Parathyroid Hormone-Related Protein Is an Essential Growth Factor for Human Clear Cell Renal Carcinoma and a Target for the von Hippel-Lindau Tumor Suppressor Gene

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ABSTRACT

Clear cell renal carcinoma (CCRC) is responsible for 2% of cancer-related deaths worldwide and is resistant to virtually all therapies, indicating the importance of a search for new therapeutic targets. Parathyroid hormone-related protein (PTHrP) is a polypeptide derived from normal and malignant cells that regulates cell growth. In the current study, we show that blocking PTHrP with antibodies or antagonizing the common parathyroid hormone (PTH)/PTHrP receptor, the PTH1 receptor, dramatically blunts the expansion of human CCRC in vitro by promoting cell death. Importantly, in nude mice, anti-PTHrP antibodies induced complete regression of 70% of the implanted tumors by inducing cell death. In addition, we demonstrate that the von Hippel-Lindau tumor suppressor protein, which functions as a gatekeeper for CCRC, negatively regulates PTHrP expression at the post-transcriptional level. These studies indicate that PTHrP is an essential growth factor for CCRC and is a novel target for the von Hippel-Lindau tumor suppressor protein. Taken together, these results strongly suggest that targeting the PTHrP/PTH1 receptor system may provide a new avenue for the treatment of this aggressive cancer in humans.

INTRODUCTION

Clear cell renal carcinoma (CCRC) represents 75% of all renal tumors. It is responsible for 2% of cancer-related deaths worldwide (200,000 cases and 100,000 deaths/year; Refs. 1, 2). The mortality rate is 30% at 5 years for localized CCRC, but this rate increases to 95% when metastases are present. At the time of diagnosis, about 20–30% of patients present with metastases, and 50–60% of patients develop metachronous metastatic disease (1, 2). Nephrectomy, the most widely used therapy for localized CCRC, is associated with poor long-term survival. Metastatic CCRC is highly resistant to chemotherapy, radiotherapy, hormonal therapy, and, to a lesser degree, immunotherapy (3). Treatment with IFNs, interleukins (ILs), or both, not only are toxic, but produced brief responses, limited to 20% of patients. Thus, effective and nontoxic therapy for CCRC is still lacking.

Parathyroid hormone-related protein (PTHrP) was initially identified as the factor responsible for the paraneoplastic syndrome, humoral hypercalcemia of malignancy (4). PTHrP has been proven to be a polypeptide normally expressed throughout the body where it displays a variety of effects, including the regulation of cellular growth, differentiation, and death (5). The perinatal lethality of PTHrP or PTH1 receptor (PTH1R) knockout mice (4) emphasizes the biological importance of this peptide system. In addition to the skeleton, the kidney appears as a specific target for PTHrP. In this organ, PTHrP is expressed in vessels, glomeruli, and tubules, and regulates vascular tone, glomerular filtration rate, as well as cell growth and differentiation (6).

PTHrP is abundantly expressed by most malignant human tumors, whether they are associated with hypercalcemia or not. Thus, in addition to its systemic hypercalcemic properties, PTHrP may have other effects in cancers. Its growth factor-like properties, together with the complex modulation of its expression by a number of growth as well as angiogenic factors such as interleukins, tumor-derived growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and vascular endothelial-derived growth factor (VEGF), point toward potential roles for PTHrP in the regulation of tumor growth and invasion (4–6). Recent studies in breast, prostate, and lung cancer (7–9) suggest such roles, and highlight the therapeutic potential of PTHrP-targeting strategies in human cancer.

CCRC is a highly vascularized tumor, which originates from the renal proximal tubular epithelium (1, 2), a target tissue for PTHrP proliferation effects (10, 11). Moreover, PTHrP is expressed in 95% of CCRC in humans, whether they are associated with hypercalcemia or not (12, 13). In 1990, Burton et al. (14) showed that PTHrP regulates the proliferation of a human PTHrP-secreting CCRC cell line (SKRC-1) in vitro. Collectively, these studies strongly suggest that PTHrP might be involved in CCRC growth and invasion.

