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# Parathyroid Hormone-related Protein (PTHrP) in Breast Cancer and Benign Breast Tissue

**R.C. Edwards, W.A. Ratcliffe, J. Walls, J.M. Morrison, J.G. Ratcliffe, R. Holder and N.J. Bundred**

**Parathyroid hormone-related protein (PTHrP) 1–86 was quantified by immunoassay in extracts of 132 breast cancers, 27 samples of normal breast tissue and four fibroadenomas. PTHrP 1–86, was detected in 68% of primary tumours (range 40–302 000 fmol/g), 33% of normal breast tissues (range 100–1800 fmol/g), and all four fibroadenomas (range 110–11 600 fmol/g). PTHrP displayed molecular heterogeneity on gel filtration chromatography, and 1–86, 1–34 and 37–67 immunoreactivity eluted as 25–27 kDa together with a peak of 19–21 kDA containing only 37–67 activity. Tumour PTHrP 1–86 levels correlated inversely with age ($P < 0.05$) and were higher in premenopausal women ($P = 0.05$). The proportion of tumours containing PTHrP was higher in axillary node positive premenopausal women ($P < 0.05$). These data suggest that oestrogen may regulate expression of PTHrP in breast cancer and that production of PTHrP may be linked to development of axillary node metastases.**

**Key words: breast cancer, hypercalcaemia, parathyroid hormone-related protein, immunoassay, fibroadenoma, prognostic factors**

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## INTRODUCTION

PARATHYROID hormone-related protein (PTHrP) is widely expressed in malignant and non-malignant tissue [1, 2], and there is overwhelming evidence that tumour-derived PTHrP is the major humoral factor responsible for hypercalcaemia of malignancy [3]. PTHrP has been identified by immunohistochemistry in a wide range of neoplastic tissues from both normocalcaemic as well as hypercalcaemic patients [1, 4], with the highest incidence of staining in tumours of squamous histological type. In breast cancer, PTHrP has been localised in 52–69% of primary tumours [5–7] compared with 92% of bone metastases and 17% of metastases at non-bone sites [8] suggesting that expression in the primary tumour favours preferential spread to bone.

PTHrP is produced by the lactating mammary gland and is secreted into milk in high concentration [9, 10]. *In vitro* PTHrP is produced by mammary epithelial cells of lactating rats [11] and a human myoepithelial cell line [12]. The detection of PTHrP in normal breast tissue by immunohistochemistry has been an inconsistent finding [5, 13].

In this prospective study, we have further examined PTHrP in primary breast cancers by comparing quantitative tissue levels measured by immunoassay with clinical prognostic factors. PTHrP was extracted from primary tumours, normal breast tissue and fibroadenomas and measured by a highly sensitive two-site immunoradiometric assay (IRMA). The extracted PTHrP was further characterised by bioassay and also by gel filtration chromatography monitored by three region-specific immunoassays.

Correspondence to W.A. Ratcliffe.
R.C. Edwards, W.A. Ratcliffe and J.G. Ratcliffe are at Wolfson Research Laboratories, Department of Clinical Chemistry, Queen Elizabeth Medical Centre, Birmingham B15 2TH; J. Walls and N.J. Bundred are at the Department of Surgery, University Hospital of South Manchester, Manchester M20 8LR; J.M. Morrison is at the Department of Surgery, Selly Oak Hospital, Birmingham B29 6JD; and R. Holder is at the Department of Statistics and Mathematics, University of Birmingham, Birmingham B15 2TT, U.K.
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## PATIENTS AND METHODS

### *Materials*

PTHrP 1–34 and [Tyr$^0$]-PTHrP 1–34 were from Peninsula Laboratories, St Helens, Merseyside, U.K.; PTHrP 1–86 was from Bachem, Saffron Walden, Essex, U.K.; PTHrP 37–67 was prepared by Alta Bioscience, University of Birmingham, Birmingham, U.K.; PTHrP 1–141 was a gift from Professor T.J. Martin, Melbourne, Australia. These peptides were labelled with $^{125}$I using chloramine T to specific activities of approximately 200, 170, 465, and 180 Ci/g for PTHrP 1–141, 1–86, 37–67 and [Tyr$^0$]-PTHrP 1–34, respectively, and purified by Sep-Pak cartridge [14]. Bio-Gel P100 was from Bio-Rad Laboratories, Watford, Herts, U.K.; and Polypep (low viscosity), molecular weight standards for chromatography, and all other chemicals were from Sigma Chemical Co., Poole, Dorset, U.K.

