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Parathyroid Hormone-related Protein$^{(50–69)}$ and Response to Pamidronate Therapy for Tumour-induced Hypercalcaemia

D.J. Dodwell, S.K. Abbas, A.R. Morton and A. Howell

A region-specific radioimmunoassay has been employed to measure levels of immunoreactive parathyroid hormone-related protein$^{(50–69)}$ (iPTHrP$^{(50–69)}$) in patients with tumour-induced hypercalcaemia (TIH). This assay is based on an antiserum raised against synthetic human PTHrP$^{(50–69)}$. The assay showed no cross-reactivity with human or bovine parathyroid hormone$^{(1–84)}$. The effect of a single dose (60 mg) of pamidronate was studied in 25 consecutive patients with TIH. All were rehydrated prior to treatment. All but 2 patients (8%) became normocalcaemic after treatment; both of these had very high levels of iPTHrP$^{(50–69)}$. Time to achieve normocalcaemia, as an index of relative resistance to pamidronate, correlated positively with pretreatment level of iPTHrP$^{(50–69)}$. Absence of radiological evidence of bone metastases also predicted relative resistance to pamidronate. In this study, iPTHrP$^{(50–69)}$-induced osteoclastic bone resorption was a more important mechanism in the causation of TIH than PTHrP-induced renal reabsorption of calcium as assessed by the renal thresholds for calcium and phosphate.
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INTRODUCTION

ALBRIGHT RAISED the possibility, in 1941, that tumour-induced hypercalcaemia (TIH) may be due to production of a parathyroid hormone (PTH)-like substance by the cancer [1]. Following this, the term "ectopic PTH secretion" came into common usage to describe the syndrome of patients with cancer who had a high plasma calcium, and it was only with recent developments in radioimmunoassay (RIA) and gene probing techniques, that doubt began to emerge about the involvement of PTH in this syndrome [2, 3].

Since that time cell culture and immunohistochemical studies have demonstrated that many malignant tumours produce a parathyroid hormone-related protein (PTHrP) which has sequence homology to, but is distinct from, PTH [4–8]. PTHrP is present in a wide variety of fetal tissues and is found in high concentrations in breast tissue and milk [9]. It has been suggested that it is important in the control of mammalian fetal plasma calcium levels [10]. Others have suggested that it may control tissue calcium levels in adults by an autocrine mechanism [11].

Although its physiological role is incompletely understood, much recent evidence implicates PTHrP in the causation of TIH [8, 9, 12]. The recent recognition of such a circulating 'humoral' factor in TIH has meant that two forms of this syndrome are now commonly described. One results from the local resorption of bone by skeletal deposits (occurring most frequently in breast cancer and myeloma) and the other from the release by the tumour of a circulating factor (commonly occurring in squamous cell lung cancer and hypernephroma) which causes distant effects on the skeleton (to promote osteoclastic bone resorption) and the kidney (to promote the renal tubular reabsorption of calcium). However, it is unlikely that these two forms of TIH are distinct clinical entities and both mechanisms may operate in some patients [12–14].

Bisphosphonates, and in particular pamidronate, are the treatment of choice for TIH and restore normocalcaemia in over 85% of unselected patients with this complication of malignancy [15–17]. Bisphosphonates inhibit osteoclastic bone resorption with little or no effect on the renal reabsorption of calcium and would therefore be of limited value in situations where excessive renal reabsorption of calcium was the predominant pathophysiological mechanism.

In this regard, Gurney *et al.* [18] found that renal tubular phosphate threshold (RPT), as an indicator of renal PTH receptor stimulation, was the best predictor of response to

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pamidronate when this drug was used in a small series of patients with TIH.

Ralston *et al.* also found that in a series of 50 rehydrated patients with TIH those with few or no bone metastases had the highest level of tubular calcium reabsorption and the highest post-treatment serum calcium levels. They also suggested that the choice of antihypercalcaemic therapy should reflect the differing causative mechanisms [19].

In order to determine the possible role of PTHrP in promoting renal reabsorption of calcium and the response to pamidronate therapy, we performed a prospective study in 25 consecutive patients admitted for the treatment of TIH.

PATIENTS AND METHODS

25 patients who presented with clinically established malignant disease and a serum calcium concentration (corrected for serum albumin) greater than 3.0 mmol/l after saline rehydration were studied. All patients were documented to have a normal serum calcium level 1–8 months prior to their presentation with TIH. No patients were receiving steroids, diuretics or drugs known to affect bone metabolism. All patients gave informed consent and the study was approved by the South Manchester Ethics Committee.

