Parathyroid hormone (PTH) / PTH-related protein (PTHrP) receptor expression and mitogenic responses in human breast cancer cell lines

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Summary Previous reports have shown the production of parathyroid hormone-related protein (PTHrP) by breast cancer cells in vitro and in vivo. We have investigated the expression of the PTH/PTHrP receptor by the human breast cancer cell lines MCF-7, ZR-75-1, T-47-D, SK-BR-3, Hs578T and MDA-MB231. Using reverse transcription–polymerase chain reaction (RT–PCR) and Southern blot analysis, we detected transcripts for the receptor in MCF-7, SK-BR-3 and MDA-MB231 cells. There was no evidence of receptor mRNA in ZR-75-1 and Hs578T cells. Furthermore, Northern blot analysis of mRNA from MCF-7 cells showed two transcripts of 1.5 and 2.4 kb which coded for the PTH/PTHrP receptor. Expression of PTH/PTHrP receptor mRNA by the breast cancer cell lines was also correlated with the detection of PTHrP transcripts. RT–PCR demonstrated PTHrP mRNA in MCF-7, ZR-75-1, T-47-D and Hs578T cells, but not in SK-BR-3 and MDA-MB231 cells. The detection of receptor transcripts was complemented by [3H]thymidine and bromodeoxyuridine incorporation studies, in which mitogenic responses to PTH and PTHrP were observed in MCF-7 cells but not in Hs578T cells. In response to both PTH(1–34) and PTHrP(1–34), quiescent MCF-7 cells proliferated in a similar dose-dependent manner (1.6–100 ng ml⁻¹). No mitogenic effects of these peptides were observed with Hs578T cells. In addition, levels of intracellular cAMP were measured in MCF-7 and Hs578T cells in response to PTHrP(1–34). In MCF-7 cells there was a significant rise in cAMP with 100 ng ml⁻¹ PTHrP(1–34). The expression of PTH/PTHrP receptor by breast cancer cells suggests that PTHrP may be a paracrine autocrine regulator of breast carcinoma.

Keywords: PTH, PTHrP receptor; PTHrP: human breast cancer

Parathyroid hormone (PTH) is secreted by the parathyroid glands and is a systemic hormone which regulates calcium and phosphate homeostasis. PTH-related peptide (PTHrP) has considerable N-terminus homology to PTH (Goltzman et al., 1989; Martin and Suva, 1989), stimulates bone resorption in vitro (Evely et al., 1990) and in vivo (Horiuchi et al., 1987) and is known to mediate humoral hypercalcemia of malignancy (Broadus et al., 1988). Initially identified in tumour cells (Burris et al., 1987; Moseley et al., 1987; Stewart et al., 1987; Strewler et al., 1987), PTHrP has now been localised in several normal tissues and a range of fetal tissues (Moniz et al., 1990) and, although the role of PTHrP has yet to be fully elucidated, it probably acts as a paracrine/autocrine regulator of cell function.

PTH and PTHrP exert their effects through cell-surface receptors which were originally thought to be principally in kidney and bone. Binding of the ligand to the receptor has multiple effects, including elevation of intracellular cAMP (Chase et al., 1969), [Ca²⁺] (Donahue et al., 1988; Schoell et al., 1991) and inositol phosphates (Cosman et al., 1989).

Expression cloning has identified a single PTH/PTHrP receptor, which binds both PTH and PTHrP at high affinity (Abou-Samra et al., 1992). In rat, transcripts for this receptor have been identified in bone and kidney but also in many other tissues, including breast (Urena et al., 1993). It has therefore been proposed that this single receptor species mediates many of the physiologically diverse actions of PTH/PTHrP throughout the body.

