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Proliferation of parathyroid cells negatively correlates with expression of parathyroid hormone–related protein in secondary parathyroid hyperplasia

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Proliferation of parathyroid cells negatively correlates with expression of parathyroid hormone-related protein in secondary parathyroid hyperplasia.

Background. Parathyroid hormone–related protein (PTHrP) is now suspected to act as an autocrine or paracrine regulator of cell growth or differentiation, although it was originally reported as a hypercalcemic substance in malignancies. This study was performed to assess the relationship between PTHrP expression and cell proliferation in human parathyroid glands.

Methods. The localization of PTH and PTHrP was studied in 42 samples of hyperplastic parathyroid from 14 long-term hemodialysis cases with immunohistochemistry and *in situ* hybridization. Results were compared with proliferative activity (proliferating cell nuclear antigen index: counts of proliferating cell nuclear antigen-positive cells/100 cells). The localization of the PTH/PTHrP receptor was also examined. Ten normal glands were studied as controls.

Results. In hyperplasia, cells positive for PTH, PTHrP, or both were observed immunohistochemically. The areas expressing PTHrP mRNA completely coincided with those positive for PTHrP immunohistochemically. Oxyphilic or transitional oxyphilic cells were consistently positive for PTHrP. PTH/PTHrP receptors were located in the cytoplasmic membrane in most parathyroid cells. Proliferating cell nuclear antigen-positive cells were rare in normal glands with an index of 0.22 ± 0.09 (mean $\pm$ SEM). They were significantly increased in hyperplastic cases but less for PTHrP-positive than for -negative cells (1.25 ± 0.16 as compared with 7.80 ± 0.52; $P < 0.0001$).

Conclusion. The observed low level of proliferation of PTHrP-positive cells suggests a functional role for PTHrP as a possible growth suppressor in the human parathyroid.

The parathyroid hormone–related protein (PTHrP) was originally isolated from human malignant tumors associated with humoral hypercalcemia [1, 2]. Three peptides composed of 139, 141, and 173 amino acid residues can be formed by alternate splicing of PTHrP messenger RNA (mRNA) [3]. The first 13 amino acid residues of the amino terminus show a high degree of homology with that of PTH, whereas amino acids 14 to 34 have important functions in binding to PTH receptors [4]. PTHrP reactions with PTH receptors in bone and kidney are responsible for the hypercalcemia observed in malignant tumor cases with elevated serum PTHrP concentrations [5]. Recently, PTHrP has been shown to be widely distributed not only in neoplasms but also in normal tissues [6]. Both PTHrP and common PTH/PTHrP receptors are expressed in the same organs, such as bone [7], brain [8], the intestine [9], the endocrine pancreas [10], and the lung [11]. Inactivation of the PTHrP gene results in an abnormal bone development [12]. Overexpression causes hyperplasia of pancreatic islets in transgenic mice [13]. Therefore, PTHrP is suspected to act as an autocrine or a paracrine regulator of cell growth or differentiation [6, 14]. Both PTH and PTHrP are expressed in the normal parathyroid gland in parathyroid hyperplasia and in parathyroid adenomas [15–19]. PTH and PTHrP have been colocalized in the same adenoma cells and are secreted through the same constitutive pathway [20]. However, knowledge of the functional role of PTHrP in this site is limited. We therefore designed this study to cast light on the relationship between PTHrP production and cell proliferation in the human parathyroid gland. For this purpose, we first determined the localization of PTHrP by immunohistochemistry and *in situ* hybridization in hyperplastic parathyroid glands and then made a comparison with the expression of the prolif-

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erating cell nuclear antigen (PCNA), a cofactor of DNA polymerase δ [21], which has been established as a sensitive marker of cell proliferation [22]. Triple immunostaining for PTH/PTHrP and PCNA was performed on the same histologic sections of parathyroid glands, allowing direct comparisons to be made at the single cell level.

METHODS

Parathyroid glands

Forty-two samples of hyperplastic parathyroid glands were obtained from 14 cases of secondary hyperparathyroidism in chronic renal failure patients maintained on long-term hemodialysis (Table 1). The study group consisted of five males and nine females. The mean age at parathyroidectomy was 50.0 ± 3.0 (mean ± SEM) years. The mean duration of hemodialysis was 14.4 ± 1.2 (mean ± SEM) years before the operation. The cause of renal failure was chronic glomerulonephritis in all cases. The concentrations of serum intact PTH were elevated in all cases before parathyroidectomy (mean ± SEM, 1484.0 ± 265.0 pg/ml; normal range, 20.0 to 53.0 pg/ml) performed in these cases because of severe renal osteodystrophy. Four parathyroid glands were resected in every case, and small fragments of parathyroid tissues were autotransplanted. In all studied cases, the resected parathyroid glands were consistently enlarged. Histologically, there were no surrounding rim-like structures, but interspersed fat tissue was evident with mild to moderate enlargement. On the basis of the histologic appearance, parathyroid hyperplasias were divided into two subtypes: diffuse (13 glands) and nodular (29 glands) [23], with mean weights and a SEM of 1.26 ± 0.34 g and 1.53 ± 0.61 g, respectively. The difference between these two values was not significant (Student's *t*-test). As a control for cell proliferation, 10 normal parathyroid glands were obtained from two cases with benign thyroid tumors and eight cases of esophageal carcinomas. These normal parathyroid glands were attached to the resected thyroid tissues or were contained in the dissected cervical lymph nodes. Serum calcium levels were in the normal range in all cases. The glands comprised both parenchymal cells and interstitial fat cells, and neither hyperplasia nor microadenomas were histologically evident. The mean age of this group was 49.7 ± 5.8 (SEM) years.