Biallelic inactivating mutations of the von Hippel-Lindau (VHL) tumor suppressor gene on chromosome 3p25–26 occur in patients with the VHL syndrome, an autosomal dominantly inherited syndrome associated with CCRC, and in most (>75%) patients with sporadic CCRC (1, 15). The products of the VHL gene (pVHL) are indicated by activation of cell death. Finally, we provide evidence that PTHrP is a novel target for VHL, and that pVHLs regulate PTHrP expression at the post-transcriptional level.

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MATERIALS AND METHODS

Cells, Cell Culture, and Stable Transfection

Normal human (h) proximal tubular cell line HK-2 (a gift from Dr. Caroline Silve, Faculté de Médecine Xavier Bichat, Paris, France) and CCCR cell lines 786–0 (American Type Culture Collection, Manassas, Virginia), UOK-126, and UOK-128 (19) were maintained in RPMI 1640 or DMEM medium (Invitrogen, Cergy-Pontoise, France) supplemented with 10% fetal bovine serum. All of the CCCR cell lines are derived from human sporadic CCCRs and encode inactive pVHL. Unless otherwise specified, cells were used at 70–80% confluence. 786–0 cells [786–0 wild-type (wt)] were transfected with PC3.1-Uni vector alone (786–0 V) or containing the hVHL cDNA in replicon-tagged at the COOH terminus (786–0 VHL), using Lipofectamine (Invitrogen). Stable clones were selected using G418 (500 μg/ml).

RNA Extraction and Reverse Transcription-PCR (RT-PCR) Analysis

Total RNA was extracted from cultured cells using the TRIzol reagent (Invitrogen), according to the manufacturer’s protocol. The relative abundance of hVHL, hPTHrP, and hPTH1R transcripts was analyzed by RT-PCR using human glyceraldehyde-3-phosphate dehydrogenase (hGAPDH) expression as a loading control. Primers for hVHL, hPTHrP, and hPTH1R were described previously (20–22). Primers for hGAPDH are: sense, 5′-GGGAAGGTGAGGTCGGGAGT-3′; and antisense, 5′-GCAGTGATGCGCATGGACTG-3′.

Analysis of PTHrP Expression

PTHrP concentrations (expressed in pm) in the conditioned medium of cultured cells were determined by RIA (Bachem, Voisins-le-Bretonneux, France) as described (21).

PTHrP and PTHIR Immunofluorescence

Cells were processed as described (23). Affinity-purified polyclonal rabbit antibodies directed against either hPTHrP (1–34; N-term Ab; Bachem) or hPTH1R (Eurogentec, Angers, France) were used at 5 μg/ml. A tetramethylrhodamine isothiocyanate-conjugated antirabbit secondary antibody was used for detection. As a competition control, the primary antibody was preincubated overnight at 4°C with 10⁶ M PTHrP (1–36) peptide (Bachem) for PTHrP staining or with 10⁻⁶ M of TLDEAERLTEEELH peptide (peptide IV; Eurogentec) for PTH1R staining. As an additional control, nonimmune rabbit IgG (Sigma-Aldrich, St. Quentin Fallavier, France) was used instead of primary antibody.

Western Blot Analysis

Protein expression was analyzed as described (22) using 10–30 μg of protein and a monoclonal mouse antihemagglutinin antibody (Roche Diagnostics, Meylan, France) to detect pVHL or the anti-hPTH1R antibody (Eurogentec). A polyclonal mouse anti-β actin antibody (Sigma-Aldrich) was used for visualization of protein gel loading.

Cell Proliferation Measurements

CCCR cell proliferation was assessed by counting adherent cells and measurement of bromodeoxyuridine incorporation (Roche Diagnostics), as described (21). Cells were grown in serum-free medium for 48 h before stimulation and then for a second 48 h with test substances at the concentrations indicated in the figures. Test substances included rabbit anti-PTHrP antibodies directed against either the NH2 terminus (N-term Ab; Bachem), the mid-region (VWR International, Strasbourg, France), or the COOH terminus (a gift from Dr. Pedro Esbrit, Fundacion Jimenez Diaz, Madrid, Spain); the PTH1R antagonist [Asn (10), Leu (11), and ω-3-Tip (12)] PTHP (7–34)amide (Bachem); and hPTH1R (1–36; Bachem), hPTHrP (38–94)amide (a gift from Prof. Andrew Fyfe Stewart, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania), or hPTHrP (107–139; Bachem).