PTHrP can be identified in normal lactating mammary tissue (Thiede and Rodan, 1988) and has been detected in primary cultures of mammary epithelial cells (Ferrari et al., 1992). In transgenic mice which overexpress human PTHrP there is profound breast hypoplasia, suggesting an important role for PTHrP in the regulation of mammosogenesis (Wysolmerski et al., 1993). The peptide has been isolated from a mammary tumour (Burris et al., 1987; Stewart et al., 1987) and identified in cultures of breast cancer cells (Walsh et al., 1992; Francini et al., 1993). In an immunocytochemical study, 60% of the human breast carcinomas investigated expressed PTHrP (Southby et al., 1990). In addition, immunocytochemistry (Bundred et al., 1991; Powell et al., 1991) and in situ hybridisation (Vargas et al., 1992) have revealed that a significant number of skeletal metastases arising from breast carcinoma express PTHrP. We have demonstrated that the human breast cancer cell line Hs578T produces PTHrP, while MCF-7 cells do not (Walsh et al., 1992). Furthermore, transcripts for PTHrP have been detected in a primary human cell line, 8701 BC, which was isolated from a primary ductal infiltrating carcinoma of the breast (Luparello et al., 1993). These findings indicate that PTHrP expression by breast cancer cells may play an important role in progression of the tumour. Expression of the PTH/PTHrP receptor may confer autocrine-paracrine regulation of the breast tumour cells by PTHrP. In this study we have investigated the expression of the PTH/PTHrP receptor by human breast cancer cell lines.

Materials and methods

Cell culture

ZR-75-1, T-47-D, SK-BR-3, MDA-MB231 (all gifts from Professor P Rudland, Department of Biochemistry, University of Liverpool, UK), MCF-7 (a gift from Dr C Green, Department of Biochemistry, University of Liverpool, UK) and Hs578T (from ECACC, Porton Down, UK) and the human osteosarcoma cell line, Saos-2 (a gift from Dr J Beresford, Bath Institute of Rheumatic Diseases, UK) were grown in minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) (Sera Lab), 2 mM L-glutamine (Gibco) and antibiotics (100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin) (Gibco).

Reverse transcription-linked polymerase chain reaction (RT–PCR) analysis of PTH/PTHrP receptor mRNA expression

Total RNA was extracted from the cell monolayers in 9 cm Petri dishes (Falcon) by the method of Chomczynski and
Saccon et al. (1997). RNA was used as a template for cDNA synthesis in a 50 μl volume containing the following reagents: 0.5 mM each of dATP, dCTP, dGTP, dTTP (Pharmacia), 2 μg of oligo-dT (Pharmacia), 20 U of RNase inhibitor (Boehringer), 10 mM dithiothreitol, 6 mM magnesium chloride, 40 μM potassium chloride, the presence of 50 μM Tris–Cl (pH 8.3) and 200 U μl−1 RNAse of mouse Moloney leukaemia virus (MMoLV) reverse transcriptase (Gibco). The reaction was incubated at 37°C for 60 min and stopped by freezing at −20°C.

PCR analysis was performed using oligonucleotides which were designed from the published sequence (Abou-Samra et al., 1992) for the PTHrP receptor. The primers which amplified a fragment of 571 bp were:

**Sense**: 5'-AGGAAAGATCCTCTGCTGCA-3'

**Antisense**: 5'-TGCTATGGATCTGGTGGGCTG-3'

Primers for the detection of human PTHrP have previously been described (Walsh et al., 1994).

The PCR reaction mixture (final volume 50 μl) contained 1 unit of Thermoprime (Advanced Biotechnologies), 0.5 μg of each oligonucleotide primer, 200 μM each of dATP, dCTP, dGTP, dTTP, 1.5 mM magnesium chloride, 10 mM β-mercaptoethanol, 10 mM Tris–HCl (pH 8.3) and 2 μl of the cDNA template. The following thermal cycle was used: a denaturation step of 94°C for 2 min followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s. The thermal cycle for amplification of PTHrP products was identical except for the annealing step, which was performed at 62°C. PCR products were analysed by agarose gel electrophoresis and visualised by ethidium bromide staining under UV light.

