, Germany). All breast adenocarcinoma cell lines (BT-474, ZR-75-1, and MDA-MB-453) were cultured at 37ºC with 5% CO₂ in

RPMi-1640 media supplemented with glutamine (200 mmol/L), bovine insulin (19 mg/L), fetal bovine serum (10%), antibiotics (penicillin, streptomycin) and antimycotics (amphotericin B) expect MDA-MB-453 cell line that cultured in Lebovitz's media (GIBCO, BRL) supplemented with the same component above mentioned. The ovarian tumor cell lines (BG-1, HBT-75, and HBT-161), lung tumor cell lines (A-427, HBT-173, and SK-MES-1) and the endometrium cancer cell line, MFE-296, were maintained in RPMI-1640 media supplemented with 10% (v/v) fetal bovine serum, 200 mmol/L glutamine, 10 mg/L bovine insulin, antibiotics (penicillin, streptomycin) and antimycotics (amphotericin B). The cells were cultured to near confluence in plastic culture flasks and then transferred to phenol red-free media containing 10% charcoal-stripped fetal bovine serum with antibiotics/antimycotics. Phenol red-free media were used since phenol red was found previously to have weak estrogenic activity (Berthois et al., 1986). And charcoal-stripped fetal bovine serum is devoid of steroid hormones.

Stimulation experiments

All tumor cell lines were then aliquoted into 24-well tissue culture plates (corning # 2582) and cultured to confluence with change in media at 3 days. Stimulations were carried out with confluent cells containing 2 mL of phenol red-free media with 10% charcoal-stripped fetal calf serum and antibiotics/antimycotics. Stimulation was initiated by adding 2 µl of each steroid dissolved in ethanol and incubating for a certain period of time (usually up to 8 days). Tissue culture supernatant (~150 µL) was removed for PSA analysis at day 8. Slight modifications of the protocol were introduced as necessary. Appropriate multiple positive and negative controls (only alcohol added) were included in each experiment. Wells with microbial contamination were excluded from the data.

Isolation of total RNA

Total RNA isolation from cell lines was performed using the TRIzol method (GIBCO BRL, Gaithesbul, MD, TRIzol reagent) following the instructions of the manufacturer. The TRIzol method is described briefly as follows: About 10^7 cells were pelleted and used for total RNA extraction. After incubating the samples and/or cell pellets for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes, 200 µL chloroform per 1 mL of TRIzol reagent was added into the solution. Following vigorous mixing, the solution was centrifuged at 12,000 x g for 15 min at 4ºC. Centrifugation separates the diphase mixture into the lower red phenol-chloroform phase and upper colorless aqueous phase, which was removed carefully. The RNA was precipitated from the aqueous phase by mixing with 500 µL of isopropanol per 1 mL of initial TRIzol reagent. The samples were incubated at room temperature for 10 min and centrifuged at 12,000 x g for 10 min at 4ºC. The supernatant was removed and the RNA pellet was washed once with 75% ethanol. After centrifugation and removal of the alcohol, the pellet was air dried and dissolved in diethyl pyrocarbonate (DCPC) treated water. The integrity of the RNA was checked electrophoretically, and the amount and purity by spectrophotometer at A₂₆₀ and A₂₈₀.

Reverse transcription

The synthesis of cDNA from the isolated total RNA was carried out with a first-strand cDNA synthesis Kit using SuperScript II reverse transcriptase (GIBCO BRL, Gaithersburg, MD). Briefly, 1-5 µg of RNA and oligo(dT) ₁₂₋₁₈ primers (500 ng), were first denatured 10

min at 70ºC , chilled on ice for 1 min and then incubated for 5 min at 42ºC in a 19 µL of reaction mixture which include 10X PCR buffer (containing 200 mmol/L of Tris-HCl and 500 mmol/L of KCl, pH 8.4) (Boehringer Mannheim), 10 mmol/L of deoxynuceotide triphosphate mix (Boehringer Mannheim), 10 mmol/L of dithiothretiol (DDT), and 25 mmol/L of $MgCl_2$. Then, 200 units (1 µL) of SuperScript II reverse transcriptase were added to the reaction mixture, incubated for 50 min at 42ºC, terminated at 70ºC for 15 min and chilled on ice. The mixture was then treated with 1 µL of RNase H for 20 min at 37ºC before proceeding to amplification of the target cDNA. Negative control reactions for RT-PCR were performed using all reagents but without added SuperScript II.