Immunohistochemistry

Double immunofluorescent staining for PTH and PTHrP was performed as detailed in our earlier report [20]. The monoclonal antibody against human (h) PTHrP (1–34) was established in our laboratory, and goat anti-hPTH antibodies were obtained from the Nichols Institute (San Juan Capistrano, CA, USA) and Nihon Mediphysics Co. Ltd. (Nishinomiya, Japan). Rhodamin (TRITC)-conjugated rabbit antimouse immunoglobulins and fluorescein (FITC)-conjugated rabbit antigoat immunoglobulins were used as second antibodies (DAKO, Glostrup, Denmark). The quantitative analysis of PTHrP-positive cells in the parathyroid glands was performed by the two independent reviewers using the following grading criteria: − = no positive cell; AF = a few positive cells (below 5%); + = 5% to 30%; and ++ = more than 30%. After taking photomicrographs, the immunofluorescently stained parathyroid sections were treated with 0.1 M glycine/HCl buffer to elute antibodies against PTH or PTHrP and were then reacted with anti-PCNA antibody (DAKO; diluted at 1:100) for two hours at room temperature (RT). After several washings in phosphate-buffered saline solution (PBS) with 0.05% Tween 20 (PBS-T), the sections were reacted with goat anti-mouse IgG (H + L) antibody conjugated with 1 nm colloidal gold particles (British BioCell International, Cardiff, UK), diluted at 1:100, for 30 minutes at RT. After several washings in distilled water, binding sites were visualized using a silver enhancing kit (British BioCell International), and the sections were counterstained with methyl green. Additional sections were immunostained routinely with avidin-biotin complex method after treatment with the anti-hPTHrP antibody. Immunostaining for PTH/PTHrP common receptors was also performed with the affinity purified polyclonal antibody (Berkeley Antibody Company, Richmond, CA, USA). After reacting with primary antibody (diluted at 1:50) and biotinylated second antibody (MaxITags; Shandon-Lipshaw, Pittsburgh, PA, USA), immunoreacted products in the parathyroid tissues were amplified by the tyramide signal amplification, known as catalyzed reporter deposition (NEL Life Science Products, Boston, MA, USA).

In situ hybridization

Expression of PTHrP mRNA was studied in 10 hyperplastic glands by *in situ* hybridization. The 33-mer oligonucleotide probe for human PTHrP, synthesized and 3′ end-labeled with digoxigenin according the report of Danks et al [24], was as follows: AAA TCG GAC GGG GTG GTT CTT TGT GTT GGG AGA (JD2). The sense probe was used as a negative control. Formalin-fixed and paraffin-embedded parathyroid tissues were examined. All solutions used for processing of thin sections, for the preparation of probes, for washing tissues, and for prehybridization and hybridization were prepared with double-distilled water treated with 0.02% diethylpyrocarbonate (Sigma, St. Louis, MO, USA). Thin sections mounted on glass slides covered with tissue adhesive (3-aminopropyltriethoxysilane; Sigma) were deparaffinized and treated with 0.3% Triton X-100 in PBS for 15 minutes at RT. After several washes in PBS, they were postfixed with 4% paraformaldehyde in PBS for five minutes and were further washed and then im-

Table 1. Clinicopathological data for the studied cases

Case	Age	Duration of hemodialysis	PTH[a]	Location[b]	Weight	Histological typing[c]	PTHrP immunostaining[d]	PTHrP in situ hybridization[d]
	years		*pg/ml*		*g*			
1	52	10.0	1100	RU	1.16	NH	−	AF
				RL	0.71	NH	−	ND
				LU	0.25	NH	+	+
				LL	0.44	NH	+	ND
2	58	5.0	2899	LL	0.07	NH	++	++
				LU	3.07	DH	−	−
				RU	ND	DH	−	AF
				RL	ND	DH	+	ND
3	45	14.0		LU	1.30	DH	+	ND
				LL	0.47	DH	+	ND
				RL	0.23	NH	AF	AF
				RU	0.28	NH	−	ND
4	60	14.0	605	RU	0.34	NH	+	ND
				RL	0.13	NH	+	ND
				LU	0.73	NH	+	ND
				LL	0.49	DH	−	ND
5	21	11.0	720	RU	1.64	DH	AF	AF
				LL	2.09	DH	−	ND
6	61	24.0	1054	LU	0.28	DH	++	+
				RU	0.22	NH	+	ND
				RL	3.24	DH	−	ND
7	49	19.0	2029	LL	ND	NH	−	ND
8	46	15.0	1307	LU	ND	NH	−	ND
				LL	ND	NH	+	ND
				RU	ND	DH	AF	ND
9	51	19.0	1056	LU	2.20	NH	+	+
				LL	0.60	NH	−	ND
				RU	0.22	DH	++	ND
				RL	0.70	NH	+	ND
10	45	16.0	1888	RU	ND	NH	+	ND
				RL	0.50	NH	−	ND
11	61	14.0	ND	RU	2.07	NH	−	ND
12	48	13.0	1271	LU	12.50	NH	AF	ND
				RU	1.76	NH	++	++
				LL	0.32	DH	−	ND
13	63	13.0	456	LU	ND	NH	++	ND
				LL	ND	NH	+	ND
				RU	ND	NH	++	ND
				RL	ND	NH	++	ND
14	42	14.0	3426	RL	2.88	NH	−	ND
				RU	0.58	NH	AF	ND
				RL	2.88	NH	AF	ND

ND is not determined.
[a] Normal range of serum intact PTH, 20.0–53.0 pg/ml
[b] Abbreviations are: LU, left upper; LL, left lower; RU, right upper; RL, right lower
[c] Abbreviations are: DH, diffuse hyperplasia; NH, nodular hyperplasia
[d] Quantitative estimation of PTHrP positive cells: (−) no positive cells; (AF) a few positive cells (below 5%); (+) 5–30%; (++) more than 30%

mersed in 3% hydrogenperoxide in methanol for 10 minutes at RT to block intrinsic peroxidase activity. After ethanol dehydration, sections were placed in a 25% formamide humid chamber and reacted with prehybridization buffer containing 25% deionized formamide in 2 × saline-sodium citrate (SSC) buffer for one hour at RT. The hybridization buffer consisted of 2 × SSC, 25% deionized formamide, 0.1% polyvinylpyrolidone (Sigma), 0.1% Ficoll (Sigma), 1% bovine serum albumin (fraction V; Boehringer Mannheim, Mannheim, Germany), 250 μg/ml salmon sperm DNA (type Iis; Sigma), and 250 μg/ml yeast tRNA (Boehringer Mannheim). The probe concentration was 2.0 ng/μl. Twenty to 30 microliters of hybridization solution were applied to each tissue section under glass coverslips, and incubation was in the 25% formamide humid chamber for 12 to 18 hours at RT. Coverslips were removed in 4 × SSC, and the sections were washed with three changes of 3 × SSC, two changes of 0.2 × SSC (prewarmed at 37°C), and finally, three changes of 2 × SSC for five minutes each. Hybridization signals were amplified and detected with the following immunohistochemical steps. After washing in PBS-T, the tissue sections were reacted with sheep antidigoxigenin antibody (Boehringer Mannheim) diluted at 1:250 with PBS containing 1% bovine serum albumin (PBS-B) for one hour at RT. After washing in PBS-T, the sections