**PTHrP receptor amplification products** were identified by Southern blot analysis. Products were separated by agarose gel electrophoresis and blotted onto Hybond N (Amersham). The PTHrP receptor probe was 571 bp (corresponding to 131–702 bp of the human PTH receptor mRNA, accession no. L04308) which had been cloned into pBluescript (a gift from Dr D Evans, Ciba-Geigy, Basle, Switzerland). The PTHrP probe was a 535 bp fragment of PTHrP which had been cloned into pBluescript and sequenced (Walsh et al., 1994). cDNA was labelled with [32P]dCTP (specific activity 3000 Ci mmol−1) using a random primed labelling kit (Boehringer). The blots were prehybridised for 2 h at 42°C in 40% formamide, 5 x SSC, 50 μM phosphate buffer (pH 7), 1 x SDS, 10 x Denhardt's and 200 μg ml−1 denatured salmon sperm DNA (Sigma). Denatured probe (5 x 106 c.p.m. per membrane) was added directly to the prehybridisation mix and incubated at 42°C overnight. Membranes were washed three times in 0.2 x SSC/1% SDS at 60°C for 20 min and exposed to X-O-mat film (Kodak) with an intensifying screen at −70°C.

**Northern blot analysis**

A 5 μg aliquot of poly(A) + RNA from MCF-7 cells was fractionated on a formaldehyde agarose gel and transferred to Hybond N (Amersham). The membrane was then prehybridised, hybridised and washed as described previously. The membrane was then exposed to X-O-mat film (Kodak) with an intensifying screen at −70°C for 5 days.

**Measurement of thymidine incorporation**

Cells were detached from the culture dish with 0.25% trypsin, resuspended in DMEM/10% FCS and seeded into a 96-well plate at 5 x 104 cells per well. The plates were incubated at 37°C for 72 h to ensure that all wells were confluent, the medium removed and the cells washed once in serum-free DMEM/0.1% bovine serum albumin (BSA) and 1% FCS. The same DMEM/0.1% FCS/1% BSA was added to each well. The wells were incubated for a further 4 days in serum-free medium, after which the medium was again replaced with 100 μl of fresh medium/0.1% BSA containing various concentrations of PTH(1–34) or PTHrP(1–34) (both Peninsula Labs). A 0.5 μCi aliquot of [3H]thymidine was then added to each well and the plates were incubated for a further 24 h. The plates were harvested with a cell harvester and [3H]thymidine incorporation measured by scintillation counting.

**Measurement of bromodeoxyuridine uptake**

Uptake of bromodeoxyuridine (BrdU) by MCF-7 cells was assessed by immunocytochemical staining using an Amer sham cell proliferation kit (Amersham, UK). PTHrP (100 ng ml−1) was added to serum-free cultures of MCF-7 cells. After 20 h incubation at 37°C bromodeoxyuridine labelling of the receptor was added. After a further 4 h incubation the medium was removed from the dishes and the cell layers were fixed in 90% ethanol/10% acetic acid. Immunocytochemical detection of BrdU was then carried out using mouse anti-BrdU followed by horseradish peroxidase-coupled rat anti-mouse immunoglobulin and 2,3-diaminobenzidine as substrate. The dishes were examined microscopically and the percentage of dark-staining nuclei recorded in each field. Three wells were used for each treatment and ten fields examined in each well.

**Measurement of intracellular cyclic AMP levels**

The cell line(s) to be investigated were seeded in DMEM/10% FCS in six-well plates and grown to confluence. The medium was then removed and replaced with serum-free DMEM containing the phosphodiesterase inhibitor 1μM 3-isobutyl-1-methyl-xanthine (IBMX) (Sigma) and PTHrP as appropriate. After 20 min incubation with or without PTHrP, the medium was removed and 0.5 ml of 60% ethanol added to each well. The cell layer was scraped into the ethanol with a plastic transfer pipette and transferred to a 1.5 ml Eppendorf tube. The sample was then acetylated and cAMP level measured by competitive radioimmunoassay as previously described (O'Reilly et al., 1986).

**Statistical analysis**

Statistical analysis was carried out by analysis of variance (ANOVA), and significance between groups determined by Duncan's new multiple range test.

