[CANCER RESEARCH 47, 6189–6192, December 1, 1987]

Progesterone-binding Cyst Protein in Human Breast Tumor Cytosol[1]

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ABSTRACT

Breast tumor cytosol has been analyzed for the presence of a progesterone-binding protein (PBCP), commonly present in benign breast cysts in huge concentrations. In 377 primary carcinomas investigated PBCP was present in measurable quantities in 60.7% using "rocket" immunoelectrophoresis. Concentrations of PBCP ranged from 0 to 12.4% of total cytosol protein with an average of 4.0 μg/mg cytosol protein. The distribution of PBCP values seems to suggest two tumor populations, one ofwhich is lacking in PBCP and the other showing a lognormal distribution. In malignant tumors PBCP levels were negatively correlated ($P = 0.024$) to estrogen receptor but not to ($P = 0.38$) progestin receptor levels. There was a highly significant ($P < 0.001$) positive correlation to cytosol albumin concentration which suggests an extracellular localization of PBCP possibly caused by restricted lymphatic drainage of tumor tissue. In benign breast tumors, mainly fibroadenomas, both PBCP incidence (81%) and average concentration (13.5 μg/mg protein) was higher than in malignant tumors. A positive correlation to sex-hormone receptor levels were observed indicating that PBCP production could be under hormonal control in this type of tumor development. In 71 metastatic tumors examined PBCP incidence was far less than in primary tumors ($P < 0.001$) and the levels seen were also considerably lower. PBCP holds promise as a marker of tumors in an early stage of development and/or with a low metastatic potential.

INTRODUCTION

Gross cystic disease is the most common benign breast disease in women with a peak incidence at 40–50 years of age. This condition is characterized by large fluid-filled cysts with diameters greater than 3 mm which are lined with dense connective tissue and occasionally by flat epithelial cells. In an early stage of development microcysts seem to be associated with apocrine metaplasia of the surrounding cells.

Protein composition of gross cystic disease fluid deviates markedly from plasma and milk and appear to resemble that of apocrine sweat glands (1). Numerous steroids and their sulfated conjugates accumulate in this fluid in concentrations greatly exceeding those found in serum (2). Pearlman *et al.* (3) in 1974 described a steroid-binding protein present in cyst fluid which was capable of binding progesterone and other hydrophobic steroids and he consequently named this protein progesterone-binding component. Others have referred to it as GCDFP[3]-25 (1) or progesterone-binding protein (4). In order to avoid confusion with other progesterone-binding proteins described in the literature we prefer to refer to it as PBCP. Although it is the major protein present in gross cystic disease fluid attention has mostly focused on another apparently cyst fluid specific protein named GCDFP-15 by Haagensen *et al.* (1). Tumor content of GCDFP-15 is correlated with the presence of estrogen receptor (4) and progesterone receptor (4, 5).

We have prepared a specific polyclonal antibody to PBCP which we have used to quantitate its presence in breast tumor cytosol by immunoelectrophoresis. This study describes the tumor distribution of PBCP in different patient categories and establishes correlations to other important cellular parameters such as the estrogen and progesterone receptor and to extracellular plasma-derived proteins such as albumin.

MATERIALS AND METHODS

PBCP was purified from breast cyst fluid by sequential chromatography on hydroxylapatite, DEAE-Sepharose CL 6B, Sephadex G-100, and concanavalin A-Sepharose 4B. Details of the purification method will be published elsewhere. The final product used for immunization was pure when assessed by gel-electrophoretic methods under intact or denaturing conditions. The protocol used for immunization was that of Vaitukaitis (6) employing multiple intradermal injections of antigen (200 μg/animal) in Freund's complete adjuvant. Three out of four animals responded with monospecific precipitating antibodies of relatively low titer when tested against cyst fluid or serum by double immunodiffusion. The IgG antibody fraction was isolated and delipidated according to Harboe and Ingild (7) and reconstituted to the original volume with isotonic phosphate buffer, pH 7.4, containing 0.2% sodium azide. PBCP was quantitated by rocket immunoelectrophoresis according to Laurell (8) using lyophilized dry weight of purified PBCP as reference standard. An optimal measuring range of 10–150 μg/ml with a lower limit of detection (*i.e.*, "rocket" <4 mm) of 50 ng (5 μg/ml in 10-μl sample) was obtained using a reconstituted antiserum concentration of 0.5/30 ml gel. Total protein was measured by a dye-binding method (9) using a 1:1 mixture of bovine albumin and IgG as reference standard. This ensured complete agreement with the standard Biuret method for protein determination. Albumin was quantitated by rocket immunoelectrophoresis using monospecific antiserum purchased from Behringwerke AG, Germany.