Table 2. Quantitative estimation of PTHrP positive cells (immunohistochemistry)

Histological typing	Total glands	PTHrP positive glands %	Quantity of PTHrP positive cells %			
			−	AF	+	++
Diffuse hyperplasia	13	7 (54)	6 (47)	2 (15)	3 (23)	2 (15)
Nodular hyperplasia	29	20 (69)	9 (31)	4 (14)	11 (38)	5 (17)
Total	42	27 (64)	15 (36)	6 (14)	14 (33)	7 (17)

Definitions are: −, no positive cell; AF, a few positive cells (below 5%); +, 5 to 30%; ++, more than 30%.

were then reacted with biotinylated rabbit antisheep IgG (H + L) antibody (Vector Laboratories, Burlingame, CA, USA) diluted at 1:200 in PBS-B for 30 minutes at RT. After three washes with PBS-T, the signals were amplified by the tyramide signal amplification [25]. The sections were incubated with horseradish peroxidase conjugated streptoavidin in the biotinyl tyramide amplification kit for 30 minutes at RT, were washed thoroughly with five changes of PBS-T for five minutes each, and were incubated with biotinyl tyramide for exactly 10 minutes at RT. The sections were then washed with four changes of PBS-T and reacted with horse radish peroxidase-labeled streptoavidin-biotin complex (Strept ABC complex/HRP; DAKO) for 30 minutes at RT and finally with 2% diaminobenzidine for five minutes.

Statistical analysis

The numbers of PCNA-positive cells were counted per 100 parathyroid cells to provide a PCNA index, estimated for PTHrP-positive and PTHrP-negative areas separately. For this purpose, counts were made in at least four different areas in each parathyroid gland. PCNA indices were statistically analyzed by two-way ANOVA using the generalized linear model procedure with SAS/STAT software (SAS Institute Inc., Cary, NC, USA), because of the imbalance in sample numbers among the study groups [26]. Significance was concluded with a P value of <0.05.

RESULTS

Distribution of PTH and PTHrP

The immunohistochemical results are summarized in Tables 1 and 2. All parathyroid glands immunohistochemically examined were positive for PTH. Of the 42 samples of parathyroid hyperplasia secondary to chronic renal failure, 27 (64%) demonstrated PTHrP staining, with no significant difference being observed between the diffuse (9/13) and nodular (20/29) types. In nodular parathyroid hyperplasia, each nodule was homogeneously comprised of one type of parathyroid cells, entirely PTH positive or PTHrP positive or positive for PTH as well as PTHrP (Fig. 1). Cells with the same immunoreactivity also showed a tendency to group together in diffuse hyperplasia samples. PTH was distributed along the cytoplasmic membranes linearly or fine granularly, whereas PTHrP showed a tendency to be distributed diffusely or coarse granularly in the cytoplasm. Histologic examination of restained hyperplasia tissues after immunohistochemical examination revealed a tendency for PTHrP-positive nodules to consist of oxyphilic cells and for PTH-positive nodules to consist of chief cells. The nodules positive for both PTH and PTHrP usually consisted of chief cells or transitional oxyphilic cells. The dominant type of parenchymal cells was that of entirely PTH positive in all hyperplastic parathyroid glands. The quantitative estimation of PTHrP-positive cells, entirely PTHrP positive, and positive for both PTH and PTHrP revealed that half of hyperplastic parathyroid glands consisted of considerable amounts of PTHrP-positive cells, 5% to 30% in one third and more than 30% in 17% of parathyroid hyperplasia (Table 2).

Distribution of PTHrP mRNA

Hybridized signals were detected in only the specimens reacted with the antisense probe for PTHrP mRNA and were negative with the sense probe. The areas expressing PTHrP mRNA in hyperplastic glands well coincided with those positive for PTHrP immunohistochemically (Fig. 2). In the PTHrP-positive areas in secondary hyperplasia cases, the intensity of detected signals for PTHrP mRNA in the cytoplasm was uneven, being strong in some cells and weak in others. Most hybridized signals were detected in the cytoplasm, mainly near the basal lamina, but weak signals were detected in nuclei as well. There was no apparent difference in the intensities of detected signals between nodular and diffuse hyperplasia cases.

Proliferative activity of parathyroid cells

PCNA-positive cells were very rare in the normal parathyroid gland, and staining could only be found in PTHrP-negative chief cells in four of the cases. The numbers of PCNA-positive cells were markedly increased in hyperplasia. Their densities differed from nodule to nodule in nodular hyperplasia, although they appeared evenly distributed within individual nodules (Fig. 3a).

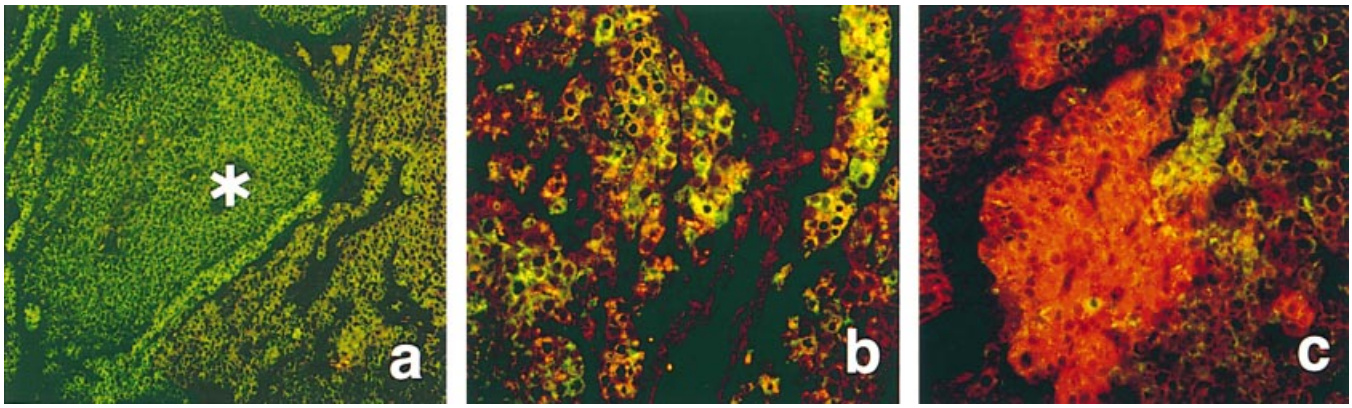

Fig. 1. Distribution of PTH and PTHrP in parathyroid hyperplasia secondary to chronic renal failure. Hyperplastic parathyroid tissues were double immunostained for PTH (green fluorescence) and for PTHrP (red fluorescence). The distribution of PTH and PTHrP was heterogeneous in nodular hyperplasia, although each nodule was homogeneously composed of one type of parathyroid cells, entirely PTH-positive cells (*a*, asterisk), positive for both PTH and PTHrP (*b*), or positive only for PTHrP (*c*). Magnification a, ×36; b and c, ×72.