**Results**

Figure 1a shows RT–PCR analysis of PTH/PTHrP receptor using cDNA from MCF-7, ZR-75-1, T-47-D, SK-BR-3, Hs578T, MDA-MB231 and Saos-2 cells as template. The quality of the cDNA had previously been verified by detection of several transcripts, including β-actin and glyceraldehyde-3-phosphate dehydrogenase (data not shown). Bands of the predicted 571 bp were visible in the PCR products from Saos-2, MCF-7 and SK-BR-3 cells. There were no products visible in the reactions using ZR-75-1, T-47-D, Hs578T and MDA-MB231 cDNA as template or in negative controls. The products from Figure 1a were blotted onto a nylon membrane and probed with a [32P]-labelled PTH/PTHrP receptor cDNA (Figure 1b). Specific hybridisation was observed with the 571 bp products from Saos-2, MCF-7 and SK-BR-3, confirming the presence of PTH/PTHrP receptor transcripts. Additionally, weak Southern blot signals were observed with MDA-MB231 and possibly T-47D cDNA. The expression of the PTH/PTHrP receptor by MCF-7 cells was also demonstrated by Northern blot analysis (Figure 2).

The breast cancer cell lines were also investigated for the expression of PTHrP mRNA. RT–PCR identified specific 535 bp products in Saos-2, MCF-7, ZR-75-1, T-47-D and Hs578T cells (Figure 3a), which was confirmed by Southern blot analysis (Figure 3b).

Further studies of MCF-7 and Hs578T were performed in order to determine whether the presence of PTH/PTHrP receptor is a hallmark of malignant breast epithelial cells.
receptor mRNA was accompanied by the expression of a functional receptor. Proliferation studies in response to PTH(1-34) and PTHrP(1-34) were performed on MCF-7 and Hs578T cells by [3H]thymidine incorporation. Neither peptide had any effect when added to rapidly proliferating cells (data not shown). However, when the cells were made quiescent by incubation in serum-free medium at confluence, MCF-7 cells (Figure 4) proliferated in a dose-dependent manner in response to PTHrP(1-34). All doses of PTHrP between 1.6 and 100 ng ml⁻¹ significantly stimulated [3H]thymidine uptake. In other experiments where PTH and PTHrP were directly compared, the two molecules were approximately equipotent in the stimulation of proliferation of MCF-7 cells (Figure 5), but neither peptide increased the uptake of [3H]thymidine by Hs578T cells (Figure 6). Additionally, to confirm the [3H]thymidine data, studies were performed on the uptake of BrdU by MCF-7 cells. Treatment of cultures for 20 h with PTHrP (100 ng ml⁻¹) resulted in 12.2 ± 3% (mean ± s.e.m.) of nuclei stained compared with 2.6 ± 1% for control cultures.

Changes in intracellular cAMP levels were measured in MCF-7 and Hs578T cells in response to PTHrP(1-34). In MCF-7 cells there was a significant increase in the level of intracellular cAMP when cultures were treated with 100 ng ml⁻¹ PTHrP(1-34) but no elevation at 10 ng ml⁻¹ (Figure 7). In experiments with Hs578T cells (data not shown) there was no significant change in the concentrations of intracellular cAMP.

Discussion

This study has demonstrated that transcripts for the PTH/PTHrP receptor can be detected by RT-PCR in the human breast cancer cell lines, MCF-7 and SK-BR-3 and the osteosarcoma cell line, Saos-2. In addition, Southern blot analysis detected receptor expression in MDA-MB231 and possibly in T-47-D cells. The receptor was not identified in ZR-75-1 or Hs578T cells. PTH and PTHrP were reportedly