Tumor tissue from mastectomies or biopsies was immediately chilled on ice and frozen in liquid nitrogen upon receipt in the laboratory. Frozen tissue (0.2–0.5 g) was finely sliced with a scalpel and homogenized at 0°C in 3-ml buffer using 3 × 20 s bursts from an Ultra Turrax homogenizer. The buffer, intended for hormone receptor assay, consisted of 5 mM phosphate buffer, pH 7.4, containing 10% glycerol and 1 mM thioglycerol. The homogenate was centrifuged for 1 h at 105,000 × *g* and the resulting supernatant, "cytosol," was used for protein analysis. Estrogen and progesterone receptors were analyzed by single-point dextran-coated charcoal assay using saturating concentrations of tritiated estradiol and promegestone (R 5020) as ligands (10).

RESULTS

Progesterone-binding cyst protein was found in greatly varying concentrations in breast tumor extracts ranging from non-detectable levels to approximately 12% of total cytosol protein. Classification of a particular cytosol as negative is dependent both on the detection limit of the method (50 ng/10 μl cytosol) and on the amount of tissue extracted. Only cytosols containing a reasonable amount of extracted protein, arbitrarily chosen to be above 1 mg/ml, have been included in the present study. There was no correlation between the PBCP concentration measured and total cytosol protein, nor was there any significant difference in total cytosol protein concentration between the patient groups studied. Differences observed in PBCP incidence (*i.e.*, PBCP > 0) between tumor groups are thus unbiased by differences in amounts of tissue extracted. PBCP

Received 3/16/87; revised 6/18/87; accepted 8/12/87.

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[1] This work was supported by the Norwegian Society for Fighting Cancer (Norsk Forening til Kreftens Bekjempelse).

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[3] The abbreviations used are: GCDFP, gross cystic disease fluid protein; PBCP, progesterone-binding cyst protein; PR, progestin-receptor; ER, estrogen-receptor; DHEA-S, dehydroepiandrosterone sulfate.

could be detected in serum in concentrations ranging from 42 to 103 μg/ml (n = 27) using double immunodiffusion as the quantitative method. This corresponds to values ranging from 0.5 to 1.2 μg PBCP/mg protein. Because of dilution with cellular protein, serum contamination would contribute insignificantly to the cytosol PBCP values reported below. In agreement with this prediction 27 serum samples diluted to give a final albumin concentration comparable to that observed in tumor cytosols (*i.e.*, a 1:20 to a 1:100 dilution), produced no "rockets" during immunoelectrophoresis. PBCP was also undetectable in cytosols prepared from ovarian tumors (n = 4), macroscopically normal livers (n = 2) as well as liver metastases (n = 3) derived from colon carcinomas.

A summary of the parameters measured is presented in Table 1. The following observations were made.

Malignant Disease. Cytosol PBCP concentration in 377 cytosols prepared from primary cancers ranged from 0–124 μg/mg cytosol protein with an average value of 4.0. Values for PBCP were clearly not normally distributed ($P < 0.001$, Smirnov-Kolmogorov test) and seem to define two patient populations (Fig. 1)—one group lacking PBCP in their tumors and the other containing PBCP with a lognormal distribution.

The age of these patients was normally distributed (Smirnov-Kolmogorov test, P = 0.076) with a mean of 63.1 ± 14.4 (± SD) years and a range of 16–96 years. Albumin concentrations expressed in percentage of cytosol protein showed a log-normal distribution (Smirnov-Kolmogorov test).

