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Expression of parathyroid hormone-related protein (PTHrP) in parathyroid tissue under normal and pathological conditions

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Summary. Parathyroid hormone-related protein (PTHrP), a factor responsible for malignancy associated hypercalcemia, plays a physiological roles such as bone development and placental calcium transport. The expression of PTHrP in adult human parathyroid tissues under normal and pathological conditions was analyzed. By immunohistochemistry, PTHrP expression was detected in 86% of normal parathyroid (12/14 cases), 74% of adenomas (14/19) and 89% of hyperplasia secondary to chronic renal failure (16/18). PTHrP protein was observed mainly in the cytoplasm of oxyphil cells, consistent with the localization of its mRNA demonstrated by *in situ* hybridization. The rate of PTHrP-positive cells was higher in areas consisting of oxyphil cells than in those of non-oxyphil cells, regardless of whether the parathyroid was normal or pathological. In the normal parathyroid, an age-related increase in PTHrP expression was observed with a relative increase in oxyphil cells, reflecting aging and deterioration of parathyroid tissue. In adenoma, cases with a predominance of oxyphil cells expressed PTHrP, whereas clear cell adenoma did not. In secondary hyperplasia, the rate of PTHrP-expressing cells was higher than in normal parathyroid or adenoma, with varying levels of expression among nodules. We speculate that PTHrP could act through the paracrine/autocrine mechanism to regulate proliferation and differentiation of normal and neoplastic parathyroid cells.

Key words: Parathyroid, Parathyroid hormone-related protein (PTHrP), Immunohistochemistry, *In situ* hybridization

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Introduction

Parathyroid hormone-related protein (PTHrP) has been isolated as a causative factor for humoral hypercalcemia of malignancy (HHM) (Suva et al., 1987). It is produced by some cancerous tissues (Kitazawa et al., 1994) such as lung cancer and renal cell carcinoma. Because of its similarity in the amino terminal portion to PTH, PTHrP simulates endocrine actions of PTH on bone and kidney through a common PTH/PTHrP receptor (Juppner et al., 1991) in HHM. On the other hand, physiological roles of PTHrP in nonneoplastic organs, including placental calcium transport (Kovacs et al., 1996), lactation (Kovacs and Kronenberg, 1997) and bone development (Karaplis et al., 1994), have been demonstrated. Moreover, PTHrP is expressed in human endocrine tissues (Asa et al., 1990; Kitazawa et al., 1991; Gutman et al., 1993), and its paracrine and autocrine action affects hormone secretion (Villanueva-Penacarrillo et al., 1999). The presence of PTHrP in the human parathyroid has been described by others (Ikeda et al., 1989; Matsushita et al., 1992) and by our group (Kitazawa et al., 1992), although its roles in parathyroid function are not clear. We have previously reported its exclusive expression in oxyphil cells in the normal parathyroid (Kitazawa et al., 1992). In this study, we further examined the expression of PTHrP in parathyroid adenoma and hyperplasia secondary to chronic renal failure.

Materials and methods

Human parathyroid tissues

Parathyroid tissue from 14 normal glands, 19 adenomas and 18 cases of hyperplasia secondary to chronic renal failure were obtained at surgery at Kuma Hospital (Kobe, Japan). Routine formalin-fixed and paraffin-embedded specimens were prepared for immunohistochemical analysis. Half the fresh tissue of 5 representative cases from each group were fixed with

4% paraformaldehyde (PFA) for *in situ* hybridization (ISH), and the rest were subjected to RNA extraction. Representative sections for each case were stained with hematoxylin and eosin (HE) for histological diagnoses by two pathologists.

