Expression of the Gene Encoding a Prolactin-inducible Protein by Human Breast Cancers in Vivo: Correlation with Steroid Receptor Status

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ABSTRACT

We have previously reported the identification and characterization of a prolactin-inducible protein (PIP) as well as the cloning of the gene encoding PIP in cultured human breast cancer cells. We now present three lines of evidence that the gene encoding PIP is also expressed by some human breast cancers in vivo: (a) detection of PIP immunoreactivity in the serum of some breast cancer patients; (b) immunohistochemical detection of PIP in breast cancer sections; and (c) the presence of PIP mRNA, detected by complementary DNA hybridization in human breast biopsy samples.

In a preliminary study using Western blot analysis authentic PIP was detected in the serum of some patients with breast cancer. Subsequently the sera of 234 unselected patients with breast cancer were assayed for the presence of PIP using a specific radioimmunoassay. Thirty-five percent of these sera contained detectable PIP (i.e., > 3 ng/ml). As well we were able to show by immunohistochemical techniques that PIP immunoreactivity was present in some human breast biopsy specimens. Levels of estrogen receptor, progesterone receptor, and PIP mRNA were determined in an unselected population of 51 human breast tumor biopsies. Sixty-one percent of these tumors had detectable PIP mRNA: a positive correlation ($r = 0.52; P < 0.01$) was found between PIP mRNA levels in breast biopsy samples and estrogen receptor content, a known prognostic indicator in human breast cancer.

INTRODUCTION

A PIP secreted by the HBC cell line T-47D has been described previously (1), and recently its cDNA was cloned. Maximum expression of this gene occurs when T-47D cells are treated with human prolactin in the presence of hydrocortisone for 4-5 days (1). While not all HBC cell lines were found to express the PIP gene, those that did were both prolactin and estrogen receptor positive. The data suggested a potential value of PIP as a marker of prolactin action in HBC. However, before this question could be addressed it was first necessary to determine if the PIP gene was expressed in vivo and if so to investigate the relationship of PIP expression to known prognostic indicators in HBC such as ER and PgR.

Since PIP is a secreted protein (1) we approached the above questions by first investigating the presence of PIP in the serum of breast cancer patients by Western blot analysis and radioimmunoassay. Next we investigated the expression of PIP by HBC biopsies using both immunohistochemical techniques and specific cDNA hybridization techniques.

MATERIALS AND METHODS

Serum Samples. Coded blood samples were obtained from an unselected population of breast cancer patients attending the Manitoba Cancer Treatment and Research Foundation. Normal human blood samples were obtained from healthy volunteers. Blood samples were clotted, centrifuged, and the serum stored at $-20^\circ$C until assay.

Human Tumor Samples. Breast tumor biopsy samples were frozen to the laboratory for routine ER and PgR assay were the source of breast tumor tissue used for either immunohistochemical studies or RNA extraction and cDNA hybridization. Other human tumor samples were kindly provided by Dr. A. Alguacil of the Department of Pathology, Health Sciences Centre, Winnipeg, Canada. Dr. R. Matusik, Department of Physiology, University of Manitoba, generously donated samples of RNA extracted from a range of human benign prostate and prostatic carcinoma specimens. All tissues had been frozen as quickly as possible following surgery and pathological examination and stored at $-70^\circ$C.

ER and PgR Assays. Samples weighing between 0.2 and 0.3 g were taken for ER and PgR assay. Available steroid receptors were assayed using a single saturating dose and a charcoal-dextran method (2) for separation of bound from free hormone. ER and PgR concentrations were expressed as fmol of steroid bound per mg of cytosol protein.

RNA Isolation and Hybridization Analysis. RNA was isolated by the guanidinium thiocyanate/cesium chloride method (3). For Northern blot analysis RNA isolated as described above was denatured in 50% (v/v) formamide and 2.2 M formaldehyde, size fractionated on a 1% (w/v) agarose-2.2 M formaldehyde gel (4) and then blotted onto nitrocellulose paper (5). Filters were baked for 2 h at 80°C under vacuum and then prehybridized in hybridization solution for at least 3 h. The filters were then hybridized with nick-translated $^{32}$P-labeled purified PIP cDNA insert, cPIP-8-3 (specific activity, 1-5 x 10$^6$ cpm/µg DNA). Hybridizations, usually for 24 h, were performed at 42°C in the presence of 50% (v/v) deionized formamide, 5% Denhardt's solution (1× Denhardt's = 0.02% w/v each of BSA, Ficoll, and polyvinylpyrrolidone), 5× SSPE (1× SSPE = 0.15 M NaCl-0.01 M NaH$_2$PO$_4$-1 mM EDTA), 250 µg/ml denatured salmon sperm DNA, and 0.1% SDS. At the end of the hybridization period the blots were washed twice in 0.1% SDS-2× SSC (1× SSC = 0.15 M NaCl-0.015 M sodium citrate) for 15-30 min at room temperature, followed by one wash in 0.1× SSC-0.1% SDS for 45-60 min at 65°C. Blots were then exposed to Kodak XAR film at $-70^\circ$C with an intensifying screen.