### *Patients*

Approval for the study was obtained from the Ethical Committees of South Birmingham Health Authority, and the University Hospital of South Manchester. Patients with invasive breast cancer ($n = 132$) were operated on at the Departments of Surgery at Selly Oak Hospital, Birmingham, and the University Hospital of South Manchester. All patients were normocalca-

emic at the time of surgery. Following its excision, 0.2–1.0 g of tumour was cut into pieces of approximately 0.5 cm, immediately frozen in liquid nitrogen and subsequently kept at −70°C until extraction. Routine histology and receptor studies were performed on tissue immediately adjacent to that selected for PTHrP studies. The following prognostic factors were recorded; age, tumour size (mm), histological grade and type (by a modification of the Bloom and Richardson method [15]), pathological axillary lymph node status, and oestrogen and progesterone receptor status. Tumour oestrogen and progesterone receptor levels were measured by Scatchard analysis using dextran-coated charcoal, and tumours containing >10 fmol/mg protein were considered positive. Patients were designated as premenopausal (less than 12 months since the last menstrual period), or postmenopausal (more than 12 months since the last menstrual period). Four fibroadenomas and 27 samples of normal breast tissue were also studied, 18 from women undergoing breast reduction, or surgery for benign conditions, five from men with gynaecomastia, and four were histologically normal tissue obtained at the time of mastectomy from sites distant to the invasive breast cancer.

*Tissue extraction*

Tissue (0.1–0.3 g wet weight) was boiled at 100°C for 10 min [16] in 10 volumes of 1 M acetic acid, cooled to 4°C, homogenised for two periods of 30 s using a Polytron rotary homogeniser (Northern Media, Hessle, North Humberside, U.K.), and centrifuged at 13 000 *g* for 5 min [16, 17]. The pellet was washed in 250 µl 1 M acetic acid and, following a second centrifugation, the supernatants were pooled, frozen and lyophilised.

If sufficient tissue was available, the efficiency of extraction of PTHrP from individual tumours was assessed by adding exogenous PTHrP 1–86 to a portion of the tissue homogenate, and determining the percentage recovery in the reconstituted extract by immunoassay. Tissue PTHrP levels were corrected for the efficiency of extraction of PTHrP, or if tissue was insufficient, according to the mean extraction efficiency of 50 tumours (see Results).

*Assays*

Lyophilised extracts of tissues were reconstituted in assay diluent consisting of 0.25% Polypep (Sigma), 0.1% Triton X-100 and 0.01% sodium azide in phosphate buffered saline, pH 7.4. PTHrP 1–86 was measured in reconstituted extracts by a two-site IRMA, and PTHrP 1–34 and 37–67 were measured in column fractions by radioimmunoassay (RIA) as described previously [14, 18]. RIAs for PTHrP 1–34 and 37–67 are specific for residues 9–18 and 52–61, respectively, while the two component antibodies in the PTHrP 1–86 IRMA recognise residues 17–27 and 52–61 [18]. The limits of detection of the PTHrP 1–86, 1–34 and 37–67 immunoassays are 0.23, 12.5 and 57 pmol/l, respectively. Reconstituted extracts were serially diluted and assayed at a range of dilutions (e.g. 2–32-fold), and results were derived from those dilutions (usually at least two) which gave a linear response in the assay.

Plasma PTHrP 1–86 was measured by two-site IRMA [14]. Blood was collected pre-operatively in the presence of EDTA as an anticoagulant, separated within 15 min, and the plasma was stored at −20°C until assay.