Immunohistochemistry

The anti-human PTHrP (1-34) monoclonal antibody, 4B3, belonging to the IgM subclass was generated and characterized as described (Kitazawa et al., 1991). Formalin-fixed and paraffin-embedded sections of the parathyroid tissue were deparaffinized with xylene and rehydrated through a series of graded alcohols. Frozen sections of 5 cases in each group were also subjected to ISH. After blocking endogenous peroxidase activity with 0.3% H_2O_2 in methanol, the sections were incubated with 4B3 in 50 mM phosphate buffered saline (PBS) at a final concentration of 5 µg/ml. The avidin-biotin-peroxidase complex (ABC) method (Hsu et al., 1981) was employed with a Vectastain ABC kit (Vector Laboratory Inc., CA, USA). Final development of the sections was carried out with 3,3'-diaminobenzidine (DAB) containing 0.03% H_2O_2. Negative controls were prepared by replacing the primary antibody with non-specific mouse IgM. Our evaluation of the semiquantitative analysis of immunostaining was as follows: (-), totally negative; (+-), less than 10% and weakly positive; (+) 10-50% and moderately positive; (++), more than 50% and strongly positive. Semiserial sections were stained with hematoxylin and eosin (HE) to distinguish oxyphil from non-oxyphil cells. The number of PTHrP-positive cells was counted in 20 areas, each consisting of either oxyphil or non-oxyphil cells. One-way analysis of variance (ANOVA) was used for statistical analysis.

In situ hybridization (ISH)

ISH was done on 4% PFA-fixed, OCT-compound-embedded frozen 7-µm-thick sections from 5 cases of each group. cDNA of PTHrP (Watanabe et al., 1990) (kindly donated by Dr. Toshiki Watanabe; Institute of Medical Science, Tokyo University, Tokyo, Japan) was metabolically labeled with bromodeoxyuridine (BrdU) as described (Kitazawa et al., 1989, 1993), and digested with restriction enzyme to yield 400 bp of DNA probe. The sections were rehydrated with PBS, refixed in 4% PFA/PBS and incubated in a hybridization medium [10mM Tris-HCl (pH 7.3), 0.6M NaCl, 1mM EDTA, 1xDenhardt's Medium/250 µg/ml yeast tRNA, 125 µg/ml sonicated salmon sperm DNA, 55% (v/v) deionized formamide/2 µg/ml of denatured probe DNA, 10% dextran sulfate] at 37 °C in a moist chamber overnight. Negative controls were prepared either with the BrdU-labeled vector DNA or with the RNase predigested specimen. After hybridization, the slides were washed for 6 hours with 50% deionized formamide/2xSSC and 2xSSC, and fixed with 4% PFA/PBS to immobilize the hybridized probe. The slides were subjected to RNase H treatment for 30 min to detect the BrdU-labeled probe in its single-stranded form (Kitazawa et al., 1993). BrdU-labeled probes were then visualized using FITC-conjugated anti-BrdU MoAb (Becton Dickinson Immunocytochemistry Systems, CA, USA) and analyzed with a confocal laser microscope (Bio-Rad, CA., USA).

Northern blot analyses

Total RNA was extracted by RNAzolTM (Tel-Test Inc., TX, USA) from the frozen samples of human parathyroid tissue (2 normal glands, 5 primary adenomas and 5 secondary hyperplasia) and from the HTLV I-infected cell line, KH2 (positive control). Each RNA (7.5 µg) was electrophoresed on a 1.2% agarose gel and transferred onto nylon membranes. cDNA of PTHrP was labeled with α^{32}P-dCTP by the random labeling method. The membranes were hybridized at 60 °C for 15hr, and washed twice in 2xSSPE at room temperature and in 0.1xSSPE at 60 °C then exposed to Kodak X-OMAT film for 4 days at -80 °C.