For dot-blot analysis RNA extracted as described above was denatured according to the protocol of White and Bancroft (6) and spotted in varying concentrations (30-0.25 µg for tumors and MCF-7 HBC cells or 2-0.0625 µg for T-47D HBC cells) onto nitrocellulose filters using a BRL dot-blot manifold. Filters were treated as described above.

Quantitation of serially diluted RNA samples was achieved by densitometric scanning of various exposures of the dot-blot autoradiograms. The line of best fit obtained by least squares regression analysis of the integrated peak areas of the signal intensity versus the amount of RNA loaded was compared to that obtained from a standard serial dilution of hormone treated T-47D RNA, arbitrarily given a value of 100, present on each dot-blot. A negative control of MCF-7 human breast cancer cell RNA was also included on each dot-blot. Consistently we have been unable to detect the presence of PIP mRNA in this cell line by Northern analysis.
Immunohistochemistry. The method of Sternberger et al. (7) was used for this purpose. Briefly, after the samples were fixed with 4% paraformaldehyde, 6-μm sections were prepared from biopsy specimens embedded in paraffin and placed on glass slides. The sections were deparaffinized and hydrated through xylene and graded alcohol. Sections were then treated with normal goat serum (diluted 1:10 with PBS) for 10 min. The sections were then incubated for 1 h with rabbit anti-PIP antiserum or preimmune rabbit serum (for control), diluted 1:500. After washing with PBS the sections were reacted with goat anti-rabbit IgG (diluted 1:20) for 20 min. The sections were washed and a solution of rabbit peroxidase-antiperoxidase diluted 1:100, was added. After incubation for 30 min, the sections were washed. Peroxidase was visualized by placing the sections in a solution of 3,3'-diaminobenzidine tetrahydrochloride (0.6 mg/ml) containing hydrogen peroxide (0.05%) in 0.05 M Tris-HCl, pH 7.4 for 1 min. After washing briefly, the sections were lightly counterstained with toluidine blue so that only the nuclei appeared light blue. After mounting with Glycergel, the specimens were examined by Normarski interference microscopy.

Affinity Chromatography and Western Blot. Immunoglobulins were isolated from rabbit anti-PIP antiserum using a Protein-A-Sepharose CL-4B column. The eluted immunoglobulins were covalently coupled to cyanogen-bromide activated Sepharose 4B gel. Breast cancer serum (0.5 ml) diluted 1:5 in TBS (0.05 M Tris-0.1 M NaCl, pH 7.8) was applied to the affinity column pre-equilibrated with TBS. The column was then washed with TBS to remove unbound serum contaminants, and PIP was eluted with 5 mM sodium thiocyanate. Eluted PIP fractions were dialyzed against distilled water, lyophilized, and resuspended in SDS cocktail (10 mM sodium phosphate buffer, pH 7.2 with 2% sodium dodecyl sulfate, 5% β-mercaptoethanol, 10% glycerol, and trace amounts of bromophenol blue). The sample was heated for 5–10 min and then subjected to 15% SDS-polyacrylamide gel electrophoresis. Electrophoretic transfer of PIP from polyacrylamide gel to nitrocellulose was carried out using the method of Towbin et al. (8). After transfer, PIP was visualized on the nitrocellulose paper by sequential treatment with 3% BSA, PIP antiserum or preimmune normal rabbit serum (1:100 dilution), peroxidase-conjugated goat anti-rabbit IgG (1:3000 dilution), and finally a 4-chloro-1-naphthol/hydrogen peroxide solution.

Radioimmunoassay for PIP. Purified PIP and rabbit anti-PIP antiserum were generated in this laboratory as described previously (1). PIP was radioiodinated by the method of Hunter and Greenwood (9). The 125I-labeled PIP was purified on a Sephadex G-75 column. Each incubation contained 50 µl of PIP standard or unknown serum, 200 µl of assay buffer (1% w/v BSA in 0.01 M PBS, pH 7.4 and 1% v/v Triton X-100), 100 µl of anti-PIP antiserum (1:10,000 dilution), and 100 µl 125I-labeled PIP (50,000 cpm). Tubes used to establish the standard curve received 50 µl of pooled normal male serum (used simply due to its greater availability in our laboratory), and additional buffer was added to the unknown serum tubes to bring the final volume to 500 µl. Tubes were incubated at 4°C for 2 days, and separation of bound from free PIP was achieved by adding 100 µl of normal rabbit serum (dilution 1:300) and 100 µl of sheep anti-rabbit γ-globulin serum (dilution 1:160) to each tube. The incubation was continued for another 18–24 h at 4°C. The immunoprecipitate was centrifuged at 3,000 × g for 30 min at 5°C, the supernatant was decanted, and the remaining pellet was counted. At the antiserum dilution used, approximately 40% of the 125I-labeled PIP was bound. The interassay coefficient of variation ranged from 3% (for values >50 ng/ml) to 44% (for values <10 ng/ml). Replicate samples within an assay differed by less than 10%. The limit of detection of the assay was 3 ng/ml.