Cell Death/Apoptosis Analysis

Cells were treated with control medium alone or with either the N-term Ab at 1.5 μg/ml or the PTH1R antagonist at 1 μM for 48 h. Acridine orange and ethidium bromide were dissolved in PBS buffer and added at 2 μg/ml to the culture medium. Cells were viewed and photographed using fluorescence microscopy, either for FITC (acridine orange) or tetramethylrhodamine isothiocyanate (ethidium bromide). Internucleosomal DNA fragmentation was detected by DNA laddering assay. Cells were resuspended in 300 μl of lysis buffer [25 mM EDTA, 1% SDS, and 1 mg/ml proteinase K (80 μg/ml)] and incubated overnight at 50°C. DNA was extracted using phenol-chloroform, precipitated with ethanol, and analyzed by agarose gel electrophoresis.

PTHrP Promoter Activity Measurement

786–0 wt cells, and 786–0 V and 786–0 VHL clones were transiently transfected with a construct containing the hPTHrP promoter (promoters P1, P2, and P3) of the hPTHrP gene ligated to a promoterless bacterial chloramphenicol acetyltransferase (CAT) gene (24). Transfection was carried out 24 h after cell seeding with 1 μg/cm² of the PTHrP promoter-CAT plasmid or the promoterless CAT plasmid, using Lipofectamine (Invitrogen). Cells were analyzed 48 h after transfection for CAT enzyme expression using a CAT enzyme ELISA kit (Roche Diagnostics). Results were normalized and expressed as pg CAT enzyme/mg of protein.

Actinomycin D Experiments

786–0 wt cells, and 786–0 V and 786–0 VHL clones were exposed to 5 μg/ml actinomycin D (Sigma) for 0–3 h. Total RNA was isolated and PTHrP expression analyzed by RT-PCR using hGAPDH for normalization of PCR reactions as described above. Lane intensity was quantified using SigmaScan software (Jandel Scientific, Erkrath, Germany) and ratio of hPTHrP on hGAPDH calculated for each time point. The relative intensity of the PTHrP mRNA signal (hPTHrP:hGAPDH ratio) in each lane was then expressed as a percentage of the signal obtained at the time of actinomycin D addition (0 h) set to 100%.

Tumor Model

Implantation. All of the animal studies were in compliance with French animal use regulations. Seven-week-old male Swiss nu nu nude mice (Ifa-Credo, St. Germain sur l’Arbresle, France) were given s.c. injections of 10⁷ 786–0 cells into the skin of the back, either on both sides (n = 10) or on the right side only (n = 20). Tumor size was measured using calipers. Two weeks after injection, mice bearing two tumors or one tumor were separated each in two groups. Mice bearing two tumors were injected i.p. daily with 40 μg of the N-term Ab or with nonspecific IgG (Sigma). Mice bearing 1 tumor were injected i.p. daily with 40 μg of the PTH1R antagonist or with the diluent. At the end of treatment, mice were anesthetized and blood was harvested for measurement of plasma electrolytes, creatinine, and PTHrP concentrations (Laboratoires Universitaires d’Analyses Médicales, Strasbourg, France). Tumors were then removed, fixed in formalin, and paraffin-embedded. Four μm-thick sections were used. Some sections were stained with H&E and others with the PTHrP N-term Ab or the anti-hPTH1R antibody using standard methods (21).

Proliferative/Apoptotic Index. The proliferative index was determined by staining tumor sections with a mouse monoclonal anti-hKi67 antibody (Dako, Trappes, France) using standard methods (21). An apoptosis detection kit, based on the terminal deoxynucleotidyl transferase-mediated nick end labeling method (Roche Diagnostics), was used to measure the apoptotic index on sections. Total and stained cells in 15 fields (0.25 mm² each) were counted to determine both indices, which were then expressed as a percentage of stained cells to total cells.

Factor VIII and Microvascular Density. Tumor sections were stained for endothelial cells with a rabbit polyclonal antihuman factor VIII antibody (Dako) using a standard immunohistochemistry method (21). Microvessel density was determined by counting, for each tumor, both vessel intersecting points and the total number of vessels in four to five fields (0.25 mm² each) showing the highest vascular density.