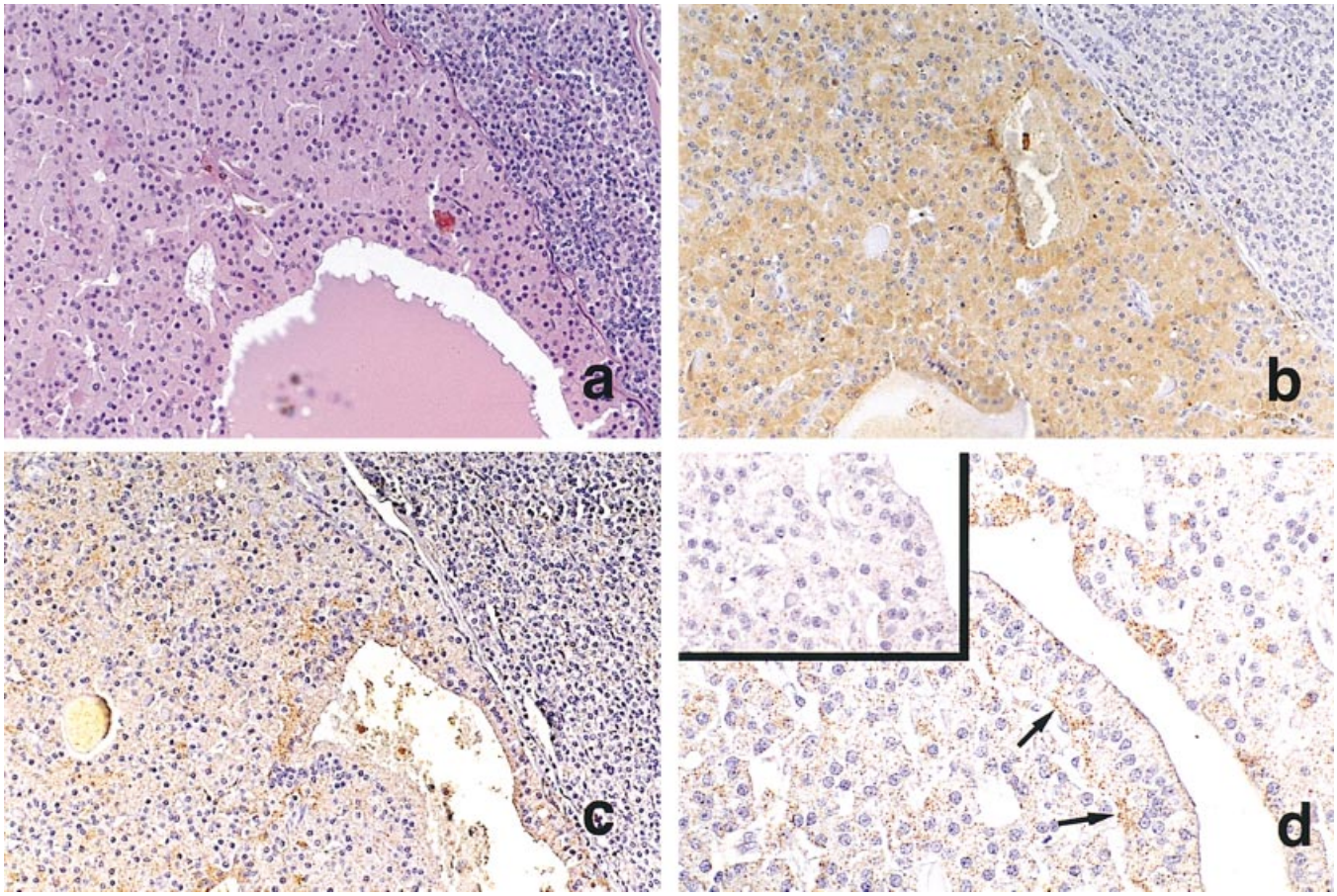

Fig. 2. *In situ* hybridization for PTHrP mRNA. Serial sections were used for histologic examination (*a*), immunohistochemistry for PTHrP (*b*), and *in situ* hybridization for PTHrP mRNA (*c*). The oxyphil cell area (a, center-left) is responsible for PTHrP production at the level of protein (b) or mRNA (c) (×125). (*d*) Higher magnification of *in situ* hybridization signals in a PTHrP-positive area. Most PTHrP mRNA appears distributed in the cytoplasm, mainly in the basal portions of the cells (arrows). The density of the signals for PTHrP mRNA is uneven with considerable heterogeneity between cells despite their homogeneous PTHrP immunostaining. *Insert*: Negative control with the sense probe (×220).