RESULTS

Presence of PIP in the Sera of Patients with Breast Cancer. Since PIP protein is a secreted protein (1) the presence of PIP in the serum of patients with breast cancer was investigated. To this end a specific radioimmunoassay for PIP was developed. Preliminary studies indicated that some sera contained a substance which inhibited the binding of 125I-labeled PIP to anti-PIP antibody. However, it was necessary to show that this immunoreactive material was indeed authentic PIP. Consequently, the immunoreactive material extracted from serum by affinity chromatography was analyzed by Western immunoblotting. This procedure confirmed the presence of PIP in breast cancer patient serum (Fig. 1). Pooled normal male serum when analyzed similarly showed no detectable PIP band (data not shown). Furthermore, those sera which were positive by Western blot analysis produced inhibition curves which were parallel to the PIP standard curve (Fig. 2).

Having shown that authentic PIP was indeed present in breast cancer patient serum, the sera of 234 unselected patients with breast cancer were then studied by radioimmunoassay. PIP was detected in the sera of 35% of these patients (Fig. 3). PIP was not detected in the sera of 11 female and 16 male healthy volunteers. This does not, however, exclude the possibility that a small subgroup of the normal population may also have detectable PIP in their serum.

The mere detection of PIP in the blood of some patients with breast cancer, however, does not clearly establish that the protein is being synthesized by the tumor.
Detection of PIP mRNA and PIP in Human Breast Cancer Biopsies. In order to investigate further the expression of the PIP gene by breast cancers in vivo, RNA was isolated from human breast tumor biopsy samples and subjected to Northern analysis. Of 20 tumors analyzed 4 were strongly positive for an approximately 900-base mRNA species equivalent to that seen in T-47D cells whereas 6 tumors were weakly positive (Fig. 4). Under the same conditions 5 samples of human benign prostatic hypertrophy, 2 samples of human prostatic carcinoma, a human bladder carcinoma, 3 human kidney tumors, 2 human colon cancers, 4 human pituitary adenomas, a human dermatofibrosarcoma, 3 samples of human placenta, 2 human uterine samples, one human thyroid sample, and one normal breast tissue sample contained no detectable PIP mRNA. However, 2 samples of nonmalignant human breast tissue showing cystic and proliferative disease had detectable PIP mRNA. Other normal primary cell lines and the "normal" human breast epithelial cell line, HBL 100 contain no detectable PIP mRNA. This study, although not exhaustive, suggests that PIP gene expression occurs in benign and malignant breast disease indicating that the phenomenon of PIP expression is not specific to mammary carcinoma.

Quantitation of PIP mRNA in human breast cancer biopsies was achieved using dot-blot analysis. A representative example of a dot-blot of RNA from samples of human breast cancer is shown in Fig. 5. Tumors such as 2, 7, 9, and 11 contained high levels of PIP mRNA, while tumors 1, 3, 4, and 12 showed detectable but definitely less quantities of PIP mRNA. Tumors such as 5 and 6 were essentially negative for PIP mRNA.

Immunohistochemical studies using PIP antibodies revealed the presence of PIP in HBC (Fig. 6), indicating that PIP mRNA was being translated in vivo. A representative tumor specimen is shown to illustrate positively stained cells, often in clusters, throughout the specimen. There was no staining when normal rabbit serum was substituted for PIP antiserum (data not shown).

Relationship of PIP mRNA to ER and PgR Status in Human Breast Cancer Biopsies. The data so far indicate that the PIP gene is transcribed and translated, and the PIP protein secreted by some HBC in vivo. The possibility exists that PIP gene expression may provide a useful tool to examine tumor cell
heterogeneity and identify more accurately those patients with hormonally dependent malignancy. In an attempt to test this hypothesis we have compared in the same tumor the level of PIP gene expression to that of ER and PgR, factors known to correlate with prognosis. A group of 51 breast tumors, selected because of sufficient size (±0.4 g) to allow all 3 determinations were extracted for RNA and analyzed by dot-blot hybridization (see Fig. 5 for a representative example of this analysis).