Prior to treatment with pamidronate, but after rehydration, a 2-h fasting urine sample was collected for estimation of creatinine, calcium and phosphate. Blood was taken for estimation of electrolytes, urea, creatinine, calcium and phosphate. Serum was stored at −70°C for estimation of iPTHrP$^{(50-69)}$. Renal phosphate threshold was calculated by the method of Walton and Bijvoet [20] and renal calcium threshold was calculated according to the method of Nordin [21].

Control subjects (10) for the measurement of normal levels of iPTHrP$^{(50-69)}$ were laboratory staff (5M, 5F) with a median serum calcium concentration of 2.46 mmol/l.

Immunoreactive PTHrP$^{(50-69)}$ was measured by a region-specific RIA using a sheep anti-human PTHrP$^{(50-69)}$ antiserum. 2 μg synthetic human (h) PTHrP$^{(50-69)}$ (kindly supplied by Professor T. J. Martin, Melbourne, Australia) was iodinated with 37 MBq ^{125}I (Amersham International, Amersham, UK) by the chloramine-T method and purified as described previously [22]. The specific activity of the tracer was 11.1–14.8 MBq/μg.

A non-equilibrium assay system was used in which 50 μl antiserum and 100 μl sample or standard intravenous buffer was incubated at 4°C for 3 days, at the end of which 100 μl ^{125}I-labelled hPTHrP$^{(50-69)}$ (500 cpm) were added and incubation was continued for a further 2 days. Separation was then achieved using dextran T70-coated charcoal (5 mg charcoal and 0.5 mg dextran T70/ml added to each tube) and both bound and free fractions were counted for radioactivity. The polyclonal antiserum was raised in a sheep against synthetic hPTHrP$^{(50-69)}$ which had been conjugated with soya bean trypsin inhibitor [23] and was a kind gift from Professor T. J. Martin. It was used at a final dilution of 1:200,000 which gave a bound/free ratio of 0.5 in the presence of ^{125}I-labelled hPTHrP$^{(50-69)}$. The buffer consisted of barbital (50 mmol/l; pH 8.6) containing 5% (v/v) outdated human blood bank plasma and 500 KIU trasylol/ml (Bayer, Newbury, UK). The final incubation volume was 250 μl. A standard curve was constructed by repetitive dilution of hPTHrP$^{(50-69)}$ (2.4–5000 pg/ml, Fig. 1). The assay was highly reproducible, with non-specific binding (NSB) of 1.93 (S.E. 0.06 n = 40), and intra-assay and interassay coefficients of variation of 4 and 10% respectively, with a detection limit of 25 pg/ml. The assay showed no crossreaction with human or bovine PTH$^{(1-84)}$ (Fig. 1). A weak, non-significant crossreactivity was observed with hPTHrP$^{(1-84)}$ (Fig. 1) which demonstrated that the assay was specific for the mid region (50–69) fragment of the PTHrP molecule. Each sample was assayed at two or more dilutions and a control tube without antiserum was included for each dilution to correct for sample NSB which was less than 6.4%.

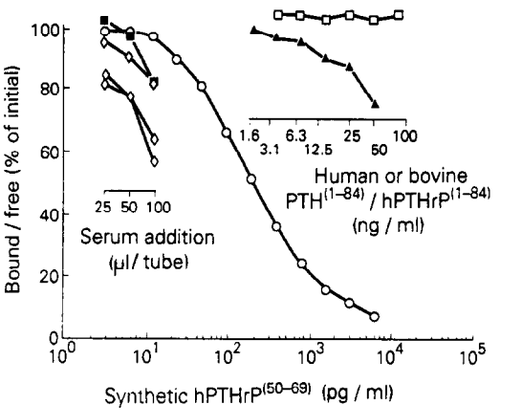

Fig. 1. Standard curve for the radioimmunoassay of human parathyroid hormone related protein$^{(50-69)}$. Effective displacement of ^{125}I-labelled hPTHrP$^{(50-69)}$ from sheep anti-hPTHrP$^{(50-69)}$ antiserum by hPTHrP$^{(50-69)}$ standards (–○–), and by dilutions of serum from 3 patients with TIH (–◇–) and a control subject (–■–). hPTHrP$^{(1-84)}$ (–▲–) showed weak crossreactivity but human bovine PTH$^{(1-84)}$ (–□–) were non-competitive in the assay.

The presence of bone metastases was established by isotope scintigraphy or skeletal radiography where appropriate. All patients were admitted to the same unit during treatment. Serum biochemistry was repeated daily for the first week after treatment and thereafter on a weekly basis.