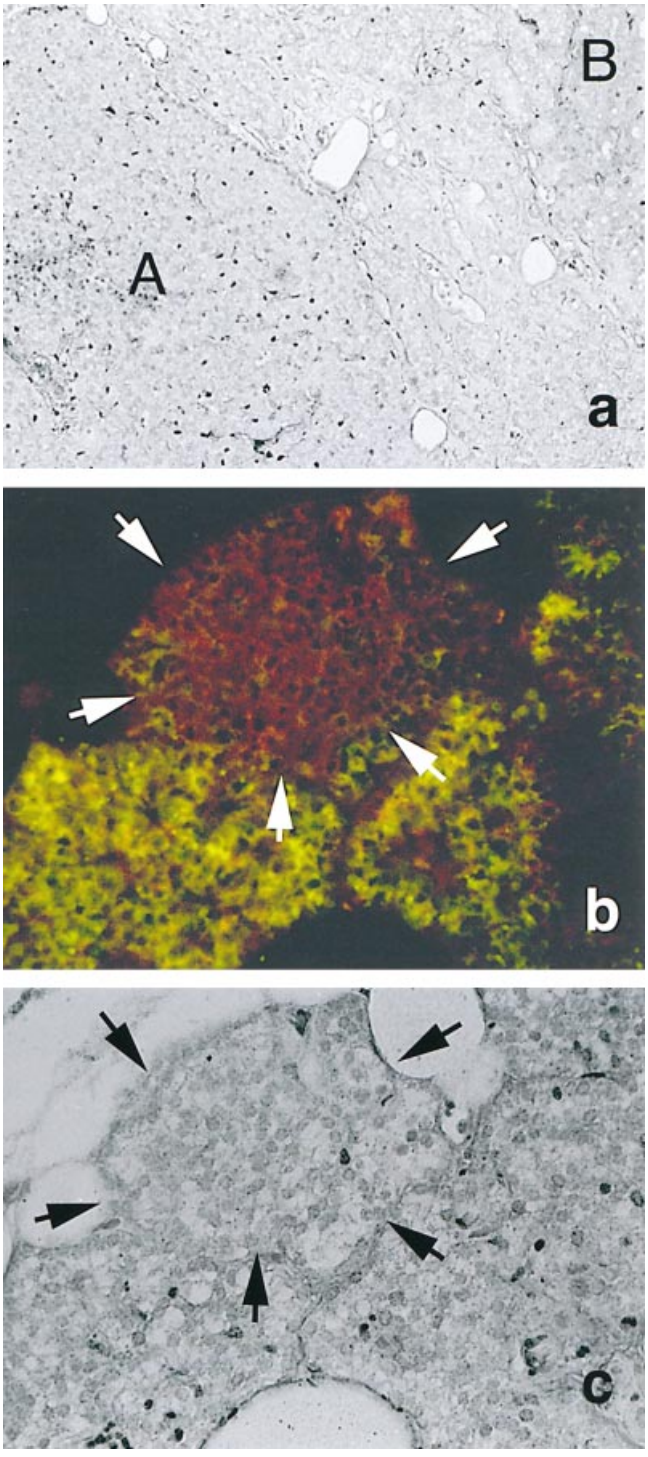

Fig. 3. (left) Heterogeneity of cell proliferation in human parathyroid glands. (*a*) Proliferating cell nuclear antigen (PCNA) immunoreactivity in a case of nodular parathyroid hyperplasia. PCNA-positive nuclei are stained black. The level of cell proliferation is heterogeneous, with the density of PCNA positive cells in the nodule on the left (A) being higher than in that on the right (B) or in the internodular area. Immunostaining with gold colloidal particle-conjugated secondary antibodies and silver enhancement (×115). (*b*) Immunohistochemistry for PTH and PTHrP. In the center of the figure, there is a small nodule consisting of a uniform population of PTHrP-positive cells (encircled with arrows), whereas the surrounding parathyroid tissue is comprised of PTH-positive cells. Double-immunofluorescence method (×230). (*c*) PCNA immunostaining of the same tissue section used for the immunohistochemistry for PTH/PTHrP in Figure 2b. The density of PCNA positive cells in the small nodule with PTHrP positive cells in Figure 2b (encircled with arrows) is extremely low comparing to the surrounding parathyroid tissue that is positive for PTH. Immunostaining with gold colloidal particle-conjugated secondary antibody and silver enhancement after double immunofluorescent staining for PTH/PTHrP (×230).

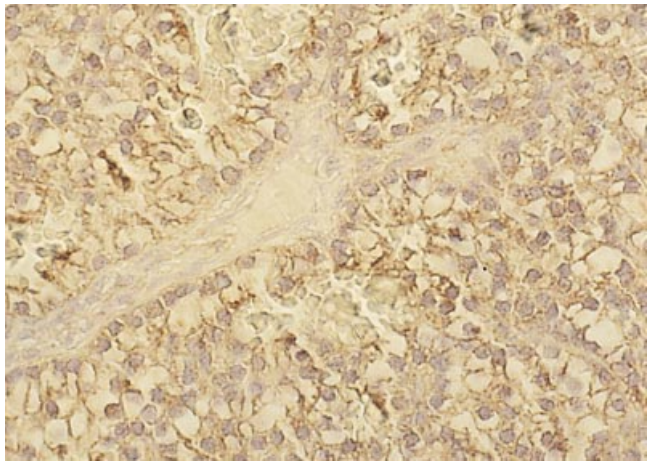

Fig. 5. The localization of PTH/PTHrP receptors in parathyroid hyperplasia secondary to chronic renal failure. PTH/PTHrP receptors are located along the cell membrane in most hyperplastic parathyroid cells. Immunoreactive products are shown in a dark brown color (4′,4′-diaminobenzidine as a chromogen). Immunostaining enhanced by the tyramide signal amplification method (×340).

The heterogeneity in cell proliferative activity exactly coincided with the heterogeneity of the immunoreactivity of the parathyroid cells for PTH or PTHrP (Fig. 3 b, c). Therefore, separate PCNA indices (counts of PCNA-positive cells/100 cells) for PTHrP-positive and -negative cell areas were generated for hyperplasia. We could only estimate a PCNA index for PTHrP-negative (PTH-positive) cells in normal parathyroid glands because the number of both PTHrP-positive and PCNA-positive cells was too small to allow analysis. The relation of histologic appearance and immunohistochemical findings for parathyroid cells to the PCNA index was statistically analyzed by two-way ANOVA using the generalized linear model procedure. The mean and SEM values for PCNA indices were 0.22 ± 0.09 ($N = 32$) in the normal parathyroid gland and 5.32 ± 0.46 ($N = 99$) in hyperplasia cases. The mean and SEM for parathyroid cells positive only for PTH with negative immunoreactivity for PTHrP were 4.99 ± 0.46 ($N = 95$; normal and hyperplasia cases), whereas the respective values for PTHrP-positive cells were 1.48 ± 0.15 ($N = 36$, hyperplasia cases). In parathyroid hyperplasia cases, the mean and SEM values for PTHrP-negative cells were 7.80 ± 0.52 ($N = 63$), and