Bioactivity was measured in serially diluted reconstituted tissue extracts using a method previously described in detail [19]. The rat osteosarcoma cell line 17/2.8 was kindly donated by Dr D.A. Heath. Results were expressed in terms of PTHrP 1–34 and were derived from a minimum of two dilutions, each in triplicate, and these gave a linear response in the assay. The number of tissues in which PTH-like bioactivity was measured was limited by the high detection limit of the bioassay (78 pmol/l) and the consequent need for larger samples of tissue.

*Gel filtration chromatography*

Gel filtration chromatography of tissue extracts, (1 ml corresponding to at least 0.1 g of tissue), was performed on a 100 × 1 cm column of Bio-Gel P-100 using the same acidic denaturing solvent used for tissue extraction (1 M acetic acid) [16, 17]. Fractions of 0.56 ml were collected at a flow rate of 2–3 ml/h, and lyophilised prior to reconstitution in 0.6 ml diluent for assay. The column was calibrated with protein molecular weight markers and iodinated synthetic fragments of PTHrP: (1) bovine serum albumin, 66 000; (2) carbonic anhydrase, 29 000; (3) $^{125}$I-labelled PTHrP 1–141, 16 000; (4) cytochrome c, 12 400; (5) $^{125}$I-labelled PTHrP 1–86, 9,903; (6) aprotinin, 6,500; and (7) $^{125}$I-labelled [Tyr$^{0}$]PTHrP 1–34, 4,180.

*Statistical analysis*

The relationship between tumour PTHrP levels or positivity and prognostic factors was assessed by Kruskal Wallis, $\chi^2$, or product moment correlation.

## RESULTS

*Extraction of PTHrP*

The efficiency of extraction was assessed from the recovery of immunoreactive PTHrP 1–86 in an extract derived from a homogenate spiked with exogenous PTHrP 1–86. The mean (±SEM) recovery for 50 tumours was 52% (±1.9%) and tissue PTHrP 1–86 levels were corrected accordingly using individual extraction efficiencies. For remaining tumours, where insufficient tissue was available to perform individual recoveries, the mean value of 52% was used as a correction factor.

*Assays*

The within-batch assay precision (CV%) of the 1–86 IRMA for assay of tumour extracts was 20, 8, 7, 6 and 7% ($n$ = 7) at 3.1, 6.9, 17, 31 and 41 pmol/l. Immunoreactivity in tissue extracts and column fractions diluted parallel to the assay standard in all immunoassays (Figure 1).

*PTHrP levels in malignant and benign tissue*

Tissue PTHrP 1–86 levels in primary breast cancers, normal breast tissue and fibroadenomas are shown in Table 1. PTHrP

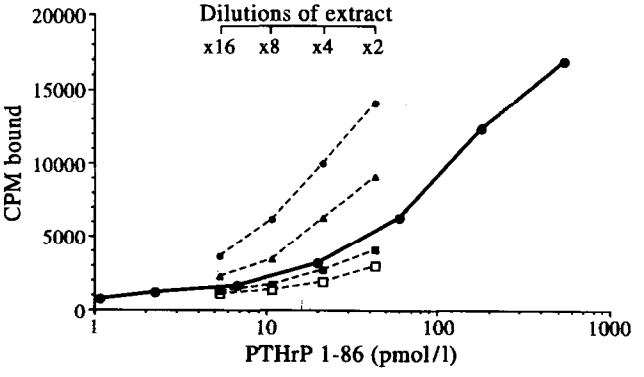


**Figure 1. Standard curve for parathyroid hormone-related protein (PTHrP) 1–86 (solid line) and responses of diluted extracts of four breast cancers (broken lines).**

