Antitumor effect of parathyroid hormone-related protein neutralizing antibody in human renal cell carcinoma in vitro and in vivo

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Functional inactivation of the von Hippel-Lindau (VHL) tumor suppressor gene occurs in 40–80% of human conventional renal cell carcinomas (RCCs). We showed recently that VHL-deficient RCCs expressed large amounts of parathyroid hormone-related protein (PTHrP), and that PTHrP, acting through the PTH1 receptor (PTH1R), plays an essential role in tumor growth. We also showed that PTHrP expression is negatively regulated by the VHL gene products (pVHL). Our goal was to determine whether blocking the PTHrP/PTH1R system might be of therapeutic value against RCC, independent of VHL status and PTHrP expression levels. The antitumor activity of PTHrP neutralizing antibody and of PTH1R antagonist were evaluated in vitro and in vivo in a panel of human RCC lines expressing or not pVHL. PTHrP is upregulated compared with normal tubular cells. In vitro, tumor cell growth and viability was decreased by up to 80% by the antibody in all cell lines. These effects resulted from apoptosis. Exogenously added PTHrP had no effect on cell growth and viability, but reversed the inhibitory effects of the antibody. The growth inhibition was reproduced by a specific PTH1R antagonist in all cell lines. In vivo, the treatment of nude mice bearing the Caki-1 RCC tumor with the PTHrP antibody inhibited tumor growth by 80%, by inducing apoptosis. Proliferation and neovascularization were not affected by the antiserum. Anti-PTHrP treatment induced no side effects as assessed by animal weight and blood chemistries. Current therapeutic strategies are only marginally effective against metastatic RCC, and adverse effects are common. This study provides a rationale for evaluating the blockade of PTHrP signaling as therapy for human RCC in a clinical setting.

Introduction

Conventional renal cell carcinoma (RCC) represents 75% of all renal cell carcinomas. In the last 20 years, the incidence rate of RCC has increased by >43% and the death rate by 16% (1). Each year, 200 000 patients are diagnosed worldwide with this neoplasm, resulting in an estimated 100 000 deaths (1,2). Approximately 20–30% of patients have metastases at initial presentation. Of the patients who present with apparent localized disease and are treated with radical or conservative nephrectomy, 50–60% will develop metastatic disease within 5–10 years. Metastatic RCC is resistant to radiotherapy and to systemic chemotherapy or biologic therapy (3,4). At present, interleukin-2 is the systemic therapy of choice for metastatic disease, but the overall response rate is only 15%. Interleukin-2 does show the most favorable immunotherapeutic response, with complete response achieved in 5% of patients, and long-term, relapse-free survival in responding patients greater than for any other therapeutic agent. The 5-year survival rate is 70% for localized RCC, but only 5% in the metastatic group. Since therapy for advanced renal cancer clearly remains inadequate, new therapeutic options for RCC are urgently needed.

Inactivation of the von Hippel-Lindau (VHL) tumor suppressor gene is responsible for hereditary human VHL syndrome. Affected individuals are predisposed to develop tumors in a variety of organs, including the kidney (5,6). The VHL gene is also inactivated in up to 80% of sporadic RCC (5–7). Linkage analysis of VHL kindreds mapped the VHL locus to chromosome 3p25–26 in humans (5). Inactivation of the VHL gene is a consequence of somatic mutations or 3p loss of heterozygosity in 30–60% of RCC patients. Hypermethylation and inactivation of the VHL promoter occurs in an additional 10–20% of patients (7–9). The VHL gene is believed to have gatekeeper properties in proximal tubular cells from which RCC originates, not only because VHL patients may have hundreds of individual discrete tumors in the kidney (10), but also because the reintroduction of the VHL gene in the VHL-deficient RCC line 786-0 suppresses their tumorigenicity in nude mice (11).