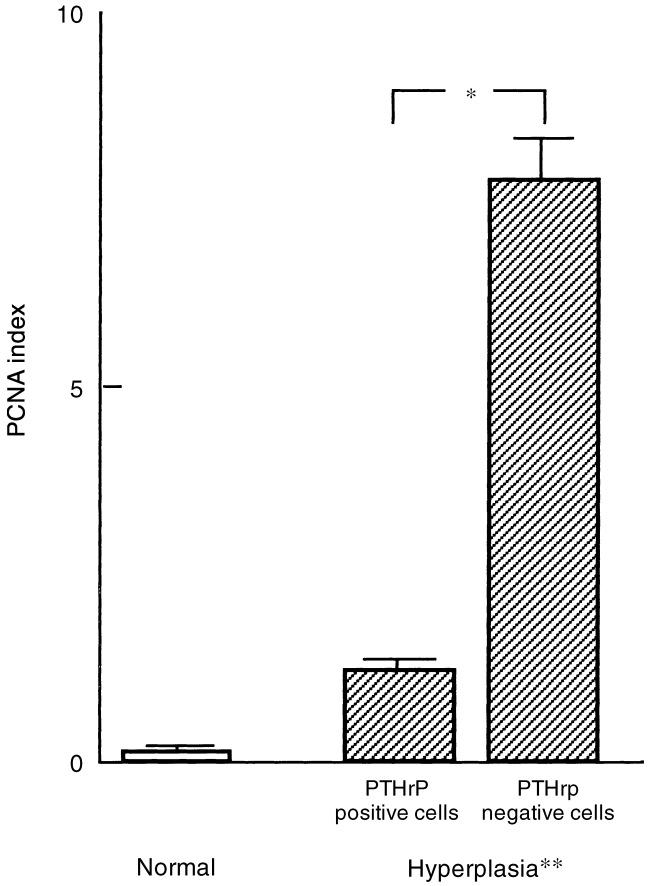

Fig. 4. Proliferation activity of the parathyroid cells. PCNA indices were measured for cells positive only for PTH (PTHrP negative cells) and for those positive for PTHrP or both PTHrP and PTH (PTHrP positive cells). The values for the PTHrP negative cells were significantly higher than those for PTHrP positive cells ($*P < 0.0001$) in hyperplasia cases. As compared with normal glands, the cases of hyperplasia demonstrated significant increase ($**P < 0.0001$). The error bars indicate SEM values.

for PTHrP-positive cells, the mean and SEM were 1.25 ± 0.16 ($N = 36$). PCNA indices for hyperplasia cases were significantly higher than the value for normal parathyroid glands ($P < 0.0001$), and the PTHrP-negative cells (cells positive only for PTH) consistently demonstrated significantly higher indices than PTHrP-positive cells ($P < 0.0001$) (Fig. 4).

Distribution of PTH/PTHrP receptor

Most parathyroid cells were immunohistochemically positive for the PTH/PTHrP receptor. The receptors were distributed along the cytoplasmic membrane of the parathyroid cells (Fig. 5).

DISCUSSION

It is well known that hyperplastic parathyroid glands in renal-failure patients with long-term hemodialysis often show a nodular appearance [27], some nodules consisting of chief cells and others of oxyphilic or transitional oxyphilic cells. It has been suggested that the functional levels of the parathyroid cells also differ from nodule to nodule. Fukuda et al have reported that vitamin D–receptor expression is suppressed in nodular parathyroid hyperplasia secondary to chronic renal failure with long-term hemodialysis [23]. They showed the receptor density to be heterogeneous but uniform within individual nodules. Because the physiological role of vitamin D is to suppress cell growth or PTH secretion in the parathyroid glands, receptor depletion may result in accelerated cell growth or vitamin D–resistant hypersecretion of PTH [28]. This study of the distribution of PTH and PTHrP revealed another distinct heterogeneity in secondary parathyroid nodular hyperplasia; that is, each nodule was homogeneously comprised of one type of parathyroid cells, exclusively PTH positive, PTHrP positive, or positive for PTH as well as PTHrP. Cells with the same immunoreactivity also showed a tendency to group together in diffuse hyperplasia cases. In addition, our sensitive *in situ* hybridization technique clearly demonstrated the distribution of the parathyroid cells expressing PTHrP mRNA to coincide well with that of the cells immunohistochemically positive for PTHrP. Therefore, it is evident that the cells in hyperplastic parathyroid glands positive for PTHrP are actively producing PTHrP. In our earlier report, we demonstrated that hyperplastic parathyroid cells from patients with chronic renal failure are able to secrete PTHrP depending on the extracellular calcium levels [19]. There are two different types of PTH receptors: one is common for PTH and PTHrP [8] and the other is specific for PTH [29]. PTH/PTHrP common receptors are known to be widely distributed in many organs and are postulated to function as paracrine or autocrine receptors [14]. In this study, we showed that most hyperplastic parathyroid cells expressed PTH/PTHrP receptors along cell membranes. Considerable amounts of parathyroid cells expressed PTHrP in hyperplastic glands. Therefore, PTHrP can act as a possible paracrine or autocrine substance in parathyroid hyperplasia secondary to chronic renal failure. The proliferative activity of the parathyroid cells evaluated with PCNA immunolabeling was significantly increased in secondary parathyroid hyperplasia in this study with considerable internodule variation. The heterogeneity in the PCNA labeling of parathyroid cells coincided exactly with the heterogeneity in their hormone production, with proliferation correlating in a negative fashion with PTHrP expression. Recently, evidence has accumulated that PTHrP acts as a paracrine or an autocrine regulator that can control cell proliferation as well as differentiation [12, 14, 30]. Several reports have appeared of inhibitory effects of PTHrP on cell proliferation in osteoblastic cells [31, 32], lymphocytes [33], keratinocytes [34–36],

vascular smooth muscle cells [37], hepatoma cells [38], and alveolar pneumocytes [39]. On the other hand, a stimulatory influence of PTHrP was noted for a rat carcinoma cell line [40], renal tubular cells [41], and a chondrocytic cell line [42]. However, there has been no report about the function of PTHrP in this regard in human parathyroid glands. From our results, it is tempting to speculate that PTHrP works as a possible suppressor of parathyroid cell growth. Thus, chief cells producing only PTH demonstrated higher PCNA indices in parathyroid hyperplasia than their oxyphilic or transitional oxyphilic counterparts producing PTHrP. Recently, Müller-Höcker et al demonstrated defects of mitochondrial respiratory chain enzymes in oxyphilic nodules in secondary hyperparathyroidism suggestive of cell aging [43]. Therefore, we cannot rule out the possibility that PTHrP-positive cells solely represent a certain subpopulation of aged parathyroid cells and that PTHrP itself is not directly relevant to cell proliferation. In either case, this is the first report showing that chief cells and oxyphil cells represent distinct subgroups that exhibit different proliferative activity in the human parathyroid gland. Further studies are needed to clarify the relationship between the expression of PTHrP and the defects of mitochondrial respiratory chain enzymes or aging. Finally, we should emphasize the superiority of our newly modified method for *in situ* hybridization with digoxigenin-labeled oligonucleotide probes. Two steps of immunologic enhancement were included, the first performed with antibodies and the second with Tyramide amplification. The method proved reliable and sensitive for routine paraffin sections. The positive staining was very clear with low background and allowed a demonstration of the intracellular distribution of mRNA in tissue sections.