*Table 1. Parathyroid hormone-related protein (PTHrP) levels in 132 breast cancers, 27 normal breast tissues and four fibroadenomas*

| | n | PTHrP 1–86 (fmol/g tissue) Median | Range |
|---|---|---|---|
| *Breast cancer* | | | |
| PTHrP detected | 90 (68%) | 190 | 40–302 000 |
| *Normal breast* | | | |
| PTHrP detected | 9 (33%) | 230 | 100–1800 |
| *Fibroadenoma* | | | |
| PTHrP detected | 4 (100%) | 5600 | 110–11 600 |

1–86 was detectable in 68% of primary breast cancers, 33% of normal breast tissue, and all four fibroadenomas examined. Absolute levels of PTHrP 1–86 in fibroadenomas and normal breast tissue were within the range found in breast cancers and did not differ significantly in tissue from normal women, males with gynaecomastia, and women with breast cancer (Table 1). The breast cancer patients had a mean age of 61 years (range 35–90 years); corresponding ages of patients from whom the fibroadenomas and normal breast tissue were obtained were 35 years (range 20–59 years) and 45 years (range 20–65 years), respectively.

Bioactive PTHrP 1–34 was not detected (<700 fmol/g) in extracts of two normal breast tissue and two breast cancers containing undetectable PTHrP 1–86. Bioactivity was detected in two tumours containing high levels of PTHrP 1–86 immunoreactivity, but not in a third containing immunoreactive PTHrP (790 fmol/g) close to the detection limit of the bioassay.

*Chromatographic characterisation of PTHrP*

Two breast cancers and two fibroadenomas containing high levels of immunoreactive PTHrP were examined by gel filtration chromatography. Typical elution profiles of PTHrP 1–86, 1–34 and 37–67 immunoreactivities are shown in Figure 2. In each case, the component of highest molecular weight eluted with an apparent mass of 25–27 kDa and contained PTHrP 1–86, 1–34 and 37–67 activities, while a second peak comprising only 37–67 activity eluted as approximately 20 kDa. The latter was the major PTHrP species in the two tumours examined (Figure 2a).

*Plasma PTHrP 1–86 levels*

Plasma PTHrP 1–86 was <0.23 pmol/l in 46 unselected patients prior to surgery, 32 of whom had tumours containing immunoreactive PTHrP.

*Relationship between tumour PTHrP and prognostic factors*

Both absolute PTHrP 1–86 concentrations and the frequency of detection of PTHrP in tumours were found to be related to the age and menopausal status of the patient. Tumour PTHrP 1–86 levels were higher in pre- than postmenopausal patients (median, range 275, 52–11 576 versus 174, 38–22 710 fmol/g; $P < 0.05$) and PTHrP 1–86 was detected in a higher proportion of tumours from pre- versus postmenopausal patients (34/41 versus 61/106; $P < 0.05$). Independent of menopausal status, there was a weak but significant inverse correlation between tumour PTHrP 1–86 levels and age ($r = -0.22$, $P < 0.05$) (Figure 3). Because of this, relationships between tumour