The protein products of the human VHL gene (pVHL) are VHL (1–213) and VHL (54–213), the latter being generated by internal translation initiation from an in-frame methionine at codon 54 (12). They have identical biological properties. pVHL is a component of an E3 ubiquitin ligase complex that targets the α subunits of HIF-1 and HIF-2 (hypoxia-induced factors) transcription factors for destruction in the presence of oxygen. As a consequence, tumor cells lacking pVHL overexpress HIF target genes of the metabolic, angiogenic and growth factor families, including VEGF, PDGF or TGF-β (12). pVHL is thus involved in cell cycle control, cell differentiation and death, regulation of extracellular pH, extracellular matrix formation and angiogenesis, all contributing to carcinogenesis. HIF-1 and -2 α proteins are frequently present in hypoxic regions of solid tumors. As a consequence of VHL deficiency, high levels of these factors are found in RCC, regardless of the oxygen tension. It follows that many of the hypoxia-responsive genes are upregulated in the majority of RCC (13). For example, RCCs are highly vascularized due to...
the overexpression of VEGF and TGF-β. Alterations of the VHL gene are considered to be an early event in RCC tumorigenesis. This is evidenced by the presence of VHL gene mutations or silencing in early pT1 stage of RCC (7.9). For example, Brauch et al. (9), using > 150 pT1 to pT3 RCC, have identified VHL mutations or silencing in 45% of RCC. Interestingly, in their study, VHL alterations, although present at all stages, were significantly associated with the pT3 stage. They hypothesized that the large range of somatic VHL alterations rates reported in the literature (40–80%, see above) might be related not only to the ethnic origin of the population studied, but also to the number of pT3 tumors included in the analyzed patient samples. Thus, depending on the population studied, 20–60% of RCC have normal VHL expression, and low levels of HIFs. This indicates that the VHL gene is not sufficient to suppress tumorigenesis and that damage to other genes is probably involved in RCC initiation and development, at least in RCC containing normal VHL.

Parathyroid hormone-related protein (PTHRp), identified as the tumor-derived agent responsible for humoral hypercalcemia of malignancy (HHM) (14), is a polypeptide derived from normal and malignant cells. It regulates cell growth, differentiation and death (15). An early study by Burton et al. (16) showed that PTHrP regulates the proliferation of the RCC line, SKRC-1. We have recently shown (17) in three VHL-deficient human RCC lines (786-0, UOK-126 and UOK-128 cells) expressing high levels of PTHR that blocking PTHR with antibodies, or antagonizing its receptor—the parathyroid hormone-1 receptor (PTH1R), inhibits tumor growth in vitro by promoting cell apoptosis. In nude mice subcutaneously implanted with 786-0 cells, anti-PTHrP treatment induced complete regression of the implanted tumors in 70% of mice, inducing cell death. In the same study, we also demonstrated that the VHL suppressor gene negatively regulates PTHrP expression at the post-transcriptional level. These studies identified PTHrP as a critical growth factor for VHL-deficient RCC expressing high levels of PTHR and suggested that PTHrP might be a new therapeutic target in patients with VHL-deficient tumors.

In the current study, our objective was to define whether blocking the PTHrP/PTH1R system might also have broader therapeutic application against RCC regardless of the VHL status and regardless of PTHrP expression levels. Using a panel of RCC lines either expressing or not expressing PVL, we provide evidence that anti-PTHrP therapy may have broad therapeutic application against human RCC.

Materials and methods

**In vitro studies**

**Cells and cell culture.** Normal human (b) proximal tubular cell line HK-2 (a gift from Dr C. Silve, Paris, France) and human RCC lines 786-0, UOK-126, UOK-128, A498, ACHN, Caki-1 and Caki-2 were maintained in DMEM medium (Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum. All cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA) except UOK-126 and UOK-128 that were generously given to us by Dr P. Anglard (Institut de Génétique et de Biologie Moléculaire et Cellulaire, Illkirch, France) (18). The cell lines are derived from human sporadic RCC, and are either VHL-deficient (786-0, UOK-126, UOK-128 and A498 cell lines) or encode active PVL (ACHN, Caki-1 and Caki-2 cell lines). Unless specified otherwise, cells were used at 70–80% confluence.