Reverse Transcriptase (RT)-PCR

Polyadenylated RNA was directly isolated from the specimens of fresh frozen samples of human parathyroid tissue (2 normal glands, 5 primary adenomas and 5 secondary hyperplasia) and from the KH2 cells (positive control) with the commercially available mRNA purification system (QuickPrep, Pharmacia, Uppsala, Sweden) and used as a template for RT-PCR. The sixth exon of the coding region of human PTHrP DNA was amplified by RT-PCR using rTth Reverse Transcriptase (Perkin-Elmer Cetus, CT, USA) (Myers and Gelfand, 1991) and the following oligonucleotide primers (Kitazawa et al., 1997):
primer 1 (sense): 5'-CGATTCTTCCTTCACCATCT-3'
primer 2 (antisense): 5'-TTTCTTTTCCTGCTCCTTGC-3'

In the reverse transcription step, primer 2-primed cDNA synthesis was done at 70 °C for 15 minutes and was followed by primer 1 and 2-primed PCR amplification. PCR amplification parameters were denaturation at 95 °C for 60 s, annealing and elongation at 60C for 60 s, for a total of 35 cycles. Each PCR aliquot (10 µl) was loaded onto agarose gel, and the presence of the 249-base pair band was determined by ethidium bromide.

Results

Immunohistochemical analysis revealed that 12 of 14 normal parathyroid tissue, 14 of 19 adenomas and 16 of 18 cases of secondary hyperplasia were positive for PTHrP (Table 1). In each case, PTHrP-positive cells were immunohistochemically evaluated in areas consisting of either oxyphil or non-oxyphil cells. The

rate of PTHrP positive cells was higher in areas of oxyphil cells than in those of non-oxyphil cells in the three groups (Fig. 1). The number of PTHrP positive cells in the normal parathyroid correlated with age (Fig. 2) and was consistent with the age-related increase in oxyphil cells.

PTHrP immunoreactivity observed mainly in the cytoplasm of the oxyphil cell lineage in each tissue was

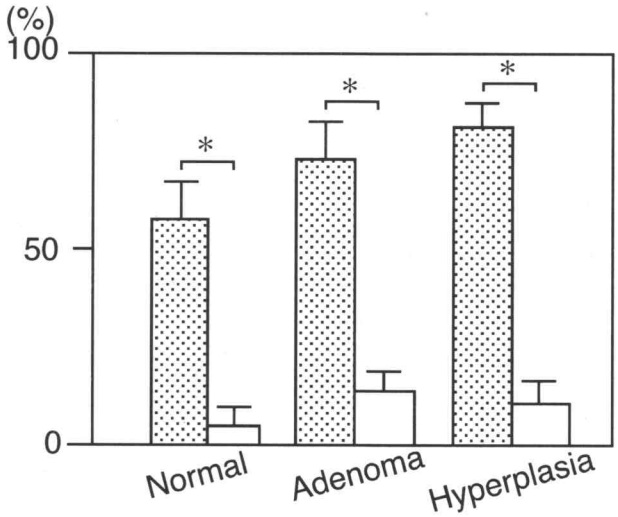

Fig. 1. Rate of cells immunohistochemically positive for PTHrP in the areas predominant with oxyphil cells (shadow bar) and non-oxyphil cells (open bar) in the three groups: normal parathyroid (normal), primary adenoma (adenoma) and hyperplasia secondary to chronic renal failure (hyperplasia). *: P<0.01 (vs. areas predominant with non-oxyphil cells).

Table 1. Immunohistochemical detection of PTHrP in normal and abnormal parathyroid tissue.

	NEGATIVE	POSITIVE			TOTAL
	(-)	(+-)	(+)	(++)	
Normal	2	10	2	0	12/14
Adenoma (1 HPT)	5	3	5	6	14/19
Hyperplasia (2 HPT)	2	2	5	9	16/18

(-): negative, (+-): less than 10% and weakly positive, (+): 10- 50% and moderately positive (++): more than 50% cells and strongly positive staining observed.