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APPENDIX

Abbreviations used in this article are: h, human; mRNA, messenger RNA; PBS, phosphate-buffered saline solution; PBS-B, phosphate-buffered saline solution with bovine serum albumin; PBS-T, phosphate-buffered saline solution with Tween; PCNA, proliferating cell nuclear antigen; PTHrP, parathyroid hormone–related protein; RT, room temperature; SSC, saline-sodium citrate.

REFERENCES

1. Suva LJ, Winslow GA, Wettenhall REH, Hammonds RG, Moseley JM, Diefenbach-Jagger H, Rodda CP, Kemp BE, Rodoriguez H, Chen EY, Hudson PJ, Martin TJ, Wood WI: A parathyroid hormone-related protein implicated in malignant hypercalcemia: Cloning and expression. *Science* 237:893–896, 1987
2. Mangin M, Webb AC, Dreyer BE, Posillico JT, Ikeda K, Weir EC, Stewart AF, Bander NH, Milstone L, Barton DE, Francke U, Broadus AE: Identification of a cDNA encoding parathyroid hormone-like peptide from a human tumor associated with humoral hypercalcemia of malignancy. *Proc Natl Acad Sci USA* 85:597–601, 1988
3. Rosol TJ, Capen CC: Mechanism of cancer-induced hypercalcemia. *Lab Invest* 67:680–702, 1992
4. Orloff JJ, de reddy D, de-Papp AE, Yang KH, Soifer NE, Stewart AF: Parathyroid hormone-related protein as a prohormone: Posttranslational processing and receptor interactions. *Endocr Rev* 15:40–60, 1994
5. Everhart-caye M, Inzucchi SE, Guinness-Henry J, Mitnick MA, Stewart AF: Parathyroid hormone (PTH)-related protein (1–36) is equipotent to PTH (1–34) in humans. *J Clin Endocrinol Metab* 81:199–208, 1996
6. Roskams T, Desmet V: Parathyroid-hormone-related peptides: A new class of multifunctional proteins. *Am J Pathol* 150:779–785, 1997
7. Tsukazaki T, Ohtsuru A, Enomoto H, Yano H, Motomura K, Ito M, Namba H, Iwasaki K, Yamashita S: Expression parathyroid hormone-related protein in rat articular cartilage. *Calcif Tissue Int* 57:196–200, 1995
8. Eggenberger M, Fluhmann B, Muff R, Lauber M, Lichtensteiger W, Hunziker W, Fischer JA, Born W: Structure of a parathyroid hormone/parathyroid hormone-related peptide receptor of the human cerebellum and functional expression in human neuroblastoma SK-N-MC cells. *Brain Res Mol Brain Res* 36:127–136, 1996
9. Li H, Seitz PK, Thomas ML, Selvanayagam P, Rajaraman S, Cooper CW: Widespread expression of the parathyroid hormone-related peptide and PTH/PTHrP receptor genes in intestinal epithelial cells. *Lab Invest* 73:864–870, 1995
10. Drucker DJ, Asa SL, Henderson J, Goltzman D: The parathyroid hormone-like peptide gene is expressed in the normal and neoplastic human endocrine pancreas. *Mol Endocrinol* 3:1589–1595, 1989
11. Hastings RH, Summers-Torres D, Cheung TC, Ditmer LS, Petrin EM, Burton DW, Spragg RG, Li J, Deftos LJ: Parathyroid hormone-related protein, an autocrine regulatory factor in alveolar epithelial cells. *Am J Physiol* 270:L353–L361, 1996
12. Amizuka N, Henderson JE, Hoshi K, Warshawsky H, Ozawa H, Goltzman D, Karaplis AC: Programmed cell death of chondrocytes and aberrant chondrogenesis in mice homozygous for parathyroid hormone-related peptide gene deletion. *Endocrinology* 137:5055–5067, 1996
13. Vasavada RC, Cavaliere C, D'Ercole AJ, Dann P, Burtis WJ, Madlener AL, Zawalich K, Zawalich W, Philbrick W, Stewart AF: Overexpression of parathyroid hormone-related protein in the pancreatic islets of transgenic mice causes islet hyperplasia, hyperinsulinemia, and hypoglycemia. *J Biol Chem* 271:1200–1208, 1996
14. Lee K, Deeds JD, Segre GV: Expression of parathyroid hormone-related peptide and its receptor messenger ribonucleic acids during fetal development of rats. *Endocrinology* 136:453–463, 1995
15. Granberg PO, Cedermark B, Farnebo LO, Hamberger B, Werner S: Parathyroid tumors. *Curr Probl Cancer* 9:1–52, 1985