Oligonucleotide primers

Two oligonucleotide primers were used to amplify a 754 base pair region of PSA cDNA. These, originally described by Deguchi et al. (1993) have the following sequence: PSA A1: 5'-TGCGCAAGTTCACCCTCA-3', PSA B1: 5'-CCCTCTCCTTACTTCATCC-3'. For actin cDNA amplification, we used the following primers, previously published (Okazaki, 1992). ACT 1: 5'-ACAATGAGCTGCGTGTGGCT-3', ACT2: 5'-TCTCCTTAATGTCACGCACGA-3'. PCR with primers A1/B1 Yields a 754 bp fragment, and with ACT1/ACT2 a 372 bp fragment.

PCR Protocol

PCR was performed in 0.2 mL thin-walled MicroAmp reaction tubes on a Perkin-Elmer Gene Amp 2400 system. Total volume was 50 µL. The reaction mixture contained PCR buffer (50 mmol/L KCl, 10 mmol/L Tris buffer, pH 8.3, 1.5 mmol/L $MgCl_2$, 10mg/L gelatin), 200 µmol/L of deoxynuceotide triphosphated, (dNTPs) 1 µmol/L of PCR primers, 2.0 units of Taq DNA polymerase (Boehringer Mannheim) and 5 µL of cDNA target (added last). The PCR was performed with one cycle at 94ºC for 5 min, 30 cycles with denaturation at 94ºC for 30 s, annealing at 60ºC for 30 s and extension at 72ºC for 7 min. 20 µL of PCR reactions were electrophoresed on 2% Agarose gels and visualized with ethidium bromide staining. Negative controls did not contain template DNA.

Labeling of PSA cDNA probe

PSA cDNA plasmid was linearized with Hind III enzyme digestion and labeled with the random primer method by incorporation of digoxigenin-labeled deoxyridine triphosphate (DIG-dUTP0, Boehringer Mannheim) using the protocol recommended by the manufacturer. PSA cDNA plasmid was first incubated for 1 h at 37ºC with 150 units of Hind-III restriction enzyme (Boehringer Mannheim) in a 10 mmol/L of Tris-HCl buffer, pH 8.0, containing 0.1 mol of NaCl, 5 mmol/L of $MgCl_2$ and 1 mmol/L of 2β-mercaptoethanol per liter. Then, the PSA cDNA plasmid was denatured by heating in a boiling water bath for 10 min and chilled on ice. 30 µL of 10X concentrated dNTP mix, including 1 mmol/L of dATP, 1 mmol/L of dCTP, 1 mmol/L of dGTP, 0.65 mmol/L of dTTP, and 0.35 mmol/L of DIG-dUTP . The whole mixture was incubated at least 60 min at 37ºC.

RNA labeling by *in vitro* transcription

The PSA PCR product was cloned in to the polylinker site of the pCR2.1 transcription vector. The recombinant pCR2.1 transcription vector contains promoters for SP6 and T7 RNA polymerizes. After linearization of the vector with Hind III restriction enzyme, the T7 RNA polymerase was used to create "run-off" transcripts using the DIG RNA Labeling Kit (Boehringer Mannheim, Germany) and the protocol recommended by the manufacture. DIG-UTP was used as a substrate and was incorporated into the transcript. The DIG-labeled RNA was used as a non-radioactive probe in southern blots. In brief description, the following reagents were added to a microfuge tube on ice: 20 µL of 10X transcription buffer, 20 µL of 10X concentrated NTP labeling mixture containing 10 mM ATP, 10 mM CTP, 10 mM GTP, 6.5 mM UTP, 3.5 mM DIG-UTP, pH 7.5, 10 µg of linearized DNA, 4000 units of T7 RNA polymerase, and 10 µL of RNase inhibitor and centrifuged briefly. The reaction mixture was incubated for 2 h at 37ºC. Longer incubation does not increase the yield of labeled RNA. DIG-labeled RNA probes have the following advantages: 1) they are of defined unity lengthy, 2) they are single strand specific and therefore 3) all labeled RNA is available for hybridization and does not renature as in the case of DNA. RNA, which is DIG-labeled according to the above protocol, allows the detection of 0.1 pg homologous DNA or RNA in a dot blot.

