Papers

Parathyroid hormone related protein and interleukin-6 mRNA expression in larynx and renal cell carcinomas from normocalcaemic and hypercalcaemic patients

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Abstract

Aims—To determine the expression of parathyroid hormone related protein (PTHrP) and interleukin-6 (IL-6) mRNAs and their possible relation in malignant tumours, derived from patients with and without hypercalcaemia, commonly associated with humoral hypercalcaemia of malignancy.

Methods—PTHrP and IL-6 mRNA expression was studied by northern blot analysis in tumour specimens from 13 consecutive patients. Six patients (two with hypercalcaemia) had squamous cell carcinomas of the larynx and seven (one with hypercalcaemia) had renal cell carcinomas.

Results—There was no relation between the histological features of the tumours and the expression of either PTHrP or IL-6 mRNAs. PTHrP mRNA was detected in all squamous cell carcinomas, expression being highest in the two patients with hypercalcaemia. In the renal cell carcinomas PTHrP mRNA was expressed only in the patient with hypercalcaemia. IL-6 mRNA was detected in nearly all tumours studied but there was no apparent relation between its expression and that of PTHrP mRNA or serum calcium concentrations.

Conclusions—PTHrP mRNA expression is increased in patients with hypercalcaemia but is not related to IL-6 mRNA expression. The results suggest a quantitative relation between PTHrP gene expression and hypercalcaemia, and imply that different mechanisms account for this expression in squamous and renal cell carcinomas.

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Keywords: Parathyroid hormone related protein, interleukin-6, squamous cell carcinoma, renal cell carcinoma.

Parathyroid hormone related protein (PTHrP) is a major pathogenic factor of the syndrome of humoral hypercalcaemia of malignancy

(HHM).¹⁻³ Earlier studies have reported the presence of PTHrP mRNA exclusively in tumours from patients with HHM.^{4,5} However, it was later demonstrated, by both immuno-histochemistry and in situ hybridisation, that epithelial tumours most commonly associated with HHM but obtained from normocalcaemic patients may also express PTHrP.⁶⁻⁹ These findings raise questions about the modulation of PTHrP gene expression in patients with hypercalcaemia and suggest that other factors may also be involved in the development of this condition.³ Previous studies in our laboratory have shown that the production and expression of PTHrP mRNA by squamous cell carcinoma cell lines could be enhanced by co-culturing these cells with fibroblasts.^{10,11} It may be that local soluble factors produced during the interaction between the epithelial and mesenchymal cells are responsible for the increased production of PTHrP. Such factors include cytokines and we recently showed that interleukin-6 (IL-6) production by fibroblasts is greatly enhanced during co-culture with keratinocytes by IL-1, which is secreted by most epithelial cells including squamous cell carcinomas.¹² Interleukin-6, which stimulates osteoclast formation,^{13,14} is also a hypercalcaemic factor¹⁵ and recently co-secretion of PTHrP and IL-6 has been reported in hypercalcaemic nude mice bearing a human renal cell carcinoma¹⁶ and in a patient with pheochromocytoma and HHM.¹⁷

To examine the possible relation between PTHrP and IL-6 in malignant tumours, we studied the expression of the two peptides by northern blot analysis in two types of tumours commonly associated with HHM. These were squamous cell carcinomas (larynx) and adenocarcinomas (renal cell) derived from patients with and without hypercalcaemia.

Methods

In this prospective study 13 patients (nine men (mean age 59 ± 4 years) and four women (mean age 70 ± 6 years)) were investigated. Six patients had histologically confirmed laryngeal squam-

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Clinical details of six patients with carcinoma of the larynx and seven with renal cell carcinoma

Patient no.	Sex	Age (years)	Primary tumour	Stage	Serum calcium (mmol/l)	Serum phosphate (mmol/l)	Tumour percentage	PTHrP/28S	IL-6/28S
<i>Squamous cell carcinoma (larynx)</i>									
1	F	68	larynx ^a	T ₄ N ₀ M ₀	2.42	1.05	50	46	—
2	M	56	larynx ^b	*	2.87	0.95	50	955	155
3	F	56	larynx ^b	T ₄ N ₂ M ₀	2.46	0.99	50	285	26
4	F	71	larynx ^c	*	2.98	0.91	90	943	2
5	M	70	larynx ^a	T ₂ N ₀ M ₀	2.43	1.02	80	239	18
6	M	62	larynx ^a	T ₃ N ₁ M ₀	2.43	1.08	60	209	5
<i>Adenocarcinoma (kidney)</i>									
<i>(nuclear grading/type)</i>									
7	M	37	renal ^a	T ₃ N ₁ M ₁	2.39	1.20	80	—	—
8	M	56	renal ^a	T ₂ N ₁ M ₁	2.96	1.27	40	261	393
9	M	69	renal ^a	T ₂ N ₁ M ₀	2.45	1.02	80	—	—
10	M	53	renal ^a	T ₁ N ₀ M ₀	2.27	1.02	90	—	—
11	F	83	renal ^a	T ₂ N ₁ M ₀	2.41	1.12	40	6	253
12	M	79	renal ^a	T ₂ N ₀ M ₀	2.34	1.20	90	—	—
13	M	46	renal ^a	T ₂ N ₂ M ₁	2.51	1.20	90	—	32