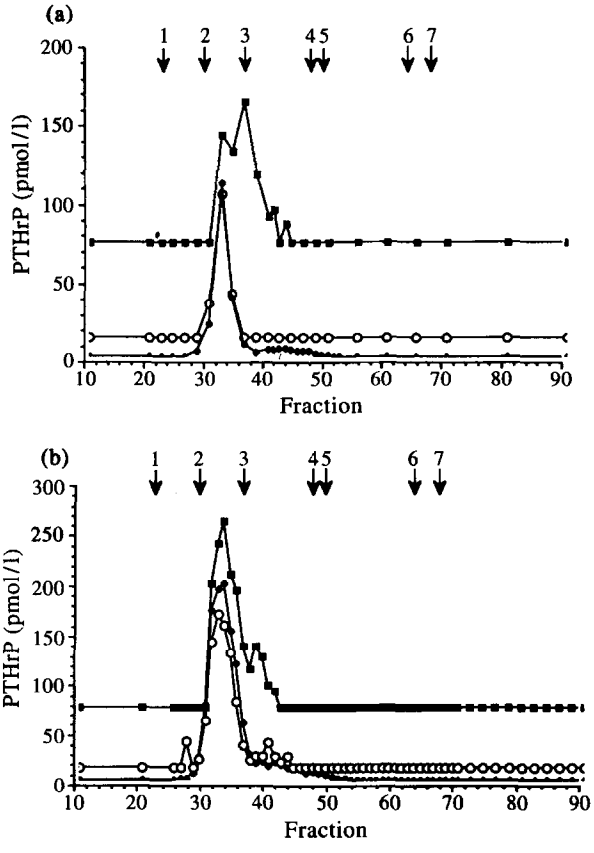


**Figure 2. Gel filtration elution profiles of extracts of a primary breast cancer (a) and a fibroadenoma (b). Gel filtration was performed on a column of Bio-Gel P-100 in 1 M acetic acid. PTHrP 1–8 (◆), 1–34 (○), and 37–67 (■) immunoreactivities were measured in column fractions by immunoassay. In (a), recoveries of 1–86, 1–34 and 37–67 activities in the fractions were 83, 36 and 62%, respectively, of the activity loaded on the column. In (b), recoveries of 1–86, 1–34 and 37–67 activities in the fractions were 99, 50 and 93%, respectively, of the activity loaded on the column. The column was calibrated with (1) bovine serum albumin, (2) carbonic anhydrase, (3) $^{125}$PTHrP 1–141, (4) cytochrome c, (5) $^{125}$I-PTHrP 1–86, (6) aprotinin, (7) $^{125}$I-PTHrP 1–34.**

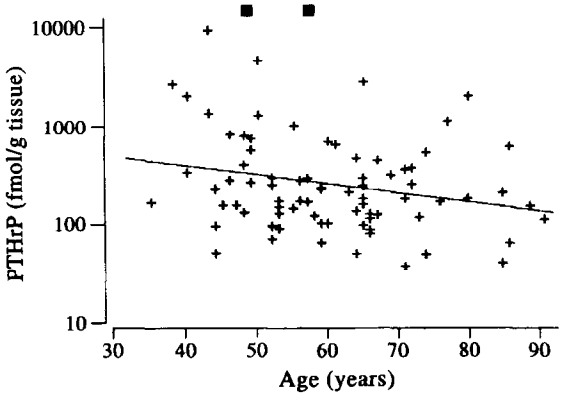


**Figure 3. Relationship between parathyroid hormone-related protein (PTHrP) levels in breast cancers and age of the patient ($P < 0.05$). Tissue levels of 22 710 and 302 000 fmol/g are denoted as ■.**

PTHrP and age and other prognostic factors were compared in groups of pre- and postmenopausal patients (Table 2). PTHrP was detected in a greater proportion of premenopausal women with positive, rather than negative, axillary nodes ($P < 0.05$) (Table 2).

## DISCUSSION

PTHrP was detected by immunoassay in 68% of breast cancers and 33% of normal breast tissues. The overlap in the tissue levels suggests that quantitation of tissue PTHrP is unlikely to be useful in diagnosis. The wide range in tissue PTHrP levels may in part reflect differences in tissue cellularity. PTHrP is produced by both epithelial and myoepithelial cells in human breast tissue [5, 6, 12, 13], and PTHrP present in endothelial cells of blood vessels [2] may also contribute to the levels measured in tissues. The PTHrP 1–86 levels found in the breast cancers were similar to those previously found in a variety of tumours from normo- and hypercalcaemic patients [17], and PTHrP 1–34 levels in tumours associated with hypercalcaemia [16, 20]. Where sufficient tissue was available to measure both immuno- and PTH-like bioactivity, these activities were closely ranked, suggesting that the PTHrP was bioactive. PTHrP immunoreactivity has not been detected in plasma from the majority of normocalcaemic breast cancer patients [6] suggesting that its production by tumour tissue is insufficient to disrupt calcium homeostasis. In contrast, plasma PTHrP levels are elevated in the majority of breast cancer patients with hypercalcaemia [6, 21], consistent with its humoral role [3].