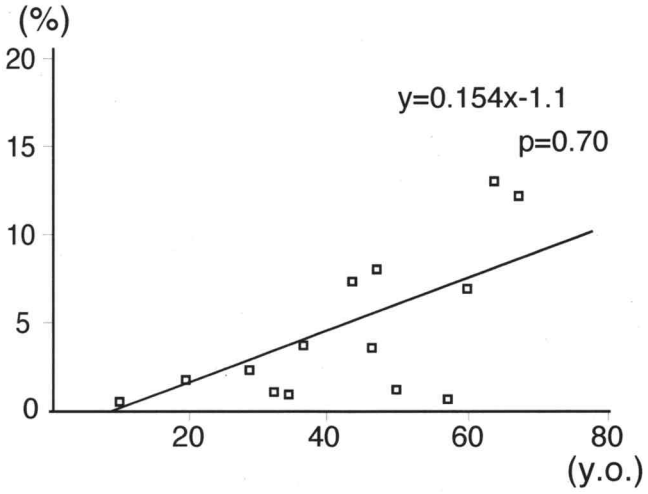

Fig. 2. Rate of PTHrP-positive cells in normal parathyroid gland in relation to age.

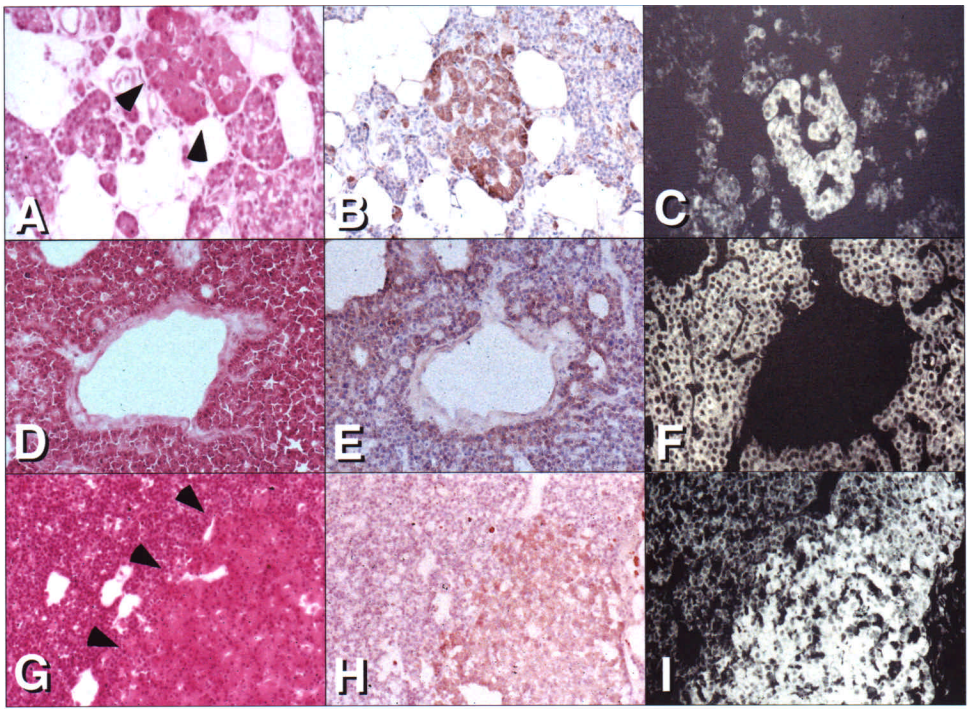

Fig. 3. PTHrP expression in normal human parathyroid tissue **(A, B, C)**, adenoma **(D, E, F)** and hyperplasia secondary to chronic renal failure **(G, H, I)**. **A, D, G:** HE staining. **B, E, H:** Immunohistochemical detection of PTHrP using a monoclonal antibody counterstained with hematoxylin. **C, F, I:** *In situ* hybridization using BrdU-labeled DNA probe analyzed with a confocal laser microscope. x 200

scarcely seen in chief or clear cells (Fig. 3B,E,H). The negative controls prepared with non-specific mouse myeloma IgM showed no staining (data not shown). The transcripts of PTHrP were clearly demonstrated in the cytoplasm of oxyphil cells by ISH (Fig. 3C,F,I) and their expression was consistent with the localization of PTHrP immunoreactivity. Signals were detected neither in the specimens hybridized with BrdU labeled vector DNA without the specific probe nor in those predigested with RNase (data not shown).