**RNA extraction and real-time quantitative RT–PCR analysis of PTHR and PTH1R.** Total RNA was extracted from cultured cells using TRIzol (Invitrogen), according to the manufacturer’s protocol. The hPTHrP and hPTH1R cDNAs were amplified using the LightCycler-FastStart DNA Master SYBR Green kit (Roche Diagnostics, Meylan, France). In addition, a standard curve was obtained for hGAPDH by serial dilutions of mixed cDNA samples. PCRs were performed in 20 μl mix containing 4 mM MgCl₂, 0.5 μM each primer set, 0.2 μM SYBR Green probe, 0.4 μM hybridization probe, 1 × FastStart buffer, 0.2 units of polymerase, dNTPs, buffer) and 2 μl cDNA (50 ng). PCRs for hPTHrP, hPTH1R and hGAPDH were as follows: 95°C for 10 min followed by 40 cycles at 95°C for 10 s, 60°C for 5 s and 72°C for 7 s (hPTHrP), 18 s (hPTH1R) or 16 s (hGAPDH). cDNA was replaced by PCR-grade water as a negative control. Each sample was run twice and quantified with the LightCycler analysis software according to the manufacturer’s protocol (Roche Diagnostics). A Relative expression of hPTHrP and hPTH1R in samples was calculated by quantifying hPTHrP and hPTH1R levels normalized to hGAPDH, and results in RCC lines were expressed as fold increase above the overexpression of hPTHrP and hPTH1R. Unless specified otherwise, cells were used at 70–80% confluence.

**Cell death analysis.** RCCs were seeded in 96-well plates (20 000 cells/well) and were exposed 48 h later to the N-terminal PTHrP-neutralizing antibody (Bachem) or non-immune rabbit IgG (Sigma) at the concentrations indicated in the figures or figure legends. Test substances were affinity-purified polyclonal rabbit antibody directed against hPTHrP (Eurogentec, Angers, France). A polyclonal mouse anti-β actin antibody (Sigma-Aldrich, St Quentin Fallavier, France) was used for visualization of protein gel loading. Visualization was performed using the ECL method (17).

**Cell proliferation.** RCC proliferation was assessed by counting adherent cells, as described (17). RCCs were seeded in 24-well plates (20 000 cells/well), grown for 48 h and then treated for 48 h with test substances at the concentrations indicated in the figures or figure legends. Test substances were affinity-purified polyclonal rabbit antibody directed against the N-terminal region of PTHrP (Bachem, Voisins-le-Bretonneux, France), non-immune rabbit IgG (Sigma) which served as control for PTHrP antibody experiments, (Tyr36)-PTHrP(1–36) (Bachem) or the PTHrP antagonist (Asn10, Leu11, D-Trp12)-PTHrP(7–34)amide (Bachem). All peptides were dissolved in 10−3 M HCl containing 0.1% bovine serum albumin (BSA) at a final concentration of 250 μM and stored at −70°C in 25 μl aliquots. Before use, peptides were further diluted to the desired concentration in 0.9% NaCl containing 0.1% BSA (diluent).

**MTT cell viability measurements.** This assay is based on the conversion of the yellow tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to purple formazan crystals by metabolically active cells and provides a quantitative estimate of viable cells (19). RCCs were seeded and treated as above for cell proliferation measurements with test substances at the concentrations indicated in the figures or figure legends. After 48 h of treatment (17), MTT (Sigma) at 0.5 mg/ml (final concentration) was added to each well for 4 h at 37°C. After this period, 1 ml of a solubilization solution (10% SDS in 0.01 M HCI) was added to each well, and plates were incubated for 24 h at 37°C. Spectrophotometric absorbance was measured at 570 nm (reference 690 nm) on 200 μl aliquots using a microplate reader.

**Implantation.** All animal studies were in compliance with French animal use regulations. Male Swiss Nu/Nu nude mice (7-week-old) (Ifa-Credo, St Germain sur l’Arbresle, France) were given injections of 10 million Caki-1 cells subcutaneously into the skin of the back (n = 20). The tumor take rate was 85%. Four weeks after injection, tumors had grown to a volume of 161 ± 27.4 mm³. The tumor-bearing mice were then separated into two groups of eight and nine animals of comparable mean tumor volume.
One group (n = 8) received daily intraperitoneal injection of 40 μg of non-immune rabbit IgG (Sigma). The other group (n = 9) received daily intraperitoneal injection of 40 μg of the N-terminal PTHrP neutralizing antibody (Bachem). We have shown in our previous study in nude mice bearing VHL-defective RCC tumors (17) that such treatment induced tumor regression. Tumor size was measured using calipers every 2–3 days (17). At the end of the treatment period (19 days), mice were euthanized with ether and blood was harvested for the measurement of plasma electrolytes, creatinine and PTHrP concentrations (Laboratoire de Biochimie Générale et Spécialisée). Tumors were then removed, fixed in formalin and paraffin-embedded. Sections of 4 μm thickness were used. Some sections were stained with hematoxylin–eosin and others with the N-terminal PTHrP neutralizing antibody or an affinity purified polyclonal rabbit antibody directed against the PTH1R (Eurogentec) as we described previously (17).