Gel electrophoresis, Southern Blotting and hybridization

Aliquots of PCR products (20 µL) were electrophoresed at 100 V for 45 min on 2% agarose minigel containing ethidium bromide. The gels were then southern blotted onto positively charged nylon membranes (Boehringer Mannheim) by overnight alkali capillary blotting with use of 0.4 mol/L NaOH. The membranes were then baked for 15-30 min at 120ºC. For hybridization, the membranes were placed in a roller bottle with 20 mL of hybridization buffer per 100 cm^2 of membrane. We used a commercial hybridization solution (DIG Easy Hyb, Boehringer Mannheim). Prehybridization was at 42ºC for 1 h. The solution was then replaced with 5 mL per 100 cm^2 of membrane of hybridization buffer containing 50 ng/mL of freshly denatured labeled PSA cDNA and/or 200 ng/mL DIG-Labeled probes. Hybridization was carried out for 12-16 h at 42ºC. Filters were subsequently washed twice with 2X SSC containing 0.1% SDS at room temperature (5 min per wash) and twice with 0.1X SSC containing 0.1% SDS at 68ºC (15 min per wash).

Detection protocol

The detection was performed as follows: The membranes were first washed briefly in a washing buffer containing 100 mM maleic acid, 150 mM NaCl, pH 7.5, and 0.3% Tween 20 for 1 to 5 min at room temperature and then were incubated in blocking solution (Boehringer Mannheim) for 30 min. Alkaline phosphatase-conjugated anti-digoxigenin antibody (Fab fragment) was diluted to a final concentration of 53 mU/mL (5000-fold) in blocking solution. The membranes were incubated in diluted antibody conjugate solution for 30 min and then washed twice for 15 min each at room temperature in washing buffer. Finally membranes were equilibrated in a buffer containing 0.1 mol/L of Tris-HCl, 0.1 mol/L of NaCl and 50 mmol/L of $MgCl_2$ pH 9.5 for 5 min at room temperature and incubated with 100-fold diluted chemiluminesent substrate in the buffer stated above, CDP-star (25 mM,Tropix, bedor, mA). While CDP-star equilibrating buffer was 0.1 M diethanolamine, 0.1 mM $MgCl_2$ pH 9.5.The chemiluminesent signal was captured on X-Ray film with 10 to 60 s exposure for CDP-star at room temperature.

Lysis procedure

The cell pellets were lysed for 30 min on ice with 1 mL of lysis buffer. Lysis buffer was 50 mmol/L Tris, pH 8.0 containing 150

mmol/L sodium chloride 5 mmol/L ethylene diamine tetraacetic acid (EDTA), 10 g/L Nonidet NP-40 surfactant, 1 mmol/L phenylmethylsulphonyl fluoride and 1 mg/L each of aprotinin and leupeptin as proteinase inhibitors. The lysate was centrifuged at 15000 g at 4°C for 30 min; the supernatant was collected and immediately assayed for PSA and total protein.

PSA measurements

PSA in the cytosolic extracts and/or tissue culture supernatant were measured with a highly sensitive and specific time-resolved immunofluorometric technique previously established and described in detail elsewhere (Ferguson et al., 1996). In brief, the PSA assay uses a mouse monoclonal anti-PSA capture antibody coated to polystyrene microtiter wells, a biotinylated monoclonal anti-PSA detection an 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 biotinylated anti-PSA detection antibody. After 1 h incubation followed by washing X6, the SA-ALP conjugate is added for 15 min followed by another washing X6. The activity of ALP is the measured by adding the substrate 5'-fluorosalicyl phosphate, incubating for 10 min and then by adding a Tb^{3+} and EDTA-containing developing solution. After 2 min, the fluorescence is measured in the time resolved fluorometric mode with the CyberFlour-615 immunoanalyzer (CyberFluor Inc., Toronto, Ontario). This assay has a detection limit of 1 ng/L of PSA and can measure PSA at levels of 1 ng/L or higher (up to 10000 ng/L) with a precision of <10%. All assays were performed in duplicate. Tissue culture supernatants and/or tumor cytosolic extracts were measured undiluted using 100 µL aliquots per assay, unless otherwise needed.