By Northern blot analysis, PTHrP transcripts measuring 1.4 and 2.2 kb were detected on KH2 cells, parathyroid adenomas and hyperplasias but not on the normal gland (Fig. 4A). A 249-base pair PCR product was detected in the mRNA extracted from parathyroid tissues in each group, as in the KH2 cell line (positive control) (Fig. 4B). Without the reverse transcription step the same samples did not exhibit the 249-base pair PCR product. In normal parathyroid tissue, the expression of PTHrP transcripts was confirmed by the RT-PCR, but not by Northern blotting.

Discussion

PTHrP expression in parathyroid tissue under normal and pathological conditions was analyzed. PTHrP immunoreactivity was detected in 86% of the normal parathyroid, 74% of adenomas and 89% of secondary hyperplasia. The localization of PTHrP transcripts, consistent with that of PTHrP protein, was demonstrated exclusively on oxyphil cells regardless of normal or pathological conditions. As previously reported, ISH (Fig. 3) and RT-PCR (Fig. 4) demonstrated PTHrP mRNA in normal parathyroid tissues; but Northern blot did not, because few PTHrP-expressing cells were present in the normal parathyroid. Thus ISH detected a small amount of PTHrP transcripts in association with the histologic subtypes of parathyroid parenchymal cells. A DNA probe metabolically labeled with BrdU (Kitazawa et al., 1989) was used for ISH. Among various nucleic acid probes, DIG-labeled antisense RNA probes transcribed in vitro are most commonly used. DNA probes, however, are more stable than RNA probes and are not subjected to unexpected degradation by contaminated RNase. Since BrdU substitutes uracil/ thymidine, advantages that its labeling index (theoretically estimated to be ~25%~) is higher than other haptens and that it is free from a conformational change of labeled nucleotides inevitable with large molecules such as DIG and Biotin can be appreciable.

The physiological role of PTHrP in parathyroid tissue has not been clarified. During fetal life PTHrP acts as a hormone to maintain placental calcium transport (Kovacs et al., 1996). The circulating PTHrP level in normal human adults is not high enough to regulate normal calcium homeostasis, although PTHrP is secreted from the parathyroid cells of rat (Sakaguchi et al., 1990) and of ovine (Masclsaac et al., 1991). Only a small number of secretory granules of PTHrP have been observed in the oxyphil cell lineage (Kitazawa et al., 1992), supposedly arising from chief cells as a metaplastic change (Roth and Raiz, 1964), suggesting that PTHrP might be involved in parathyroid cell differentiation and aging in normal adults. An age-related increase in PTHrP expression was observed in the normal parathyroid (Fig. 2), probably related to an age-related increase in oxyphil cells. Based on a recent report that PTHrP enhances the secretory response of PTH to hypocalcemia (Lewin et al., 2000), we speculate that, an age-related increase in PTHrP adjusts for the deterioration of parathyroid.

Primary hyperparathyroidism (adenoma) is clonal proliferation of parathyroid cells caused by the overexpression of the cyclin D1 gene (PRADA1) (Rosenberg et al, 1993). The level of PTHrP expression was homogeneous in each adenoma, probably due to less variability of the histological phenotype. Adenomas with a dominance of oxyphil cells expressed PTHrP (11/19 cases), consistent with another report (Matsushita et al., 1992), whereas clear cell adenomas did not (5/19 cases). Although PTHrP enhances breast cancer cell growth (Falzon and Du et al., 2000), no significant difference in