Statistics

All of the values are expressed as mean ± SE. Values were compared using multifactorial ANOVA followed by Student-Newman-Keul’s test for multiple comparisons. P < 0.05 was considered significant.
RESULTS

CCRC Cell Lines Constitutively Express PTHrP, and the PTH1R Transcripts and Proteins. Using RT-PCR, we detected PTHrP and the PTH1R transcripts in all of the cell lines (Fig. 1A). CCRC cell lines showed higher PTHrP and PTH1R expression than HK-2 cells, although the difference between the HK-2 and UOK-126 cells was less marked for PTH1R. 786–0 and UOK-128 cells exhibited higher levels of expression of both transcripts as compared with UOK-126. 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 RIA for PTHrP (Fig. 1B) and Western blot for the PTH1R (Fig. 1C). Using immunofluorescence in cultured cells, immunoreactivity for PTHrP and the PTH1R were both diffuse (indicating membrane/cytoplasm distribution) and nucleolar (Fig. 1D). The pattern of staining was similar in all of the cell lines. Cells stained in the presence of excess synthetic peptides (CTL; Fig. 1D) or in the absence of primary antiserum (data not shown) showed no staining. Thus, these three CCRC lines constitutively express both PTHrP and the PTH1R. In addition, PTHrP expression was significantly higher in CCRC cells compared with normal cells.

PTHrP or PTH1R Blockade Inhibits CCRC Cell Growth. Post-translational processing of native PTHrP gives rise to several mature secretory forms, including the NH<sub>2</sub>-terminal PTHrP (1–36), the mid-region PTHrP (38–94), the COOH-terminal PTHrP (107–139/141),
and (in humans) the distal COOH-terminal PTHrP (141–173), as well as combinations of these peptides, all having their own (patho)physiological functions (4, 6). The PTHrP (1–36) fragment interacts with the PTH1R, but specific receptors for mid-region and COOH-terminal PTHrP fragments have not been identified (4, 6).

We tested the effect of antibodies directed against each PTHrP region on CCRC cell growth. It should be noted that the anti-PTHrP antibodies used here not only recognized the fragment against which they were directed but also the full-length PTHrP molecule. All three of the antibodies substantially decreased both cell number and bromodeoxyuridine incorporation (Fig. 2A) in 786–0 cells. A PTH1R antagonist also markedly reduced cell number and bromodeoxyuridine incorporation in 786–0 cells (Fig. 2B), indicating that the effect of secreted PTHrP involved the interaction of a secreted NH2-terminal-containing PTHrP with the PTH1R. Identical results were obtained using UOK-126 and UOK-128 cells (data not shown).

However, exogenously added PTHrP (1–36; Fig. 2C) or PTHrP (38–94)amide (Fig. 2D) had no effect on growth in 786–0 cells. Of interest, high concentrations (10−7 for cell density and 10−6 m for bromodeoxyuridine incorporation) of PTHrP (107–139) slightly decreased cell growth by 10–20% (Fig. 2E). Identical results were obtained in UOK-126 and UOK-128 cells, with the exception that PTHrP (107–139) was ineffective in UOK-126 cells, even at high concentrations (data not shown). Taken together, these observations strongly suggest that either a large NH2-terminal containing fragment or full-length PTHrP is required to induce PTH1R-dependent stimulation of CCRC cell growth.

PTHrP or PTH1R Blockade Induces Cell Apoptosis. That PTHrP, in addition to its mitogenic effects, might also be a survival factor for CCRC cells is suggested by cell culture observations, which showed that exposure of 786–0 cells to PTH1R antagonist produces cell detachment (Fig. 3A). This effect was already observable after 24 h of exposure and complete after 48 h because 72 h treatment did not result in additional cell detachment (data not shown). To determine whether PTHrP/PTH1R system blockade induces cell death, we additionally examined simultaneous ethidium bromide and acridine orange staining in control and treated CCRC cells. Ethidium bromide permeates dead cells only and, therefore, is a marker of cell death, whereas acridine orange freely permeates and visualizes all of the cells present in the field. Exposure to the PTH1R antagonist markedly increased the number of ethidium bromide-stained 786–0 cells as compared with untreated cells (Fig. 3B). Analysis of genomic DNA in treated 786–0 cells revealed internucleosomal DNA fragmentation (180-bp laddering pattern) specific for apoptosis (Fig. 3C). Cell detachment, ethidium bromide staining, as well as internucleosomal DNA fragmentation were seen in all of the CCRC cell lines, regardless of how the PTHrP/PTH1R system was interrupted (data not shown). Collectively, these results strongly suggest that PTHrP acts as an essential mitogenic and survival factor in CCRC cells.