**Proliferative/apoptotic index.** The proliferative index was determined by staining tumor sections with a mouse monoclonal anti-hKi67 antibody (Dako, Trappes, France), as previously described (17). An apoptosis detection kit, based on the TUNEL method (Roche Diagnostics), was used to measure the apoptotic index on sections. To determine both indices the number of total and stained cells in 15 fields (0.25 mm² each) were quantified in a blinded fashion by an experienced urologic histopathologist (Dr Véronique Lindner, Department of Pathology, University Hospital, Strasbourg, France). Both indices were expressed as a percentage of stained cells to total cells.

**Factor VIII and microvascular density.** Tumor sections were stained for endothelial cells using a rabbit polyclonal anti-human factor VIII antibody (Dako), as previously described (17). Microvessel density was determined in a blinded manner as described above by counting, for each tumor, both vessel intersecting points and the total number of vessels in 4–5 fields (0.25 mm² each) showing the highest vascular density.

**Statistics.** All values are expressed as mean ± SEM. Values were compared using multifactorial analysis of variance followed by the Student–Newman–Keuls’s test for multiple comparisons. P < 0.05 was considered significant.

**Results**

**PTHrP and PTH1R expression in RCC lines**

Using real-time quantitative PCR analysis, we detected PTHrP (Figure 1A, upper panel) and the PTH1R transcripts (Figure 1A, lower panel) in all cell lines. RCC lines showed higher PTHrP expression than HK-2 cells. Interestingly, in VHL-deficient A498 cells, PTHrP levels were similar to those observed in wild-type VHL-expressing RCC lines. PTH1R expression was not higher in all RCC lines compared with HK-2 cells and did not show any difference in relation with the VHL status of the cells. Similar results were obtained with cells grown in the absence of serum (data not shown). This pattern of expression was confirmed at the protein level by IRMA for PTHrP (Figure 1B, upper panel) and western blot for the PTH1R (Figure 1B, lower panel).

Thus, all RCC lines employed constitutively express both PTHrP and the PTH1R. In addition, PTHrP expression was significantly higher in RCCs compared with normal cells, regardless of the VHL status of the cells.

**Effect of PTHrP/PTH1R blockade on RCC growth and survival in vitro**

Since PTHrP is overproduced by all RCC lines studied here (and most described in the literature), and because our previous studies suggested that either a large N-terminal-containing fragment or full-length PTHrP is required to induce PTH1R-dependent stimulation of RCC growth (17), we choose to expose cells to various concentrations of a PTHrP antibody recognizing all N-terminal-containing PTHrP peptides, including full-length PTHrP or to a potent and specific PTH1R antagonist, i.e. (Asn¹⁰, Leu¹¹, D-Trp¹²)PTHrP(7–34)amide (20). Cells were then analyzed for growth, viability and apoptosis. The antibody decreased cell number (Figure 2A) and viability (Figure 2B) in a concentration-dependent manner and increased cytoplasmic histone-associated DNA fragment specific for apoptosis (Figure 2C) in all cell lines tested, including HK-2 cells. These results suggest that endogenous PTHrP acts on RCC growth and survival by inhibiting cell apoptosis. In all cell lines, except Caki-2 cells, growth was decreased by 40–80% at the highest concentration use of the antibody. Increasing PTHrP antibody concentrations to 50 μg/ml also decreased cell growth in Caki-2 cells by 80% (data not shown). PTHrP(1–36) at 10⁻⁶ M alone did not affect cell number (Figure 3A, left panel) and viability (Figure 3A, right panel), but reversed or significantly decreased the inhibitory effect of the PTHrP antibody on cell number (Figure 3B, left panel) and viability (Figure 3B, right panel) in all cell lines tested. These results demonstrate that the effect of the antibody relates specifically to PTHrP blockade. The absence of effect of PTHrP on cell growth is reminiscent of what we had observed previously in VHL-deficient RCC lines (17). Growth inhibition was reproduced by blocking the PTH1R in all cell lines tested, including HK-2 cells. Indeed, the PTH1R antagonist at 10⁻⁶ M decreased both cell number (by 20–47%) (Figure 4A) and cell viability (by 15–60%) (Figure 4B) by inducing cell apoptosis (Figure 4C). This concentration of the PTH1R antagonist was chosen since it had maximally inhibited cell growth in our previous study on VHL-deficient RCCs (17). These results further argue the auto/paracrine nature of the PTHrP-induced cell survival.