PTHrP may have a number of physiological actions in normal breast tissue. In lactation, PTHrP is secreted into milk in high concentration, and also enters the maternal circulation where it may act to mobilise calcium from the maternal skeleton [10, 22]. Myoepithelial cells cultured *in vitro* produce PTHrP, and effects of PTHrP on intracellular calcium and cyclic 3′-5′adenosine monophosphate (cAMP) in these cells suggests that it may function to regulate the flow of milk [12]. It may also regulate blood flow to the mammary gland during suckling [23], and have a role in the active transport of calcium into milk [24]. In the newborn, immunoreactive PTHrP from milk crosses the gut and may affect calcium homeostasis and/or tissue growth [25].

PTHrP has previously been localised by immunohistochemistry in hyperplastic mammary tissue [13, 26], and this study also found increased frequency of expression of PTHrP in fibroadenomas compared with normal breast tissue (100% versus 33%), and higher tissue PTHrP levels than in normal breast tissue and breast cancers (median 5600 fmol/g versus 230 and 190 fmol/g, respectively). The PTHrP levels in fibroadenomas may reflect the higher cellularity of the tissue and the younger ages of the patients. Studies of PTHrP secretion by normal and malignant keratinocytes have suggested that a relationship exists between the differentiated state of the cell and production of PTHrP, with increased production associated with induction of differentiation [27]. In renal cell carcinoma, there is evidence that PTHrP functions as an autocrine growth factor [28]. It remains to be established whether PTHrP has a similar role in the pathogenesis of benign and malignant diseases of the breast.

PTHrP has previously been localised by immunohistochemistry in 52–69% of primary breast cancers [5, 6, 13]. This is the first study to quantify PTHrP in malignant tissue and examine its relationship with prognostic factors. PTHrP 1–86 was detected by immunoassay in 68% of breast cancers, whereas we have previously localised PTHrP 37–67 activity by immunohistochemistry in 52% of breast cancers [6]. This difference may reflect the specificities of the antibodies employed and the differences in the detection limits of the methods. In contrast, PTHrP gene expression has been identified in the majority of breast cancers using amplification of complementary DNA by the polymerase chain reaction [29]. In this study, both the

*Table 2. Distribution of clinical and pathological variables according to parathyroid hormone-related protein status of breast cancers in pre- and postmenopausal women*

| | Premenopausal | | Postmenopausal | |
|---|---|---|---|---|
| | Number assayed | % with detectable PTHrP | Number assayed | % with detectable PTHrP |
| *Histology* | | | | |
| Lobular | 5 | 80 | 12 | 58 |
| Ductal | 29 | 86 | 72 | 64 |
| *Histological grade* | | | | |
| I | 1 | 100 | 11 | 45 |
| II | 16 | 75 | 40 | 58 |
| III | 13 | 100 | 29 | 79 |
| *Axillary node status* | | | | |
| Negative | 12 | 42 | 37 | 65 |
| Positive | 23 | 91* | 36 | 67 |
| *Oestrogen receptor* | | | | |
| Negative | 8 | 88 | 21 | 71 |
| Positive | 15 | 87 | 42 | 59 |
| *Progesterone receptor* | | | | |
| Negative | 14 | 79 | 35 | 66 |
| Positive | 9 | 100 | 28 | 61 |

* $P < 0.05$.

frequency of expression and the concentration of PTHrP in tumours correlated with age and menopausal status, with higher levels in younger, premenopausal women. This is consistent with the correlation found between amplified complementary DNA for PTHrP in breast cancers and the age of the patient [29], and suggests that expression of PTHrP in breast cancer is increased by oestrogen as in the rat uterus [30].