PTHrP or PTH1R Blockade Induces CCRC Regression and Cell Apoptosis in Nude Mice. To assess the biological significance of PTHrP/PTH1R system blockade in CCRC, we used a xenograft athymic mouse model. In initial experiments, CCRC tumor-bearing nude mice were treated i.p. twice a week with 40 μg/mouse of either the N-term Ab or the PTH1R antagonist for 25 days. This protocol, whereas inhibiting significantly tumor growth, was not efficient enough to either completely block tumor growth or induce tumor regression (data not shown). Therefore, to optimize the efficiency of the anti-PTHrP/PTH1R treatments, additional experiments were performed using the same dose except that injections were performed daily for 17 days, as detailed in “Materials and Methods.” The average total tumor volume was 281 ± 88 mm3 and 95 ± 13 mm3 in mice bearing 1 or 2 tumors, respectively, 2 weeks after s.c. implantation of the cells. The mice were then treated for 17 days with either N-term Ab or nonspecific IgG (Fig. 4A). Impressively, 7 tumors disappeared, whereas 3 others regressed by 50–80% to an average volume of 22.9 ± 14 mm3 in N-term Ab-treated mice compared with IgG-treated animals. In contrast, 17-day treatment with the PTH1R antagonist did not cause tumor regression but abolished subsequent tumor growth (Fig. 4B). Interestingly, however, 2 of the 10 tumors

Fig. 2. Inhibition of clear cell renal carcinoma (CCRC) cell growth in response to parathyroid hormone-related protein (PTHrP)/parathyroid hormone 1 receptor (PTH1R) system blockade. A, effects of anti-PTHrP antibodies on cell number (left) and bromodeoxyuridine incorporation (right) in 786–0 cells. IgG, nonspecific IgG; 5 μg/ml; N-term, antibody against the NH2 terminus of PTHrP (N-term Ab); 1.5 μg/ml; mid-region, antibody against the 34–53 region of PTHrP; 2 μg/ml; C-term, antibody against the 107–139 region of PTHrP. 5 μg/ml. Mean; bars, ±SE, n = 8–12 for cell number and n = 4 for bromodeoxyuridine (BrdUrd); *, P < 0.05; **, P < 0.01 from controls. B, concentration-dependent effects of the PTH1R antagonist on cell number (left) and BrdUrd incorporation (right) in 786–0 cells. Mean; bars, ±SE, n = 12 for cell number and n = 11–15 for BrdUrd; *, P < 0.05; ***, P < 0.01 from controls. C, concentration-dependent effects of PTHrP (1–36) on cell number (left) and BrdUrd incorporation (right) in 786–0 cells. Mean; bars, ±SE, n = 12–16 for cell number and n = 8 for BrdUrd. D, concentration-dependent effects of PTHrP (38–94)amide on cell number (left) and BrdUrd incorporation (right) in 786–0 cells. Mean; bars, ±SE, n = 8 for cell number and n = 12 for BrdUrd. E, concentration-dependent effects of PTHrP (107–139) on cell number (left) and BrdUrd incorporation (right) in 786–0 cells. Mean; bars, ±SE, n = 16 for cell number and n = 8 for BrdUrd.

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regressed significantly by 90% and 60% after treatment with the PTH1R antagonist.