Taken together, these results show that blockade of the PTHrP/PTH1R signaling pathway induces tumor cell death through apoptosis, regardless of the VHL status of the cells and PTHrP expression level.

**Effect of PTHrP blockade on growth of wild-type VHL-expressing RCC tumors in nude mice**

To evaluate the biological significance of PTHrP blockade in wild-type VHL-expressing RCC, we used the xenograft athymic mouse model. Based on our previous studies in nude mice bearing VHL-deficient RCC tumors (17), and in order to compare the current studies with our prior studies, mice were treated with daily intraperitoneal injections of 40 μg/mouse of the PTHrP antibody or the non-immune IgG, as detailed in Materials and methods, for a period of 19 days. Treatment with the PTHrP antibody significantly inhibited RCC tumor growth by 80% in all animals compared with IgG-treated animals (Figure 5A and B). No histopathological differences were observed between either group on hematoxylin–eosin staining (data not shown). Areas of necrosis were present in the center of all tumors. Tumors obtained from IgG-treated mice strongly expressed both the PTHrP (Figure 5C, upper left panel) and the PTH1R (Figure 5C, upper right panel). Obvious staining for PTHrP and PTH1R was observed in the cytoplasm of all cells and in the nuclei of many cells. No apparent differences were noted from mice treated with the PTHrP antibody in the staining pattern of tumors for PTHrP (Figure 5C, lower left panel) and PTH1R (Figure 5C, lower right panel). The proliferative index was not different between tumors obtained from PTHrP antibody-treated mice (Figure 6A, upper and lower right panels) and IgG-treated mice (Figure 6A, upper and lower left panels). Quantification is given on the histogram (Figure 6A). In marked contrast, there was a significant increase in the apoptotic index in tumors obtained from PTHrP antibody-treated mice (Figure 6B, upper and lower right panels) compared with IgG-treated mice (Figure 6B,
Fig. 1. PTHrP and PTH1R expression in RCC lines and in HK-2 cells in vitro. (A) Real-time quantitative PCR analysis of hPTHrP (top) and PTH1R (bottom) mRNA in the various cell lines. GAPDH was used as the housekeeping gene for quantification of both transcripts as detailed in Materials and methods. PTHrP and PTH1R expression in RCC lines were expressed as fold increase above HK-2 cells set to 1. Results are shown as mean ± SEM, n = 3–12 and n = 3–7 for PTHrP and PTH1R quantifications, respectively; *P < 0.05; **P < 0.01 from HK-2 cells. (B) IRMA measurements of immunoreactive PTHrP in conditioned medium of each cell line (top). Results are shown as mean ± SEM, n = 4; **P < 0.01 from HK-2 cells. Western blots of RCC lysates incubated with antibodies against the hPTH1R and β-actin (bottom). Shown are representative radiographs of at least three independent experiments.
Fig. 2. Effect of PTHrP blockade on RCC growth in vitro. Effects of the PTHrP antibody at various concentrations on cell number (A), cell viability (B) and cytoplasmic histone-associated DNA fragments (C) in all RCC lines and in HK-2 cells. IgG, non-specific IgG, 5 μg/ml. The effects in each cell line have been compared with cells treated in control (Ctl) using the vehicle (pure water) that has been used to dissolve and dilute PTHrP antibody and IgG. For the analysis of the effects of the various treatments on cell number (A) and cell viability (B) the control has been set to 100%. For the analysis of cell apoptosis (C), the control (representing basal apoptosis) has been set to 1 according to the manufacturer’s protocol. Results were expressed either as percentages from control (A and B) or as fold increase above control level (C) Results are shown as mean ± SEM, n = 4–16 for cell number and MTT and n = 3–7 for histone-associated DNA fragments. *P < 0.05; **P < 0.01 from the corresponding control.
upper and lower left panels). Quantification is given on the histogram (Figure 6B) showing that PTHrP antibody treatment increased the apoptotic index by 35% (P < 0.05). Tumor neovascularization shows no difference between antibody-treated mice (Figure 6C, upper and lower right panels) and IgG-treated mice (Figure 6C, upper and lower left panels). Quantification of tumor neovascularization is given on the histograms representing the number of vessel intersecting points (Figure 6C, upper histogram) and the total number of vessels as a function of surface area (Figure 6C, lower histogram).