The primary malignant tumors investigated showed an expected incidence (10) of steroid receptors; 27.5% (105/382) being classified as ER-negative and 44.0% (168/382) as PR-negative, using a cutoff limit of 10 fmol/mg cytosol protein. PBCP was inversely correlated to ER content (Spearman rank, $R = -0.103$; P = 0.024). It was not significantly correlated to the level of progesterone receptor (R = 0.016, P = 0.38). PBCP-positive and PBCP-negative tumors showed nearly identical incidences of receptor-positive tumors amounting to 72.1 *versus* 73.6% for ER and 54.9 *versus* 57.6% for PR, respectively. As would be expected from the correlation analysis PBCP-negative tumors were generally richer in ER (average, 98.2 fmol/mg; SD, 128 fmol/mg) than PBCP containing tumors (average, 77.9 fmol/mg; SD, 127.8 fmol/mg). This difference did not reach statistical significance (P = 0.072, Mann-Whitney U test). Progesterone receptor concentration was quite similar in the two groups (54.8 *versus* 42.5 fmol/mg, P = 0.41 by Mann-Whitney U test).

PBCP was highly significantly correlated ($P < 0.001$) to albumin content expressed in percentage of total cytosol protein (Spearman rank, R = 0.236; n = 369). PBCP values decreased with age (Fig. 2) but this decline did not reach statistical significance (R = 0.081, P = 0.06). There was no decrease with age in the incidence of PBCP (*i.e.*, PBCP > 0) in the tumors examined, the overall incidence being 60.7%. The 40–50-year age group showed the highest incidence (71.1%) but this was not significantly different from overall incidence (χ^2 test, P = 0.21) or from the age group (70–80 years) with lowest incidence (55.9%) (χ^2 test, P = 0.10).

Table 1 *Characteristics of tumor populations*

Parameter	Primary cancer (n = 377)	Benign tumors (n = 58)	Metastatic disease (n = 71)
Patient age	63.1 ± 14.4	44.4 ± 15.0	64.6 ± 11.7
PBCP (μg/mg protein)	4.0 ± 11.0	13.5 ± 28.3	0.8 ± 2.6
Incidence (>0)	60.7%	81.0%	23.9%
Range	0–124	0–118	0–19
Cytosol protein (mg/ml)	4.3 ± 2.1	3.8 ± 1.7	3.8 ± 1.8
Albumin (% of soluble protein)	22.1 ± 11.8	34.2 ± 12.4	18.2 ± 9.9
Estrogen receptor (fmol/mg)	88.9 ± 153.2	11.8 ± 29.5	57.4 ± 86.3
Incidence (>10 fmol/mg)	72.5%	24.1%	64.8%
Range	0–1666	0–192	0–537
Progesterone receptor (fmol/mg)	47.1 ± 100.1	32.9 ± 47.3	28.8 ± 85.6
Incidence (>10 fmol/mg)	56.0%	56.9%	35.2%
Range	0–1183	0–237	0–647

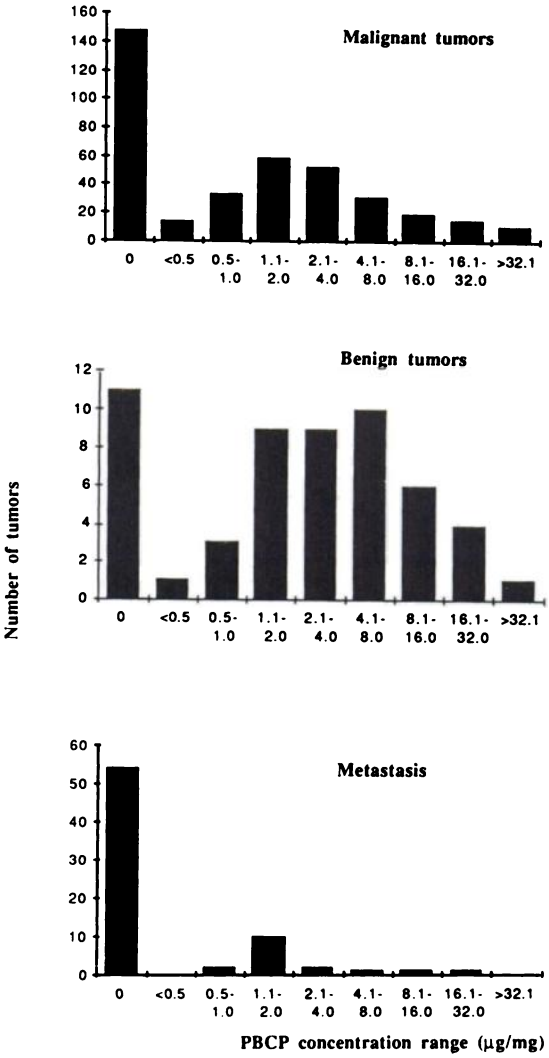

Fig. 1. Histogram showing distribution of tumor cytosol PBCP values (μg/mg cytosol protein) among patient groups. *Abscissa*, logarithmic scale.