Figure 1 (a) Total RNA was isolated from confluent cultures of Saos-2, MCF-7, ZR-75-1, T-47-D, SK-BR-3, Hs578T and MDA-MB231 cells, and used in RT-PCR with primers specific for the PTH/PTHrP receptor (see Materials and methods). Lane 1, 1 kb marker (Gibco-BRL); lane 2, RT-PCR of Saos-2 cDNA showing specific amplification of a 571 bp product; lane 3, MCF-7 PCR products; lane 4, ZR-75-1 PCR products; lane 5, T-47-D PCR products; lane 6, SK-BR-3 PCR products; lane 7, Hs578T PCR products; lane 8, MDA-MB231 PCR products; lane 9, PCR of no DNA negative control. (b) Southern blot of a probed with a 3²P-labelled PTHrP receptor cDNA. Lane 1, 1 kb marker (Gibco-BRL); lane 2, RT-PCR of Saos-2 cDNA showing specific amplification of a 571 bp product; lane 3, MCF-7 PCR products; lane 4, ZR-75-1 PCR products; lane 5, T-47-D PCR products; lane 6, SK-BR-3 PCR products; lane 7, Hs578T PCR products; lane 8, MDA-MB231 PCR products; lane 9, PCR of no DNA negative control.

Figure 2 Northern blot hybridisation of 5 µg of MCF-7 poly(A)⁺ RNA with a 3²P-labelled PTHrP receptor probe.

Figure 3 (a) Total RNA was isolated from confluent cultures of Saos-2, MCF-7, ZR-75-1, T-47-D, SK-BR-3, Hs578T and MDA-MB231 cells, and used in RT-PCR with primers specific for PTHrP (see Materials and methods). Lane 1, 1 kb marker (Gibco-BRL); lane 2, RT-PCR of Saos-2 cDNA showing specific amplification of a 571 bp product; lane 3, MCF-7 PCR products; lane 4, ZR-75-1 PCR products; lane 5, T-47-D PCR products; lane 6, SK-BR-3 PCR products; lane 7, Hs578T PCR products; lane 8, MDA-MB231 PCR products; lane 9, PCR of no DNA negative control. (b) Southern blot of a probed with a 3²P-labelled PTHrP cDNA. Lane 1, 1 kb marker (Gibco-BRL); lane 2, RT-PCR of Saos-2 cDNA showing specific amplification of a 571 bp product; lane 3, MCF-7 PCR products; lane 4, ZR-75-1 PCR products; lane 5, T-47-D PCR products; lane 6, SK-BR-3 PCR products; lane 7, Hs578T PCR products; lane 8, MDA-MB231 PCR products; lane 9, PCR of no DNA negative control.
detected in cultures of breast cancer cells isolated from a patient with humoral hypercalcaemia of malignancy (Francini et al., 1993), and we have previously detected PTHrP transcripts in MCF-7 and HS578T cells and immunoreactive peptide in HS578T cells but not in MCF-7 cells (Walsh et al., 1992). In the data presented here transcripts for PTHrP were detected in MCF-7, ZR-75-1, T-47-D and HS578T cells. The failure to detect PTH/PTHrP receptor mRNA in ZR-75-1 and HS578T cells may be due to constitutive lack of expression or, alternatively, down-regulation as a result of PTHrP production, as has been suggested from studies of rat vascular tissue in which high PTHrP mRNA levels are associated with low PTH/PTHrP transcripts and vice versa (Okano et al., 1994). Further study is required to determine if endogenous production of PTHrP can modulate the expression of PTH/PTHrP receptor in breast cancer cells.

PTH(1-34) and PTHrP(1-34) modulated the proliferation of MCF-7 cells but had no effect on HS578T cells. Furthermore, in response to PTHrP(1-34) (100 ng ml⁻¹), intracellular levels of cAMP were elevated in MCF-7 cells but not HS578T cells. PTH(1-34) and PTHrP(1-34) stimulated [³H]thyidine incorporation by MCF-7 cells equipotently, suggesting that both hormones mediate their effects through the same receptor. PTH and/or PTHrP has previously been reported to stimulate the proliferation of other cell types, including osteoblasts (MacDonald et al., 1986), human renal carcinoma cells (Burton et al., 1990) and Walker 256 carcinoma cells (Benitez-Verguizas and Esbrit, 1994). These results indicate that PTH and PTHrP may be growth factors for breast cancer cells and may play a significant role in the progression of malignancy. PTH and