A number of comparisons were made in this group of 51 patients with breast cancer; these findings are presented in Table 1. Statistically significant positive correlations were found between (a) ER concentration and the patient’s age at the time of biopsy and (b) between ER concentration and relative PIP mRNA expression.

The mean ER concentration of this population of breast cancer patients was 42.5 ± 8.1 (SE) fmol/mg protein with values ranging from 1.2–249 fmol/mg protein. The mean PgR concentration was 67.9 ± 18.1 fmol/mg protein with the range of values being 0.6–574 fmol/mg protein.

**DISCUSSION**

The results presented in this communication show that the expression of PIP gene occurs in human breast cancer in vivo and is not merely a tissue culture phenomenon. While PIP expression in vitro has been shown to be regulated by prolactin in the presence of hydrocortisone, we do not as yet know if PIP expression in vivo is similarly regulated. The value of PIP as a marker of prolactin action in HBC in vivo awaits further study. However, the statistically significant correlation between ER concentration and relative PIP mRNA concentration in biopsy samples is particularly interesting since previous studies have shown a significant correlation between ER and prolactin receptor levels in HBC cell lines (10), and a similar correlation between ER and prolactin receptor has been found in human biopsy samples by some (10, 11) but not all investigators (12). This could be taken as evidence to suggest that PIP expression by HBC may also correlate with the presence of prolactin receptors. Due to the limited amount of tumor tissue available we were unable to measure prolactin receptors in this group of tumors. Nevertheless we have previously shown that only prolactin receptor positive HBC cell lines express the PIP gene.

It is unknown at this stage if there is any functional significance associated with the correlation found between ER and PIP expression in tumor samples. Estrogens at physiological concentrations do not seem to have any effect on PIP expression in T-47D cells in vitro. However, estrogens have been reported to modulate prolactin receptor levels in HBC cell lines (10, 13, 14) as well as in rodent hepatic tissue (15). Conversely prolactin has been shown to modulate ER in HBC cells (16). Probably the most likely explanation for our observation is that ER-positive tumors arise from hormonally sensitive cell types which also possess prolactin receptors and express PIP.

Using MCF-7 as a negative control we have arbitrarily assigned any hybridization signals greater than that seen for RNA from MCF-7 on dot-blot as PIP mRNA positive. The number of PIP mRNA positive tumors was then 61% in this tumor population. This figure is strikingly similar to that obtained from many studies which have measured 50–72% of prolactin receptor positive tumors in human breast cancer (10, 17, 18), although some groups have reported lower percentages.
of prolactin receptor positivity (19). The significant correlation between ER concentration and patient age at biopsy is consistent with other studies (20–23) and suggests that the population of breast cancer patients used by us is similar to others in which steroid hormone receptor levels have been reported. The correlation between ER and PgR levels in our group of tumors fails to achieve statistical significance. This is probably due to the small numbers in this study. Other studies, in which an unselected population of tumors has been studied and in which a significant correlation was found between ER and PgR concentrations, had larger numbers, usually greater than 100 patients (20, 22–24). Interestingly the correlation coefficients achieved in many of these studies were similar to the r values found in our study.

The detection of PIP expression by cDNA hybridization would appear to be a more sensitive technique than measurement of PIP by radioimmunoassay in serum (61 versus 35% PIP positivity, respectively). However, many variables determine the serum concentration of PIP, e.g., half-life of PIP in serum and the sensitivity of the assay. The population of patients used for the radioimmunoassay studies was different from that used for the tumor RNA studies, and no significant correlation of PIP serum levels and ER concentration of the original tumor (obtained from patients records) was found. This is not surprising since in many cases the steroid receptor assay had been done a year or more before a serum sample was taken for PIP assay. Also the effect of various forms of treatment that the patients had been subjected to during the time intervals on PIP serum levels is unknown. In contrast the measurement of PIP mRNA, ER, and PgR were performed on the same tumor biopsy samples. Although PIP mRNA was not detected in a small number of human tumors other than breast cancer such as prostate, bladder, colon, uterus, thyroid, and kidney cancer it was, however, detected in some benign breast tissue. Although this study is not exhaustive it tends to suggest that the expression of PIP occurs only in benign and malignant breast diseases. It is possible that the expression of PIP in combination with the known prognostic factor ER and/or PgR, may more accurately identify those patients with hormonally dependent malignancy. The usefulness of PIP expression as a prognostic index in human breast cancer, however, awaits further study correlating measurements with disease free interval and survival data.

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ADDENDUM

After submission of this manuscript we have found that the predicted amino acid sequence of PIP obtained from its cDNA nucleic acid sequence is the same as that of a protein which had previously been purified from human breast cyst fluid. This protein is called GCDFP-15 and has been detected by radioimmunoassay in the blood of some patients with breast cancer and patients with gross cystic disease of the breast (25).

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