PTHrP concentrations in plasma were 0.6 ± 0.1 pM (n = 6) in nude mice without tumor. PTHrP concentrations were significantly increased to 2.7 ± 0.3 pM (P < 0.001) in Caki-1 tumor-bearing mice. Plasma concentrations of electrolytes, creatinine and albumin, as well as the body weight showed no difference between animals treated in control or with the PTHrP antibody over the period of treatment (Table I). Although circulating PTHrP was elevated in tumor-bearing mice, plasma calcium levels were not different from normal nude mice (data not shown). This result is similar to the ones observed in our previous study (17), and suggests that the increase in PTHrP circulating level obtained in these mice was not high enough to affect calcium homeostasis.

These results demonstrate that blocking the PTHrP signaling pathway is effective against progression of wild-type VHL expressing RCC tumors through an increase in cell apoptosis. In addition, anti-PTHrP treatment has no apparent toxicity.

**Discussion**

Studies in human breast, prostate and lung cancers strongly suggest that PTHrP behaves as a factor that regulates tumor growth and invasion (21–23). Our previous studies on human RCC revealed a critical role for this factor in the growth of these tumors as well (17). Indeed, using three different human RCC lines deficient in VHL tumor suppressor gene and expressing high levels of PTHrP, we showed that PTHrP participates in tumor growth and that PTHrP expression is negatively regulated at the level of mRNA stability by pVHL. These results suggest the potential for anti-PTHrP treatment therapy in human directed against VHL-deficient RCC expressing high levels of PTHrP. Since normal VHL expression is documented in 20–60% of patients (see Introduction), the goals of the current study were to explore the PTHrP/PTH1R status in a panel of RCCs expressing or not expressing pVHL, and to determine whether blocking the PTHrP/PTH1R
system might be of therapeutic value against RCC independent of the VHL status and of PTHrP expression levels.

In the current study, we report that in addition to VHL-deficient RCCs, PTHrP expression is also significantly increased in RCCs that express pVHL. Thus, PTHrP appears to be upregulated in human RCCs whether they express pVHL or not. In two independent previous studies, PTHrP was shown to be overexpressed in 95% of human RCC (24,25). In these two studies, a total of 40 RCC tumors were investigated by immunohistochemistry either for N-terminal PTHrP (ClI IgG, black circles) or the antibody against the N-terminus of PTHrP (PTHrP antibody, open circles). Results are shown as mean ± SEM, n = 8 and 9 tumors in the IgG group and PTHrP antibody group, respectively; **P < 0.01 from ClI IgG. In this study, tumor growth was inhibited by 80% in mice treated with the PTHrP antibody. (B) Photographs show the implanted tumors in a representative mouse at the first day of drug injection (left, day 0), in a representative IgG-treated mouse (middle, day 19) and in a representative mouse treated with the PTHrP antibody (right, day 19). (C) Sections of tumors from IgG- (upper panels) or PTHrP antibody (lower panels) -treated mice shown in (A) immunostained with an antibody against either PTHrP (left panels) or the PTH1R (right panels). A strong staining is seen in the cytoplasm of tumor cells. Staining of PTHrP and of the PTH1R is also obvious in nuclei in many cells throughout the tumors (magnification 400×).
PTHrP expression in RCC. Although pVHL plays an important role in regulating PTHrP expression level (17), other factors are obviously involved. In accord with this hypothesis, in our previous studies, the reintroduction of the VHL gene in VHL-deficient RCCs decreased PTHrP levels by only 50%, leaving them far higher than levels in normal cells (17). A variety of growth and angiogenic factors have been shown to regulate PTHrP expression, including EGF, PDGF, TGF-α.