Benign Disease. Tissue extracts from benign tumors showed an average PBCP value of 13.5 ± 28.3 μg/mg cytosol protein (mean ± SD, n = 58). Again, PBCP values did not appear normally distributed (skewness, 2.87; kurtosis, 7.36). PBCP was detectable in 81.0% (47/58) of the tumors. This incidence is significantly different (P = 0.0032) from the overall incidence observed in malignant tumors (χ^2 test). As would be expected there is a considerable difference in average age between the two patient groups (44.4 *versus* 63.1 years). If PBCP incidence in benign tumors is tested against that of the 40–50-year age group with malignant tumors this difference becomes statistically insignificant (χ^2 test, P = 0.25).

The albumin concentration in benign tumor cytosol averaged 32.2 ± 12.3% (±SD) of total protein which is significantly higher than that found in malignant tumors (Student's t test on logarithmically transformed values, $P < 0.005$).

Average concentrations of estrogen and progestin receptors

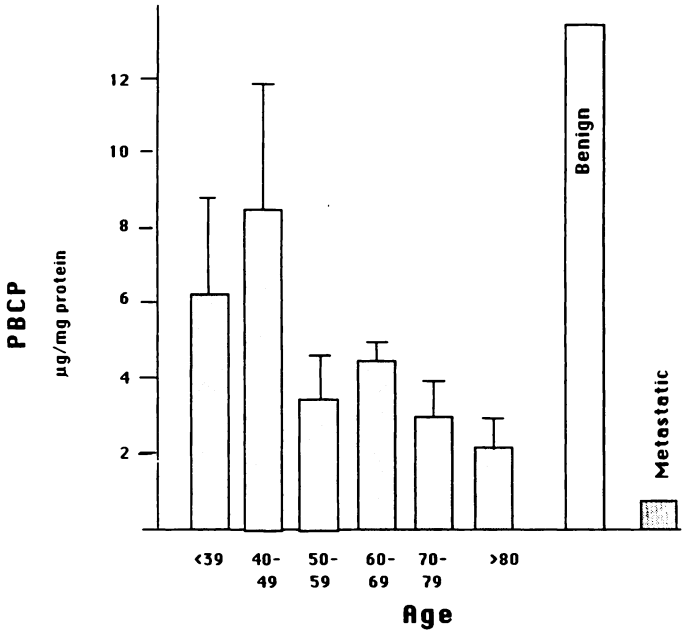

Fig. 2. Distribution of PBCP in primary mammary carcinoma according to patient age. PBCP was determined in tumor cytosol by "rocket" immunoelectrophoresis. *Bars on the right*, average values found in benign and metastatic tumors, respectively.

were 11.8 ± 29.5 and 32.9 ± 47.5 (±SD) fmol/mg soluble protein, respectively. The incidence of ER- and PR-negative tumors (*i.e.*, <10 fmol/mg protein) was 75.9% (44/58) and 43.1% (25/58), respectively. In this patient group PBCP appeared to be positively correlated to receptor values, the Spearman rank correlation coefficients being 0.32 ($P = 0.014$) and 0.29 ($P = 0.029$) for ER and PR, respectively. These results should be interpreted with caution due to the high incidence of low (0) receptor values observed, especially for the estrogen receptor.

The positive correlation between PBCP and albumin observed in malignant tissue could not be reproduced in benign tumors ($R = -0.062$, $P = 0.65$). PBCP was uncorrelated to total cytosol protein concentration ($R = 0.25$, $P = 0.06$) and to the age of the patient ($R = 0.15$, $P = 0.25$).

Metastatic Disease. Cytosols prepared from soft tissue metastasis, mainly from axillary lymph nodes or locoregional cutaneous lesions, contained detectable levels of PBCP in only 17 out of 71 samples investigated. This incidence is significantly lower ($P < 0.0001$) than in primary tumors or in benign disease. Among the few positive cytosols observed PBCP concentrations were generally low (range, 0.4–18.8 µg/mg protein) yielding an average value of 0.78 µg/mg protein, *i.e.*, 1/5 of the level present in primary cancer. Due to this low PBCP incidence no meaningful correlations to other tumor parameters could be carried out. Incidence and content of estrogen receptor were close to those of primary tumors whereas progesterone receptor levels were significantly lower than in benign and primary tumors (Table 1).