Patients were treated with 60 mg pamidronate in 1l normal saline over 8 (11 patients) or 24 (14 patients) h after 24–48 h of saline rehydration (3l daily plus unrestricted oral fluids). All patients were felt to be clinically rehydrated prior to treatment with pamidronate. No concurrent antitumour therapy was given until normocalcaemia had been achieved. Intravenous fluids (3l daily) were continued until normocalcaemia was achieved or for 4 days, whichever occurred first.

Statistical analyses of static parameters between the groups was performed by the use of the Wilcoxon two-sample rank-sum test. Correlation was tested using Pearson's product moment method [24].

RESULTS

PTHrP

Median serum concentration of iPTHrP$^{(50-69)}$ in control subjects was 53 (range 31–77) pg/ml.

Pretreatment characteristics of patients with TIH are shown in Table 1. iPTHrP$^{(50-69)}$ was elevated in most (76%, 19/25) patients. Median level of iPTHrP$^{(50-69)}$ was 114 (range 38–410) pg/ml. No significant differences were seen in iPTHrP$^{(50-69)}$ levels between tumour types but levels tended to be higher in patients with squamous cell lung cancer (median 86, range 38–260 pg/ml) than with breast cancer (median 59, range 41–118 pg/ml). Elevated levels were also seen in 2 patients with non-Hodgkin lymphoma (140 and 343 pg/ml), and patients with myeloma (143 pg/ml), squamous carcinoma arising in the perineum (107 pg/ml), squamous carcinoma of the cervix

Table 1. Patient characteristics

Age (median, range)	61 (19–81)		
Sex (M:F)	13:12		
Tumour type (no., %)		Raised PTHrP	Bone metastases
Breast	6 (24)	5 (83)	6 (100)
Squamous lung	10 (40)	8 (80)	3 (30)
Non Hodgkin lymphoma	2 (8)	2 (100)	0
Unknown primary	2 (8)	0	0
Others*	5 (20)	4 (80)	2 (40)
Pretreatment biochemical parameters (median, range)			
Serum creatinine (mm/l)	0.12 (0.06–0.43)		
Serum calcium (mm/l)	3.6 (3.05–4.7)		
Renal calcium threshold (mm/l)	2.1 (1.7–2.61)		
Renal phosphate threshold (mm/l)	0.76 (0.25–1.56)		
PTHrP (pg/ml)	114 (38–410)		

*One each of myeloma, squamous carcinoma of the perineum, soft tissue sarcoma, squamous carcinoma of the cervix and metastatic neuroendocrine tumour.

(112 pg/ml) and metastatic neuroendocrine tumour (410 pg/ml). 6 patients (2 with carcinoma from an unknown primary site, 2 with squamous cell lung cancer, 1 with breast cancer and the other with metastatic soft tissue sarcoma) had levels of iPTHrP$^{(50-69)}$ within the normal range. iPTHrP$^{(50-69)}$ was significantly higher in patients without apparent bone metastases (median 107, range 38–410 pg/ml) than in those with bone metastases (median 60, range 41–170 pg/ml, $P = 0.025$, Fig. 2).

Biochemical parameters

Median renal phosphate threshold (RPT) was 0.76 (range, 0.25–1.56) mm/l and median renal calcium threshold (RCT) was 2.1 (1.7–2.61) mm/l. There were no correlations between RPT or RCT and initial serum calcium, tumour type, iPTHrP$^{(50-69)}$ or the presence or absence of bone metastases. No correlation was seen between RCT and RPT. These parameters continued to demonstrate no significant correlations even if RCT or RPT values were omitted in patients with a raised serum creatinine after saline rehydration.

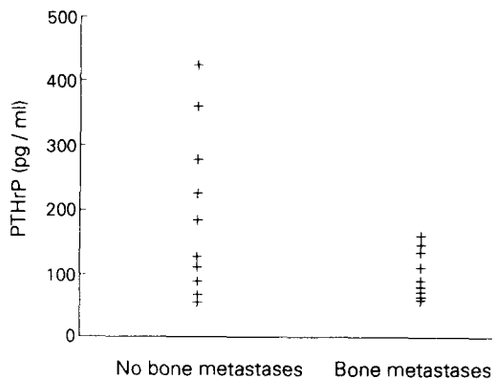

Fig. 2. Serum level of iPTHrP$^{(50-69)}$ in patients with and without scintigraphic or radiographic evidence of bone metastases ($P = 0.025$).