An initial retrospective study linked the detection of PTHrP in primary breast cancers by immunohistochemistry to the progesterone receptor status of the tumour and a favourable index of prognosis [5], although in a later study, the frequency of detection was similar in primary breast cancers associated with a widely differing prognosis [7]. The present study, quantifying tumour PTHrP levels, has failed to confirm a relationship with either oestrogen or progesterone receptor status, although the link found between PTHrP positivity and axillary node involvement is consistent with the positive correlation found between PTHrP gene expression and node involvement [29]. Earlier studies also suggested a link between expression of PTHrP and the subsequent development of metastases. Powell and colleagues found a higher frequency of immunostaining for PTHrP in bone metastases compared with primary breast cancers or metastases at non-bone sites [8], and PTHrP gene expression was present in a higher proportion of tumours which metastasised to bone rather than non-bone sites [31]. Longer term follow-up of patients in the present prospective study will indicate whether expression of PTHrP in the primary tumour does indeed correlate with the subsequent development of bone metastases.

PTHrP from several sources including milk [32], conditioned media [33], and tumours has been shown by chromatography [17, 19, 21] and pulse-chase studies [34] to be heterogeneous with respect to size. Acidic denaturing conditions were used in the present study for extraction and chromatography to prevent proteolysis *in vitro*. Two major peaks were resolved in both fibroadenomas and breast cancers, the smaller component, eluting as approximately 21 kDa, contained only 37–67 immunoreactivity, consistent with proteolytic cleavage of the amino-terminus of PTHrP. Mid-region subfragments of PTHrP derived by post-translational proteolytic processing at residue 37 may represent the major intracellular and secreted forms of the peptide [35, 36]. The larger species, containing 1–34, 1–86 and 37–67 activity, eluted with an apparent mass of 25–27 kDa, similar to that of PTHrP from tumours from other sites [17]. The biological significance of the heterogeneity of tissue PTHrP is at present unclear. PTHrP has been considered to be a polyhormone or a precursor for peptides with differing biological actions and proteolysis may serve to regulate these actions [37].

In summary, median PTHrP levels were similar in primary breast cancers, and normal breast tissue, although the highest levels were found in breast cancers. Tumour PTHrP levels, and the frequency of detection of PTHrP in tumours, correlated inversely with age, were higher in premenopausal women, and correlated positively with axillary node involvement. Factors which regulate expression of PTHrP in breast cancer, and its role in tumour growth and metastatic spread remain to be established.


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# Levels of Circulating Intercellular Adhesion Molecule-1 in Patients with Metastatic Cancer of the Prostate and Benign Prostatic Hyperplasia

J.M. Wolff, R.N. Stephenson, G.D. Chisholm and F.K. Habib

**Current reports suggest a role for intercellular adhesion molecule-1 (ICAM-1) in the progression of malignancy. The availability of a new antibody makes it possible to measure circulating ICAM-1 (cICAM-1) in human body fluids including serum; this might help in monitoring tumour burden and in providing additional prognostic information. In this study, serum levels of cICAM-1 were measured by an ELISA assay in patients with benign prostatic hyperplasia (BPH; $n=20$) and metastatic cancer of the prostate (CaP; $n=25$). Serum ICAM-1 concentrations were also measured in a group of healthy men ($n=8$). The mean ± S.E.M. cICAM-1 level for BPH was 339.52 ± 15.30 ng/ml compared with 263.55 ± 18.54 ng/ml for CaP. Even though the difference between the two groups was significant ($P<0.005$), there was a marked overlap between the individual values in both groups, thus minimising the prognostic value of these measurements in prostate cancer. Endocrine therapy had no notable effect on the serum levels of cICAM-1. The mean ± S.E.M. cICAM-1 concentrations in serum from a younger group of healthy volunteers was 204.1 ± 10.38 ng/ml, and this value was significantly lower than that measured in serum from either BPH or CaP. We also undertook some immunohistochemical studies to examine the distribution of ICAM-1 in prostate tissue. We observed focal epithelial cell membrane staining which was exceedingly patchy in both the BPH and cancer specimens. On the basis of these studies, we suggest that cICAM-1 levels do not provide additional information on patients with metastatic CaP.**

**Key words: ICAM-1, BPH, prostate cancer**
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