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**Figure 4** Effect of PTHrP on [³H]thyidine incorporation by MCR-7 cells. MCF-7 cells were seeded into a 96-well plate at 5 x 10⁴ cells per well and grown to confluence. The cells were then incubated in serum-free medium for 4 days. The medium was then replaced with fresh medium containing 0.4-100 ng ml⁻¹ PTHrP(1-34) and 0.5 μCi of [³H]thyidine. The plates were incubated for a further 24 h, cells harvested and [³H]thyidine incorporation measured by scintillation counting. All data represented as means ± s.e.m. (n = 6). Asterisk denotes significance (P < 0.05).

**Figure 5** Effect of PTH (■) and PTHrP (□) on [³H]thyidine incorporation by MCF-7 cells. MCF-7 cells were seeded into a 96-well plate at 5 x 10⁴ cells per well and grown to confluence. The cells were then incubated in serum-free medium for 4 days. The medium was then replaced with fresh medium containing 0.4-100 ng ml⁻¹ PTH(1-34) or PTHrP(1-34) and 0.5 μCi of [³H]thyidine. The plates were incubated for a further 24 h, cells harvested and [³H]thyidine incorporation measured by scintillation counting. All data represented as means ± s.e.m. (n = 6). Asterisk denotes significance (P < 0.05).

**Figure 6** Effect of PTH (■) and PTHrP (□) on [³H]thyidine incorporation by HS578T cells. HS578T cells were seeded into a 96-well plate at 5 x 10⁴ cells per well and grown to confluence. The cells were then incubated in serum-free medium for 4 days. The medium was then replaced with fresh medium containing 0.4-100 ng ml⁻¹ PTH(1-34) or PTHrP(1-34) and 0.5 μCi of [³H]thyidine. The plates were incubated for a further 24 h, cells harvested and [³H]thyidine incorporation measured by scintillation counting. All data represented as means ± s.e.m. (n = 3).

**Figure 7** Effect of PTHrP(1-34) on intracellular cAMP in MCF-7 cells. Cells were grown to confluence in six-well plates and incubated with IBMX/PTHrP for 20 min. Cell layers were harvested as described in Materials and methods and cAMP determined by RIA. All data represented as mean ± standard deviation (n = 6). Asterisk denotes significance (P < 0.05).
PTHrP have been shown to stimulate the production of matrix-degrading enzymes, including plasminogen activator (PA), in other cell types (Hamilton et al., 1984). If PA production was linked to PTH/PTHrP receptor activation in breast carcinoma cells this would have important implications for breast metastasis. In addition, other work has shown that PTHrP expression by MDA-231 cells is stimulated by an extracellular matrix produced by bone cells (Guise et al., 1994).

PTH/PTHrP receptor transcripts have an apparent widespread tissue distribution (Urena et al., 1993), and we have shown that breast cancer cells express the receptor and respond to PTH/PTHrP. There is some evidence that primary hyperparathyroidism may be associated with increased incidence of malignancies (Farr et al., 1973). A mitogenic effect of PTH on cancer cells could be one mechanism to explain this observation, but further work is required to confirm this.

Subclones of the 8701 BC primary breast cancer cell line can be subdivided according to their ability to express PTHrP mRNA, with PTHrP-positive clones displaying a more aggressive growth behaviour than PTHrP-negative clones in vitro (Luparello et al., 1993). In addition, an RT–PCR study of 38 normocalcaemic breast cancer patients with long-term follow-up demonstrated higher levels of PTHrP expression in patients who subsequently developed bone metastases (Bouizaur et al., 1993). In the data presented here we have shown the presence of PTH/PTHrP receptor mRNA in four out of six breast cancer cell lines, and one of these, MCF-7, was shown to proliferate in response to PTH(1–34) and PTHrP(1–34). We conclude that PTHrP may be an important autocrine/paracrine factor in the progression of breast cancer.

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