^aSpecimens obtained from primary tumours, ^bfrom primary metastases to lymph nodes and ^cfrom a recurrent metastatic tumour. *Recurrent malignant disease. N_x=absence in regional lymph nodes.

ous cell carcinomas and seven had renal cell carcinomas, one associated with Von Hippel-Landau's disease (retinal cerebellar haemangioblastosis). Tumour tissue specimens suitable for northern analysis were obtained at surgery. Tumours were classified according to the American Joint Committee on Cancer staging system. A nuclear grading system was also applied to the renal cell carcinomas.¹⁸

All patients underwent bone scintigraphy before surgery and none had skeletal metastases. Parathyroid hormone was undetectable in the plasma of the hypercalcaemic patients, excluding the possibility of concomitant primary hyperparathyroidism.

Immediately after excision of the tumours, tissue samples were taken for pathological examination and for the determination of PTHrP and IL-6 mRNA expression by northern blot analysis. For the latter, specimens were immediately placed in liquid nitrogen and kept at -70°C until analysis. Of the laryngeal tumours, three specimens were obtained from the primary tumours (patients 1, 5 and 6), two from regional lymph nodes (patients 2 and 3) and one from a subcutaneous tumour mass in the neck (patient 4). All renal cell carcinoma specimens were taken from the primary tumours.

Total cellular RNA was isolated from tumours by grinding (one minute; Polytron, Kinematica,

Figure 1 Northern blots of PTHrP and IL-6 mRNA expression in six patients with laryngeal carcinoma (lanes 1-6). Lanes 2 and 4 contain mRNA from patients with hypercalcaemia. SCC-4 and IL-1 stimulated human dermal fibroblasts were used as positive controls for PTHrP and IL-6 mRNA expression, respectively. Lane C1 contains mRNA from a patient with parathyroid carcinoma, hypercalcaemia and metastases.

Figure 2 Northern blots of PTHrP and IL-6 mRNA expression in seven patients with renal cell carcinoma (lanes 7–13). The patient in lane 8 was hypercalcaemic. SCC-4 and IL-1 stimulated human dermal fibroblasts were used as positive controls for PTHrP and IL-6 mRNA expression, respectively. Lane C2 contains normal kidney tissue.

Switzerland) in ice-cold lithium chloride (3.3 M)/urea (6.6 M). After overnight incubation at -20°C , the homogenate was centrifuged at $10\,000 \times g$ for 30 minutes at 4°C and the pellet was resuspended in 10 mM Tris/0.5% SDS (sodium dodecyl sulphate (pH 7.7)). Total RNA was extracted with three cycles of phenol:chloroform:isoamyl alcohol (25:24:1) and subsequently precipitated at -20°C in 70% ethanol/0.1 M sodium acetate (pH 5.2). The pellet was resuspended in 10 mM Tris/1 mM EDTA (pH 7.6). Total RNA was quantified by spectrophotometry at 260 nm. RNA samples were analysed using electrophoresis on a 1% denatured agarose gel containing 7.5% formaldehyde and transferred to a nylon membrane (Hybond N, Amersham, UK).¹⁹ The membranes were hybridised with a ^{32}P labelled cDNA probe for PTHrP²⁰ (kindly provided by M Karperien, Hubrecht Laboratories, The Netherlands) and human IL-6²¹ (kindly provided by Dr RA de Paus, University Hospital Leiden, The Netherlands) at 60°C in 7% SDS, 0.5 M NaHPO_4 (pH 7.2) and 10 mM EDTA.²² The 28S rRNA probe was kindly provided by Dr C Backendorf, Gorlaeus laboratory, Leiden, The Netherlands. The blots were washed with $2 \times \text{SSC}$ (0.30 M NaCl, 0.0310 M sodium citrate), 1% SDS for one hour at 60°C . Autoradiographs were prepared using Kodak XAR-5 film and intensifying screens at -70°C . The intensity of the bands obtained was quantified photodensitometrically (Desaga CD60).

A squamous cell carcinoma cell line (SCC-4) was used as the positive control for PTHrP mRNA expression¹¹ and human dermal fibroblasts previously stimulated with IL-1 served as

a positive control for IL-6 mRNA expression.¹² Malignant tissue from a patient with parathyroid carcinoma (not producing PTHrP) and normal renal tissue taken from a healthy area of a diseased kidney were used as additional controls.

All tissue specimens were evaluated by an experienced pathologist. An estimation was made of the total number of cell nuclei, lymphocytes and mesenchymal cells per tumour specimen.

Calcium, phosphate and albumin concentrations were determined in a blood sample taken the day before surgery using automated techniques and serum calcium concentrations were adjusted to an albumin of 42 g/l.