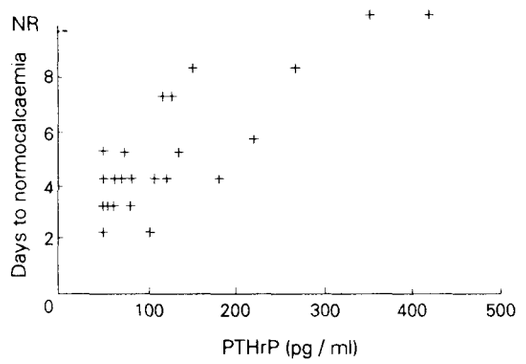

Fig. 3. Number of days taken to achieve a normal serum calcium according to iPTHrP$^{(50-69)}$ ($R = 0.76$, $P<0.001$). ($r = 0.53$, $P = 0.04$, if non-responders are excluded).

There was no significant relationship between initial serum calcium levels after rehydration and iPTHrP$^{(50-69)}$ or tumour type.

Median serum creatinine after saline rehydration was 0.12 (0.04–0.43) mm/l with 4 patients having values above the normal range. After achieving normocalcaemia, all but 1 patient (with an initial serum creatinine of 0.43 mm/l falling to 0.23 mm/l) attained a normal serum creatinine. No significant changes were seen in other electrolyte values after treatment with pamidronate.

Response to pamidronate

2 (8%) patients failed to achieve normocalcaemia after pamidronate. Both of these patients—1 with a neuroendocrine tumour (serum calcium 4.3 mm/l, RCT 2.14 mm/l) and the other with non-Hodgkin lymphoma (serum calcium 3.36 mm/l, RCT 1.89 mm/l)—had no evidence of bone metastases but had the highest levels of iPTHrP$^{(50-69)}$ (410 and 343 pg/ml, respectively). The first patient was retreated several times with pamidronate but remained hypercalcaemic and the second patient died suddenly from a pulmonary embolus while still hypercalcaemic.

The remaining 23 (92%) patients became normocalcaemic between 2 and 8 (median 4) days after treatment with pamidronate. There was a significant inverse relationship between iPTHrP$^{(50-69)}$ level and response to pamidronate (Fig. 3, $P<0.001$). Response to pamidronate was independent of tumour type, RPT, RCT or initial serum calcium level. There was a tendency for patients without bone metastases to demonstrate resistance to pamidronate although this did not achieve conventional statistical significance ($P = 0.08$). There was no difference in response to pamidronate between the two infusion rates used in this study.

DISCUSSION

The causative role of PTHrP in humoral TIH is in little doubt. High levels of serum iPTHrP$^{(1-34)}$ [12, 25, 26] and urinary iPTHrP$^{(126-141)}$ [27] have been reported in patients with TIH. Evidence also exists for chromatographic [28] and immunohistochemical [9, 23] characterisation of multiple forms of PTHrP in different human tumours associated with hypercalcaemia. In the present study we have used a region-specific radioimmuno assay (RIA) to demonstrate the presence of high concentrations of serum iPTHrP$^{(50-69)}$ in patients with TIH. The antiserum used in this study was raised against a unique

PTHrP sequence with no homology to PTH [29] and therefore, showed no cross reaction with human or bovine PTH (Fig. 1). The same antiserum has previously been used for the immunohistochemical localisation of $PTHrP^{(50-69)}$ in parathyroid adenomas [23]. The weak crossreactivity of the antiserum with $PTHrP^{(1-84)}$ demonstrated that the assay was region-specific and that a much shorter midregion fragment of the PTHrP molecule circulates in high concentrations in some patients with TIH.

With respect to the response to pamidronate therapy for TIH, all but 2 patients became normocalcaemic in this study. Both of these had very high levels of $iPTHrP^{(50-69)}$. Additionally in those patients who did achieve normocalcaemia, time to attain a normal serum calcium level (as an indicator of relative resistance to pamidronate) correlated closely with $iPTHrP^{(50-69)}$. The absence of apparent bone metastases also tended to suggest resistance, but response to pamidronate was independent of all biochemical parameters, including renal thresholds to calcium and phosphate. This is at variance with the study of Gurney *et al.* [18] who found that RPT, but not RCT, was predictive of failure of pamidronate therapy in a group of patients with hypercalcaemia. They suggested that a low RPT was due to a circulating humoral factor—putatively PTHrP—which by interacting with renal tubular "PTH" receptors, promoted reabsorption of calcium and contributed to the failure of antiresorptive therapy. However, the differing doses of pamidronate used by Gurney *et al.* may have contributed to this result. Their study is also unusual in that they report that only 7 of 15 patients achieved normocalcaemia, whereas most studies indicate that pamidronate is effective in >85% of patients [15–17].