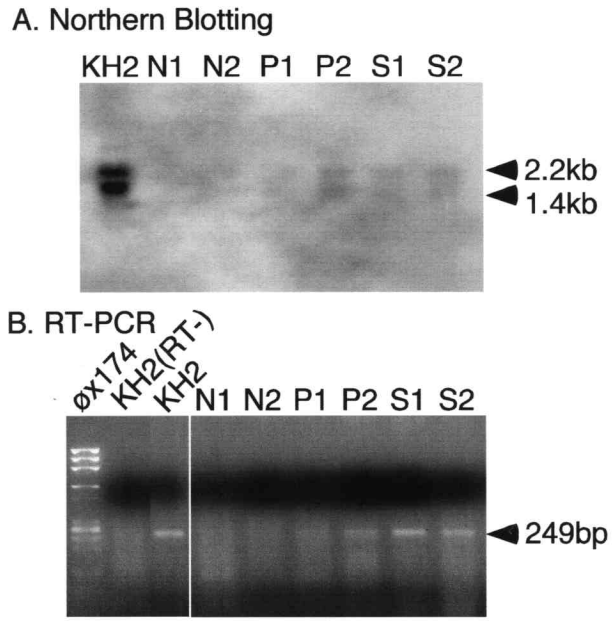

Fig. 4. A. Northern blot analysis for PTHrP. Representative 2 cases of each group (N1, N2: normal, P1, P2: primary adenoma and S1, S2: secondary hyperplasia) are demonstrated. Total RNA (7.5 mg/lane) extracted from parathyroid tissues and HTLV I-infected cell line (KH2) was blotted and hybridized with ^{32}P-labeled human PTHrP cDNA. Transcripts of PTHrP, measuring 1.4 and 2.2 kb, were detected on KH2 cells, parathyroid adenoma and hyperplasia but not on normal glands. **B.** Detection of PTHrP transcripts by RT-PCR. A quantity of 10 µl of each aliquot was loaded onto agarose gel and stained with ethidium bromide. A 249-base pair RT-PCR product was detected in the mRNA extracted from parathyroid tissues (N1, N2: normal, P1, P2: primary adenoma and S1, S2: secondary hyperplasia) as well as in the mRNA from KH2 cells.

the size of adenomas was observed between our PTHrP-positive and -negative cases.

Long-standing renal failure with a decrease in serum $1\alpha,25(OH)_2$vitamin D3 and calcium stimulates the polyclonal proliferation of parathyroid cells and the hypersecretion of PTH (secondary hyperparathyroidism) (Salusky et al., 1995); diffuse hyperplasia at the initial phase is followed by nodular hyperplasia. Most cases of secondary hyperplasia in this study were multinodular hyperplasia with oxyphil cell foci. The rate of PTHrP-expressing cells was higher than that in the normal parathyroid (Table 1), and a nodule-to-nodule difference in PTHrP expression level was also observed. Since the correlation between PTHrP expression and cell proliferation in secondary hyperplasia is negative (Matsushita et al., 1999), albeit that PTHrP overexpression causes pancreatic islet hyperplasia in mouse (Porter et al., 1998), we speculate that PTHrP might suppress proliferation of parathyroid cells and promote differentiation into the oxyphil cell lineage. Moreover, the calcium sensing receptor (CaSR), determining the set point of Ca for PTH secretion (Brown et al., 1993), decreases in parathyroid hyperplasia (Kifor et al., 1996) and correlates negatively with cell proliferation (Yano et al., 2000). Further study is needed to clarify the CaSR-mediated regulation of PTHrP production and its role in the local calcium metabolism in chronic renal failure.

This study demonstrates PTHrP mRNA expression in parathyroid tissue under normal and pathological conditions, by ISH and RT-PCR, and the correlation of PTHrP expression with the metaplastic change of parathyroid cells into the oxyphil phenotype. Our results suggest that PTHrP may act through the paracrine/autocrine mechanism to regulate proliferation and differentiation of parathyroid cells.

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