Measurement of steroid hormone receptor

The concentration (fmol/mg) of estrogen receptor (ER) and progesterone receptor (PR) in all tumor cell lines were measured with the Abbott enzyme immunoassay kit (Abbott laboratories, North Chicago, IL) at the Sunnybrook Health Science Center.

RESULTS

Different carcinoma cell lines were first measured for the presence of the steroid hormone receptors, estrogen and progesterone receptor (ER and PR). The cell line, tissue of origin, ER and PR concentrations (fmol per mg of total protein) were summarized in Table 1.

In order to investigate the mechanism of differential expression and regulation of PSA gene stimulation experiments were further conducted using the steroid receptor-negative breast carcinoma cell lines BT-20, HBT-100, and the steroid hormone receptor-positive carcinoma cell lines BT-474, ZR-75-1, MDA-MB-453, and MFM-233. The same experiment was carried out using BG-1 (ovarian) MFE-296 (endometrium), HBT-161 (ovarian), HBT-173 (lung), A-427 (lung), SK-MES-1 (lung), NCI-H460 (lung), MIA PaCa (pancreas), and Colo320 (Colon) cell lines. None of the compounds was able to induce detectable PSA production (Figure 2). Of
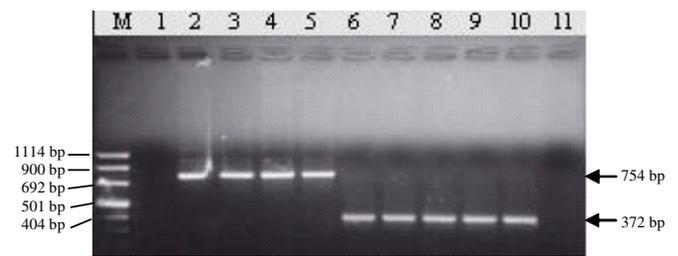

Figure 1. RT-PCR for mRNA extracted from BT-474 cell line. M: molecular weight markers. Sizes in base pairs (bp) are shown in left panel. Lane 1. PCR negative control (no template added). Lanes 2-4 are positive for PSA mRNA. Lanes 6-10. RT-PCR for actin and lane 11 negative control.
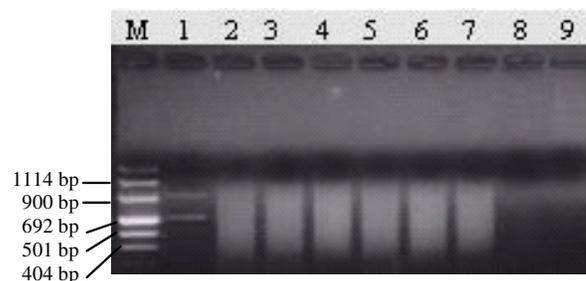

Figure 2. RT-PCR for mRNA extracted from HBT-173 cell line. M: Molecular weight. Lanes: (1) HBT-173, (2) BT-20, (3) HBT161, (4) A-427, (5) SK-MES-1, (6) HBT-75, (7) HBL-100 and lanes 8-9 are negative control.

all the carcinoma cell lines (Table 1) tested for PSA production, only steroid hormone receptor positive breast carcinoma BT-474, ZR-75-1 and MDA-MB-453 cell lines were able to produce PSA at the protein and the mRNA level in response to stimulatory effects of steroid hormones. And among steroid receptor positive cell lines, BT-474 had strong response to stimulatory effect at the protein and mRNA level rather than ZR-75-1 and MDA-MB-453 which were detected only on southern blot hybridization (Figures 1 and 3). Therefore, we were able to establish a highly sensitive tissue culture system which produces the phenomenon of PSA production by breast cells. The breast carcinoma BT-474 cell line was cultured in the absence of any stimulating steroid and in the presence of the stimulating steroid norgestrel at a concentration of 10^{-8} M.