Results

Two of the six patients with laryngeal carcinomas and one of the seven patients with renal cell carcinomas were hypercalcaemic. The tumours of hypercalcaemic patients had no distinctive histological features when compared with those of normocalcaemic patients (table).

All laryngeal tumours expressed PTHrP mRNA (lanes 1–6; fig 1) but the highest expression was detected in the tissues from the two patients with hypercalcaemia (lanes 2 and 4; fig 1). Similarly, IL-6 mRNA expression was detected in all but one (lane 1) of the laryngeal tumours. By contrast, PTHrP mRNA was not detected in six of the seven patients with renal cell carcinoma but it was expressed in the patient with hypercalcaemia (lane 8; fig 2). IL-6 mRNA expression varied in the renal cell carcinomas. The normal renal tissue (lane C2) and the parathyroid carcinoma (lane C1) did not express either PTHrP or IL-6 mRNA.

In the laryngeal carcinomas there was no apparent relation between PTHrP and IL-6 mRNA expression and the relative number of tumour cells per specimen (table); the number of specimens studied, however, is too small to allow definite conclusions to be drawn. Interestingly, in the renal tumours the highest IL-6 mRNA expression was found in two clear cell carcinomas (patients 8 and 11), which also showed the abundant tumour invasion by mononuclear cells typical of tumour infiltrating lymphocytes. No such tumour infiltrating cells were detected in any of the laryngeal tumours.

Discussion

Previous studies using different detection techniques have provided conflicting data about a possible relation between PTHrP expression and hypercalcaemia in patients with malignant diseases. Cellular immunostaining with antibodies directed against PTHrP was positive in nearly all squamous and renal cell carcinomas examined but bore no relation to hypercalcaemia.⁶⁻⁹ Using *in situ* hybridisation, increased PTHrP mRNA expression was detected in hypercalcaemic patients with carcinoma of the cervix²³ but not in another series of squamous cell carcinomas.⁹ These findings led the former authors to suggest that increased gene transcription contributes to the development of hypercalcaemia while the latter authors concluded that the clinical expression of HHM depends on the rate of secretion of PTHrP rather than on gene expression. In the present study, using northern blot analysis, we found that all laryngeal carcinomas expressed PTHrP mRNA but this expression was highest in the two patients with hypercalcaemia. Our data, therefore, support the hypothesis that the potential of a given squamous cell carcinoma for inducing HHM may reside in the quantitative capacity of the cells to express the PTHrP gene.²⁴ In contrast to the findings in laryngeal tumours, PTHrP mRNA expression was not detected in any of the tumours from the normocalcaemic patients with renal cell carcinomas but it was clearly expressed in the patient with HHM. The possibility of low PTHrP gene expression in the renal cell carcinomas which could not be detected by northern analysis seems unlikely as only about 25% of cultured renal cell carcinoma cells appear to produce PTHrP and previous studies using more sensitive RNase protection analysis failed to detect PTHrP mRNA expression in a number of renal cell carcinoma cell lines.²⁴

The present results, obtained in two histologically distinct malignancies associated with the syndrome of HHM, underline the importance of the relation between PTHrP and hypercalcaemia in malignant disease. These results also raise questions about the modulation of PTHrP gene expression by malignant cells. This is particularly relevant for laryngeal tumours in which PTHrP mRNA was detected in all tumours studied. We examined the possible involvement of IL-6 as this cytokine has previously been reported to be co-secreted with PTHrP in hypercalcaemic nude mice bearing a

human renal cell carcinoma and in a patient with HHM due to pheochromocytoma,^{16,17} is recognised as a hypercalcaemic factor¹³⁻¹⁵ and is produced by fibroblasts during co-culture with squamous carcinoma cells, an interaction previously shown to stimulate PTHrP production *in vitro*.¹⁰ All of the laryngeal carcinomas expressed both PTHrP and IL-6 mRNAs. However, no association between the degree of expression of these two factors and serum calcium concentrations could be demonstrated. The detection of both PTHrP and IL-6 mRNAs in epithelial tumours known to be associated with HHM is intriguing and further studies of their relative importance in the development of hypercalcaemia are warranted. IL-6 mRNA was also detected in renal clear cell carcinomas, confirming previous findings.^{25,26} Expression was greatest in the tumours with the highest degree of infiltration by tumour infiltrating lymphocytes. This suggests an immunological response rather than an epithelial-mesenchymal cell-cell interaction. It may be that in squamous cell carcinomas which already express the PTHrP gene, local factors released from mesenchymal cells in response to tumour products modulate the production of the protein, while in the renal cell carcinomas different mechanisms are responsible for the expression of PTHrP by malignant cells and consequently for the development of hypercalcaemia.

In conclusion, our results confirm the importance of PTHrP in the pathogenesis of HHM by demonstrating the relation between the quantity of PTHrP gene expression and serum calcium concentrations. They also reveal clear differences between human squamous cell and renal cell carcinomas, suggesting that different mechanisms are responsible for PTHrP expression in these tumours.

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