PTHrP may contribute to the hypercalcaemia of malignancy by stimulating renal reabsorption of calcium or by promoting osteoclastic bone resorption. Human PTHrP has been shown to be of approximately equal efficacy to PTH in stimulating osteoclastic bone resorption in an experimental model [30], but was only 15% as effective as PTH in stimulating cAMP production in a bovine renal membrane bioassay [31]. The study we report indicates that although $iPTHrP^{(50-69)}$ appeared to predict failure of, or relative resistance to, antiresorptive therapy, this was not due to excessive renal tubular reabsorption of calcium, as assessed by RPT or RCT. An alternative hypothesis is that standard doses of anti-resorptive agents, such as pamidronate, are unable to overcome the powerful stimulation of osteoclastic activity by very high levels of $iPTHrP^{(50-69)}$. In this regard higher doses of pamidronate have been demonstrated to correct "resistant hypercalcaemia" refractory to a standard dose (ARM, unpublished and Ref. 32).

It is of interest that, although traditionally PTHrP has been implicated in the pathogenesis of hypercalcaemia in patients with hypernephroma and squamous cell lung cancer, we have demonstrated high levels of $iPTHrP^{(50-69)}$ in patients with a variety of tumour types including myeloma, lymphoma and breast cancer. PTHrP mRNA has been identified, by *in situ* hybridisation, in seven tumours associated with TIH (including a patient with breast cancer), but not in tumours rarely complicated by this syndrome [4]. However, there are limited data on the expression of PTHrP in haematological malignancies. Burtis *et al.* found elevated $PTHrP^{109-138}$ levels in 5 of 8 patients with "osteolytic" hypercalcaemia (from breast cancer and myeloma) although this finding was, in part, attributed to diminished renal function in these patients [9]. Henderson *et al.* also reported that 2 of 6 patients with various haematological malignancies and hypercalcaemia had an elevated PTHrP [12].

The pathophysiology of hypercalcaemia in breast cancer has recently been re-evaluated and it is now evident that, despite the high prevalence of bone metastases in this disease, "humoral" mechanisms are more commonly involved than previously realised [12–14]. PTHrP has also been identified, by immunohistochemical studies, in tumour tissue, in 60% of a series of 99 normocalcaemic breast cancer patients [33]. However, the large number of differing PTHrP detection methods currently used make it difficult to compare the relative biological and pathological effects reported between both clinical and experimental studies. Standardisation of these assays and detection methods would allow more firm conclusions to be reached.

Nevertheless, parathyroid-hormone like peptides would appear to be associated with many diverse types of malignancy and this and other studies [9, 11, 12] indicates that tumour production of PTHrP is a more prevalent pathogenetic mechanism in TIH than previously thought. However, PTHrP-stimulated osteoclastic bone resorption is likely to be a more important mechanism than excessive renal reabsorption of calcium in the causation of TIH in most cases. Bisphosphonate therapy and fluid replacement should remain the mainstay of treatment.

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The Importance of Added Albumin During Continuous Intravenous Infusion of Interleukin-2 with Alpha-interferon

James Cassidy, Christopher Poole, Elizabeth Sharkie, Will P. Steward and Stanley B. Kaye

We treated 14 patients (4 malignant melanoma/10 renal carcinoma) with a combination of continuous infusion interleukin-2 (IL-2) and subcutaneous alpha-interferon. Variable concentrations of albumin were added to the infusion of IL-2. The toxicity of this regimen seems to be related to the percentage of albumin added to the IL-2 infusion. Partial responses were observed in 3 cases. Interestingly, 1 patient's response appeared dependent on the addition of human serum albumin. The mechanism of these effects is unknown, but the use of albumin with IL-2 should be carefully investigated in future studies.
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INTRODUCTION

TREATMENT WITH either interleukin-2 (IL-2) or interferon (IFN) can occasionally induce remissions in patients with malignant melanoma or renal cancer. Laboratory data indicate that the combination of these two agents may be synergistic [1]. We have therefore performed a feasibility study of this combination in such patients. During the course of the study the opportunity was taken to vary the conditions of the infusion system for IL-2. Early experience had suggested that, because of adherence to the plastic infusion set, IL-2 should be administered with albumin [2]. However, the necessity for this has not been clearly demonstrated. We therefore report our experience with 14 patients, and suggest that the addition of albumin may have a significant effect on the toxicity and efficacy of IL-2.

PATIENTS AND METHODS

Patients with histologically confirmed metastatic renal cell carcinoma and malignant melanoma were entered into the study after fully informed consent had been obtained. Eligibility criteria included performance status 0-1 (WHO scale), adequate bone marrow values [white blood cells > 4.0; platelets > 100]

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