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**Fig. 6.** Histopathological analysis of tumors from IgG- and PTHrP antibody-treated mice. (A) Left, tumor sections of IgG- (upper and lower left panels) or PTHrP antibody (upper and lower right panels)-treated mice immunostained with antibody against Ki67 (black nuclei; upper panels, magnification 200×; lower panels, magnification 400×). Right, quantification of Ki67 staining (proliferative index). Results are shown as mean ± SEM, n = 8–9. (B) Left, tumor sections of IgG- (upper and lower left panels) or PTHrP antibody (upper and lower right panels)-treated mice immunostained for DNA fragmentation (TUNEL) (black nuclei; upper panels, magnification 200×; lower panels, magnification 400×). Right, quantification of TUNEL staining (apoptotic index). Results are shown as mean ± SEM, n = 8–9; *P < 0.05 from IgG-treated mice. Anti-PTHrP treatment induced cell apoptosis. (C) Left, tumor sections of IgG- (upper and lower left panels) or PTHrP antibody (upper and lower right panels)-treated mice immunostained for factor VIII (upper panels, magnification 200×; lower panels, magnification 400×). Right, upper histogram, quantification of crossing vessels intersecting points per surface area (0.25 mm²). Results are shown as mean ± SEM, n = 8–9. Right, lower histogram, quantification of vessels per surface area (0.25 mm²). Results are shown as mean ± SEM, n = 8–9.
of each treatment. Phosphate, potassium, creatinine, albumin and urea in plasma were measured. Animal weights were measured at the beginning (Day 0) and at the end (Day 19) of each treatment.

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<tr>
<th>Group</th>
<th>Calcium (mM)</th>
<th>Sodium (mM)</th>
<th>Phosphate (mM)</th>
<th>Potassium (mM)</th>
<th>Creatinine (µM)</th>
<th>Albumin (g/l)</th>
<th>Urea (mM)</th>
<th>Weight g (Day 0)</th>
<th>Weight g (Day 19)</th>
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<tr>
<td>Control IgG (n = 8)</td>
<td>2.7 ± 0.02</td>
<td>168.13 ± 1.89</td>
<td>3.01 ± 0.09</td>
<td>5.45 ± 0.26</td>
<td>19.5 ± 0.78</td>
<td>10.06 ± 1.99</td>
<td>4.1 ± 0.4</td>
<td>32.15 ± 0.71</td>
<td>32.61 ± 0.9</td>
</tr>
<tr>
<td>PTHrP antibody (n = 9)</td>
<td>2.44 ± 0.24</td>
<td>166.75 ± 2.2</td>
<td>2.87 ± 0.16</td>
<td>5.55 ± 0.19</td>
<td>20.75 ± 1.62</td>
<td>11.78 ± 2.06</td>
<td>4.11 ± 0.35</td>
<td>31.61 ± 0.56</td>
<td>31.84 ± 0.44</td>
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Mice treated for 19 days in control (IgG) or with the PTHrP antibody were anesthetized and the blood was collected. Concentrations of calcium, sodium, phosphate, potassium, creatinine, albumin and urea in plasma were measured. Animal weights were measured at the beginning (Day 0) and at the end (Day 19) of each treatment.

and -β and VEGF in different cell types (14,26–31). Since these factors and/or their receptor are also up-regulated in human RCC and participate in RCC tumorigenesis (1,2,32,33), it is tempting to hypothesize that one or more of these factors participate (s) in the control of PTHrP expression in RCC. However, these factors and/or their receptors are also negatively regulated either at the transcriptional or post-transcriptional level, or both, by pVHL, making their potential involvement in the regulation of PTHrP expression in RCC complex (12,33–36).

The fact that PTHrP is constitutively upregulated in RCC implies that factors, such as oncogenes, that regulate its expression are present in RCC. Alternatively, tumor suppressor factors that negatively regulate PTHrP expression might be repressed in RCC. The identity of these additional factors has not been investigated. In addition to a variety of genetic changes, oncogenes or proto-oncogenes such as c-met (hepatocyte growth factor/scatter factor receptor), C-erbB1 (EGF receptor), c-jun and members of the ras-family have been implicated in RCC tumorigenesis (37), but their possible relation with PTHrP expression remains uncertain. Rare kindreds with familial RCC not linked to the VHL tumor suppressor gene have been described, suggesting that additional familial RCC susceptibility genes exist (37). One possible candidate is p53 tumor suppressor. RCC tumors rarely acquire p53 mutation, although p53 functions are repressed in RCC. However, these factors and/or their receptor are also up-regulated in RCC tumorigenesis (12,33–36).