DISCUSSION

The PSA gene is a member of the human kallikrein gene family and is known to be tightly regulated by androgens in the male prostate (Ojasoo et al., 1988; Glover and Darber, 1989). The epithelial cells of the prostate gland are rich in AR and some stromal cells also contain AR as

Table 1. Steroid hormone receptor levels in cell lines tested for PSA production.

| No.	Cell lines	Tissue of origin	Estrogen receptor (ER) (f mol/mg)	Progesterone receptor (PR) (f mol/mg)
1.	ZR-75-1	Breast	112	482
2.	MDA-MB-453	Breast	0	0
3.	BT-474	Breast	29	389
4.	MFM-233	Breast	1	1
5.	HBL-100	Breast	0	0
6.	BT-20	Breast	0	0
7.	BG-1	Ovarian	36	420
8.	HBT-75	Ovarian	0	1
9.	HBT-161	Ovarian	N/A	N/a [2]
10.	HBT-173	Lung	1	3
11.	A-427	Lung	0	1
12.	SK-MES-1	Lung	N/A	N/A
13.	NCI-H460	Lung	N/A	N/A
14.	MFE-296	Endometrium	1	1
15.	MIA PaCa	Pancreas	1	0
16.	Colo 320	colon	1	0
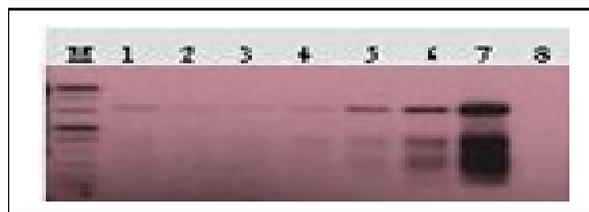
A **B**

Figure 3. PCR products were run on an agarose gel, Southern-blotted and hybridized with a DIG-labeled RNA probe. **(A)** M, Biotinylated molecular weight markers; lanes 1 to 6, MDA-453 cells were stimulated with testostrone, estrogen, B-estradiol, tamoxifen, triaminolone acetonide, norgestrel, respectively; Lane 7, positive control; Lane 8, negative control. **(B)** M, Biotinylated molecular weight markers. Lanes 1 to 7, RZ-75-1 cells were stimulated with testostrone, norgestrel, aldostrone, triaminolone acetonide, corticostrone and progesterone, respectively; lane 8, positive control.

well as the enzyme 5α-reductase, which reduces testosterone to dihydrotestosterone (Ojasoo et al., 1988). The PSA gene has an HRE to which the activated AR binds (Glover and Darber, 1989; Nordeen et al., 1989; Kafrissen, 1992; Murtha et al., 1993; Smith et al., 1995). The PSA gene is up-regulated by androgens and androgen agonists and is down regulated by antiandrogens.

Various non-prostatic carcinoma cell lines were first cultured and measured for the presence of steroid hormone receptors, ER and PR. Further using tissue culture system these cell lines were tested for PSA production. We have shown that the steroid hormone receptor-positive breast carcinoma cell line BT-474 is capable of producing PSA protein under appropriating stimulation by steroid hormones. T47-D and MCF-7 cells

do not produce PSA at the protein and mRNA level in the absence of steroid hormones (Diamandis et al., 1994; Yu et al., 1994). However, BT-474, ZR-75-1, and MDA-MB-453 do not produce PSA protein but constitutively express PSA mRNA in the absence of any stimulation (Figures 1 and 3). The PSA mRNA produced by BT-474 cells is identical to the sequence of PSA mRNA from prostate cells. In contrast, the breast carcinoma cell line BT-20, which is devoid of steroid hormone receptors did not produce PSA after stimulation. We have thus postulated that PSA production by breast cells is dependent on the steroid hormone/steroid hormone receptor system. We further demonstrated that the receptors and hormones are necessary but not sufficient for PSA production. When we stimulated the steroid hormone receptor-positive cell lines ZR-75-1 (breast

carcinoma) none was able to induce PSA production. The presence of estrogen and progesterone receptors in these cell lines was confirmed by analysis with established enzyme immunoassay kit (Table 1).

Apparently, either post-receptor defect is present in these cell lines is tissue specific. These possibilities were not studied further.

Taken together, our data suggest the following: The breast carcinoma cell line BT-474 has the necessary receptors and other transcriptional machinery to produce PSA. Once stimulated by a steroid hormone, Bt-474 cells synthesize detectable intracellular protein within 8 h and secrete detectable protein within 24 h. PSA gene regulation is under the control of androgens and progestins through the independent action of the androgen and progesterone receptors (positive regulation). Our data, showing multihormone regulation of the PSA gene, are in accord with those of Glover and Darbe (1989) and Zava et al. (1979) who concluded the same using T-47D cells transfected with the mammary tumor virus long terminal repeat sequences.

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