DISCUSSION

The presence of PBCP in tumor cytosol seems to be very much dependent on the localization of the tumor with a high incidence in tumors at the primary site and low incidence in locoregional recurrences. A positive correlation to tumor albumin values could suggest an extracellular localization of PBCP possibly related to the presence of microcysts. Both gross cystic disease and microcysts of the breast have a marked peak incidence at 40–50 years and is quite infrequent in postmenopausal women (11). This could explain the somewhat higher incidence of PBCP-positive tumors observed in the younger age group. However, the absence of a significant age correlation in PBCP both with respect to incidence and concentration seems to rule out microcysts as a major source of PBCP and strongly suggest that PBCP is produced by cancer cells, at least in primary tumors.

The lack of a positive correlation with steroid receptors in cancer tissue may suggest that PBCP is synthetized by a steroid-hormone independent mechanism or at least one that is not mediated through steroid receptors. Both PBCP and albumin show a negative correlation to the estrogen receptor suggesting that PBCP-positive tumors are either of low cellularity or show a high relative interstitial volume. These results are quite different from those reported by Silva *et al.* (4, 12), who showed, using a radioimmunoassay, that high cytosol concentration of cyst proteins were associated with high values for the estrogen and progestin receptor. The percentage of cancer cells showing positive immunohistochemical staining for a different cyst protein (GCDFP-15) was found by Le Doussal *et al.* (5) to correlate to the cytosol concentration of the progestin receptor. The positive correlation between PBCP and progestin receptor observed in benign tumors in the present investigation suggests that PBCP could be regulated by a hormonal mechanism in benign tumors. There is some evidence that the serum concentration of cyst proteins are under the control of androgens (13) but as long as the major source of production of immunoreactive serum PBCP is unknown the significance of this observation is difficult to assess.

Breast cysts are known to accumulate large quantities of steroid hormones and hormone precursors both in the form of free and conjugated steroids (2), especially sulfates (14). As a steroid-binding protein present in very high concentration in cyst fluid (10–30 µM),[4] PBCP may explain the accumulation of some steroids by way of passive diffusion. Alternatively, PBCP can be regarded as a marker of organelles that have developed the capacity of accumulating steroids and hence created their own microenvironment. Of special significance may be the accumulation of estrogen sulfate and DHEA-S. The former is a direct precursor of potent estrogens through the intermediate action of steroid sulfatases which, reportedly, are abundant in breast cancer cells (15, 16). Breast tissue from cancer patients has been shown to have higher than circulating levels of estrogens, suggesting local synthesis or the presence of an active transport mechanism. DHEA-S may also be converted to Δ-5-androstene-3β,17β-diol which may act as an estrogen (17). This diol could also block the binding to receptor of tritiated estradiol used in receptor assay leading to underestimation of ER. This could contribute to, but hardly explain, the negative correlation observed between ER level and PBCP concentration.

Recent results from Vermeulen's laboratory (18) suggest that DHEA-S have a direct influence on the activity of 17β-dehydrogenase, the enzyme converting estrone to estradiol. Local accumulation of DHEA-S may thus also modulate estrogen activity at this site. It is conceivable that cancer cells producing PBCP are growing under a liberal access to steroid hormones

[4] O. A. Lea, results to be published.

and may thus have developed special vulnerability to changes in hormonal environment.

The presence of PBCP in a tumor shows that these cells are highly differentiated and capable of secretory protein synthesis. This may have prognostic significance since highly differentiated tumors generally tend to grow more slowly and be more sensitive to hormonal manipulation. Breast tumors have been reported (19) to accumulate a number of blood-derived proteins of molecular radius smaller than 60 Å either as a result of increased capillary permeability or more likely, decreased lymphatic drainage at the site of the tumor. The present finding of a far higher albumin and PBCP content in benign compared to malignant tumor cytosol may reflect differences in tumor envelopment which are relevant to the process of metastasis. Work is in progress in this laboratory to determine if PBCP and albumin content are correlated to established prognostic factors such as tumor grade or differentiation (20), lymph node status, and to responsiveness to various forms of endocrine therapy.

ACKNOWLEDGMENTS

The authors wish to thank Hanne Winther and the EDB section of The University of Bergen for running some of these data on their computer.

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