Our previous results (17) suggested that the secreted PTHrP species which acts as a survival factor for RCC is most likely an N-terminal-PTHrP fragment, and that it acts through the PTH1R. For this reason we performed experiments with a PTHrP antibody recognizing all N-terminal-containing PTHrP species. Using three different methods, we show that PTHrP acts on RCC growth by preventing cells from entering the apoptotic pathway. The inhibitory effect of the PTHrP antibody on cell growth was blocked or significantly inhibited in all cell lines by exogenous PTHrP(1–36), demonstrating that the effect of the antibody relates specifically to PTHrP blockade. Interestingly, high concentrations of exogenous PTHrP(1–36) did not affect cell growth in all cell lines. These data are reminiscent of what we observed in our previous study on VHL-deficient RCC lines expressing high levels of PTHrP (17), and further suggest that all RCC lines secrete PTHrP above a maximally effective level. Finally, using a potent, specific competitive PTH1R antagonist, we show that PTHrP induces tumor cell survival in all cell lines tested through its interaction with the PTH1R. The fact that PTHrP acts as a survival factor for RCC irrespective of the VHL status of the tumor suggest that PTHrP acts so through a common pathway in cells with active or inactive VHL. Interestingly, Caki-2 cells were more resistant to the inhibitory effect of the antibody, despite the fact that these cells produce a similar amount of immunoreactive PTHrP as some of the other cell lines that were more sensitive to the antibody. Since PTH1R expression is also low in this cell line, one possibility is that the PTHrP/PTH1R system is less active in these cells. Another possibility is that PTHrP acts through a signaling pathway defective in this cell line. The molecular mechanism by which PTHrP induces cell survival in RCC tumors is currently under investigation in our laboratory.

In the present study, we used the same dose and protocol for injection of the PTHrP antibody in mice bearing Caki-1 tumor as the one used previously in mice bearing the VHL deficient renal epithelial cells NRK-52E of proximal origin and human proximal tubule cells HK-2 in culture (45–47). Using either exogenous PTHrP fragments or specific PTHrP neutralizing antibodies, it has been demonstrated that PTHrP is a potent mitogenic factor for proximal tubule cells both in vitro and in vivo (45–47). Our own results described here in HK-2 cells confirm the results obtained by Garcia-Ocana et al. (47) in the same cell line. Our data further suggest that PTHrP participates in the growth of this cell type in vitro through the inhibition of cell apoptosis. PTHrP was shown to increase DNA synthesis in proximal tubule cells through cAMP and PKC-dependent pathways (48) and has been suggested to participate in an autocrine way in the regenerative (protective) and/or damage processes following renal injuries, including renal ischemia, tubulo-interstitial nephropathy, chronic and acute renal failure and glomerulonephritis (49).
786-0 tumor (17), i.e. 40 μg/mouse daily. Although the treatment was very efficient in inhibiting Caki-1 tumor progression, we did not observe the tumor regression in Caki-1 tumors, previously observed in 786-0 tumors. Since Caki-1 cells express lower levels of PTHrP than 786-0 cells, the lower efficiency of the PTHrP antibody on Caki-1 tumors was surprising. However, this result is corroborated with the data observed in vitro using the PTHrP neutralizing antibody. Thus, it is tempting to suggest that the lower efficiency of the PTHrP antibody on Caki-1 tumor growth results from a PTHrP/PTH1R system being less central to cell survival in this cell line. However, we cannot rule out the possibility that increasing the dose of the PTHrP antibody would have led to regression of these tumors as well. Unfortunately, it was not possible to increase the dose for cost reasons. Similarly, although it could have been of value to study a larger panel of RCC tumors in vivo to evaluate the efficacy of antibody blockade for a number of tumors, this was not possible. Another finding that contrasts somehow with our previous results is the lack of effect on tumor neovascularization. Indeed in our previous study, anti-PTHrP/PTH1R treatment slightly but significantly increased neovascularization of 786-0 tumors, suggesting that PTHrP has antiangiogenic activity. The lack of effect of the PTHrP antibody on tumor vascularization in the current study might be a result of the lower PTHrP expression by Caki-1 tumors, or the presence of pVHL in Caki-1 cells. Knowing the pathogenesis of human RCC, it will be important in the future to examine the presence of PTHrP in RCC metastases, independent of VHL status, and also whether anti-PTHrP treatments might be effective against metastatic spread in RCC. Together with our recent studies obtained in VHL-deficient RCC tumors expressing high levels of PTHrP (17), the data presented herein constitute a starting point for clinical evaluation of anti-PTHrP strategies against human RCC.

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References


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