16. Ikeda K, Weir EC, Mangin M, Dannies PS, Kinder B, Deftos LJ, Brown EM, Broadus AE: Expression messenger ribonucleic acids encoding a parathyroid hormone-like peptide in normal human animal tissues with abnormal expression in human parathyroid adenomas. *Mol Endocrinol* 2:1230–1236, 1988
17. Danks JA, Ebeling PR, Hayman JA, Diefenbach-Jagger H, Collier FM, Grill V, Southby J, Moseley JM, Chou ST, Martin TJ: Immunohistochemical localization of parathyroid hormone-related protein in parathyroid adenoma and hyperplasia. *J Pathol* 161:27–33, 1990
18. Matsushita H, Hara M, Nakazawa H, Shishiba Y, Matuhasi T: The presence of immunoreactive parathyroid hormone-related protein in parathyroid adenoma cells. *Acta Pathol Jpn* 42:35–41, 1992
19. Matsushita H, Hara M, Honda K, Kuroda M, Usui M, Nakazawa H, Hara S, Shishiba Y: Inhibition of parathyroid hormone-related protein release by extracellular calcium in dispersed cells from human parathyroid hyperplasia secondary to chronic renal failure and adenoma. *Am J Pathol* 146:1–8, 1995
20. Matsushita H, Usui M, Hara M, Shishiba Y, Nakazawa H, Honda K, Torigoe K, Kohno K, Kurimoto M: Cosecretion of parathyroid hormone and parathyroid hormone-related protein in human parathyroid adenoma cells. *Am J Pathol* 150:861–871, 1997
21. Almendral JM, Huebsch D, Blundell PA, Macdonald-Bravo H, Bravo R: Cloning and sequence of the human nuclear protein cyclin: Homology with DNA-binding proteins. *Proc Natl Acad Sci USA* 84:1575–1579, 1987
22. Galand P, Degraef C: Cyclin/PCNA immunostaining as an alternative to tritiated thymidine pulse labeling for marking S phase cells in paraffin sections from animal and human tissues. *Cell Tissue Kinet* 22:383–392, 1989
23. Fukuda N, Tanaka H, Tominaga Y, Fukagawa M, Kurokawa K, Seino Y: Decreased 1,25-dihydroxyvitamin D_3 receptor density is associated with a more severe form of parathyroid hyperplasia in chronic uremic patients. *J Clin Invest* 92:1436–1443, 1993
24. Danks JA, McHale JC, Clark SP, Chou ST, Scurry JP, Ingleton PM, Martin TJ: In situ hybridization of parathyroid hormone-related protein in normal skin, skin tumors, and gynecological cancers using digoxigenin-labeled probes and antibody enhancement. *J Histochem Cytochem* 43:5–10, 1995
25. Hacker GW, Hauser-Kronberger C, Zehbe I, Su H, Scheichl A, Dietze O, Tubbs R: In situ localization of DNA and RNA sequences: Super-sensitive in situ hybridization using streptoavidin-nanogold™-silver staining: Minireview, protocols and possible applications. *Cell Vision* 4:54–65, 1997
26. Hochi Y, Kido T, Nogawa K, Kito H, Shaikh ZA: Dose–response relationship between total cadmium intake and prevalence of renal dysfunction using general linear models. *J Appl Toxicol* 15:109–116, 1995
27. Matsushita H: Pathology of the parathyroid glands, in *Bloodworth's Endocrine Pathology* (3rd ed), edited by Lechago J, Gould VE, Baltimore, Williams & Wilkins, 1997, pp 249–272
28. Brown AJ, Dusso A, Lopez-Hilker S, Lewis-Finch J, Grooms P, Slatopolsky E: 1,25-(OH)2D receptors are decreased in parathyroid glands from chronically uremic dogs. *Kidney Int* 35:19–23, 1989
29. Usdin TB, Gruber C, Bonner TI: Identification and functional expression of a receptor selectively recognizing parathyroid hormone, the PTH2 receptor. *J Biol Chem* 270:15455–15458, 1995
30. Lanske B, Karaplis AC, Lee K, Luz A, Vortkamp A, Pirro A, Karperien M, Defize L, Ho C, Mulligan RC, Abou-Samra AB, Juppner H, Segre GV, Kronenberg HM: PTH/PTHrP receptor in early development and Indian hedgehog-regulated bone growth. *Science* 273:663–666, 1996
31. Kano J, Sugimoto T, Fukase M, Fujita T: The activation of cAMP-dependent protein kinase is directly linked to the inhibition of osteoblast proliferation (UMR-106) by parathyroid hormone-related protein. *Biochem Biophys Res Commun* 179:97–101, 1991
32. Martinez ME, Garcia-Ocana A, Sanchez M, del Medina S, del-Campo T, Valin A, Sanchez-Cabezudo MJ, Esbrit P: C-terminal parathyroid hormone-related protein inhibits proliferation differentiation human osteoblast-like cells. *J Bone Miner Res* 12:778–785, 1997
33. Adachi N, Yamaguchi K, Miyake Y, Honda S, Nagasaki K, Akiyama Y, Adachi I, Abe K: Parathyroid hormone-related protein is a possible autocrine growth inhibitor for lymphocytes. *Biochem Biophys Res Commun* 166:1088–1094, 1990
34. Holick MF, Ray S, Chen TC, Tian X, Persons KS: A parathyroid hormone antagonist stimulates epidermal proliferation and hair growth in mice. *Proc Natl Acad Sci USA* 91:8014–8016, 1994
35. Kaiser SM, Sebag M, Rhim JS, Kremer R, Goltzman D: Antisense-mediated inhibition of parathyroid hormone-related peptide production in a keratinocyte cell line impedes differentiation. *Mol Endocrinol* 8:139–147, 1994
36. Kremer R, Woodworth CD, Goltzman D: Expression and action of parathyroid hormone-related peptide in human cervical epithelial cells. *Am J Physiol* 271:C164–C171, 1996
37. Jiang B, Morimoto S, Fukuo K, Yasuda O, Chen S, Ogihara T: Role of parathyroid hormone-related protein in the proliferation of vascular smooth muscle cells. *Miner Electrolyte Metab* 21:157–160, 1995
38. Li H, Seitz PK, Selvanayagam P, Rajaraman S, Cooper CW: Effect of endogenously produced parathyroid hormone-related peptide on growth of a human hepatoma cell line (Hep G2). *Endocrinology* 137:2367–2374, 1996
39. Hastings RH, Summers-Torres D, Yaszay B, Lesueur J, Burton DW, Deftos LJ: Parathyroid hormone-related protein, an autocrine growth inhibitor of alveolar type II cells. *Am J Physiol* 272:L394–L399, 1997
40. Benitez-Verguizas J, Esbrit P: Proliferative effect of parathyroid hormone-related protein on the hypercalcemic Walker 256 carcinoma cell line. *Biochem Biophys Res Commun* 198:1281–1289, 1994
41. Garcia-Ocana A, De Miguel F, Penaranda C, Albar JP, Sarasa JL, Esbrit P: Parathyroid hormone-related protein is an autocrine modulator of rabbit proximal tubule cell growth. *J Bone Miner Res* 10:1875–1884, 1995
42. Henderson JE, He B, Goltzman D, Karaplis AC: Constitutive expression of parathyroid hormone-related peptide (PTHrP) stimulates growth and inhibits differentiation of CFK2 chondrocytes. *J Cell Physiol* 169:33–41, 1996
43. Müller-Höcker J, Aust D, Napiwotzky J, Munscher C, Link TA, Seibel P, Schneeweiss SG, Kadenbach B: Defects respiratory chain in oxyphil chief cells normal parathyroid in hyperfunction. *Hum Pathol* 27:532–541, 1996