Because 70% of the tumors disappeared in the N-term Ab group and because the remaining tumors were very small, histopathological analysis was not possible, but was performed instead on tumors of PTH1R antagonist-treated animals and their respective controls. No histopathological differences were observed between control and treated groups on H&E staining. Areas of necrosis were present in the center of all of the tumors (data not shown). Tumors obtained from control mice strongly expressed PTHrP and the PTH1R (Fig. 4C). Obvious staining for PTHrP and PTH1R was observed in the cytoplasm of all cells and in the nucleoli of many cells (Fig. 4C). Although difficult to quantify, no apparent differences were noted in the staining pattern of the two tumor groups (data not shown). The proliferative index was not different between control and treated groups (Fig. 4D). In marked contrast, however, treatment with the PTH1R antagonist doubled the apoptotic index (Fig. 4E). Moreover, the number of vessel intersecting points and the total number of vessels as a function of surface area were significantly increased (Fig. 4F).

PTHrP concentrations in plasma were 0.6 ± 0.1 pm in normal mice without tumor. PTHrP concentrations were significantly increased to 5.1 ± 0.9 pm (P < 0.05) and 3.4 ± 0.4 pm (P < 0.01) in mice bearing 1 or 2 tumors, respectively. On the other hand, plasma concentrations of electrolytes, as well as the body weight, revealed no difference between animals over the period of treatment (Table 1).

These studies demonstrate that interrupting the PTHrP/PTH1R system not only is effective against CCRC through an increase in tumor cell apoptosis, but also appears to have no apparent toxicity.
pVHL Suppress Endogenous PTHrP mRNA and Protein Levels in Stably Transfected CCRC Cells. To determine whether PTHrP belongs to the family of genes regulated by pVHL, we stably transfected VHL-deficient 786–0 wt cells with a hVHL expression vector or the vector alone and measured PTHrP expression by RT-PCR (Fig. 5A) and RIA (Fig. 5C). 786–0 VHL clones 2 and 3 expressed the highest amount of PTHrP. In accordance with previous studies, pVHL appeared as two bands of Mr ~28,000 and Mr 23,000 daltons (Fig. 5B), corresponding, respectively, to full-length VHL (1–213) and to VHL (54–213; Ref. 18). The expression of hPTHrP was negatively correlated with the expression of hVHL at both mRNA (Fig. 5A) and protein (Fig. 5C) levels. Whereas 786–0 V cells and 786–0 V clones expressed similar amounts of PTHrP, the expression of hPTHrP was decreased by 50% in 786–0 VHL clones. On the other hand, hPTH1R expression was unaffected by VHL at both mRNA (Fig. 5A) and protein levels (Fig. 5D). Thus, PTHrP is down-regulated by the VHL proteins, suggesting that PTHrP is a target for pVHL.

PTHrP Expression Is Regulated by pVHL at the Post-Transcriptional Level. PTHrP expression has been shown to be regulated at the transcriptional and/or post-transcriptional levels by growth-, angiogenic- (e.g., interleukins and TGF-B), and vasoactive factors (e.g., angiotensin II; Refs. 4–6). To analyze whether PTHrP is reg-
ulated by pVHL at the transcriptional level, we transiently transfected 786–0 wt cells, as well as 786–0 V clones 3 and 4, and 786–0 VHL clones 2 and 3 with a CAT expression vector containing the 5'-flanking hPTHrP promoter regions, and measured CAT enzyme in cell extracts. The reintroduction of the VHL gene in 786–0 V clones 3 and 4, and 786–0 VHL clones 2 and 3 exposed to the transcriptional inhibitor actinomycin D, indeed indicated that the hPTHrP mRNA half-life was 38.3 ± 5 h and 40.1 ± 3.4 h in 786–0 wt and 786–0 V, respectively, but was significantly decreased by 50% to 20.9 ± 2.6 h in 786–0 VHL (Fig. 5F; *P < 0.01). Therefore, pVHL decreased PTHrP expression in CCRC by destabilizing PTHrP mRNA.

**DISCUSSION**

Previous findings focusing on breast, prostate, and lung cancers (see “Introduction”) strongly indicated that PTHrP not only behaves as a tumor hypercalcemic factor, but also as a factor regulating tumor growth. Here we report that the expression of both PTHrP and PTH1R are common features of CCRC cells and that PTHrP expression is significantly increased in tumor cells versus normal cells. Most importantly, the findings reported herein strongly support the view that the PTHrP/PTH1R system is essential for CCRC cell survival. Indeed, the addition of either PTHrP antibodies or blockade of the PTH1R rapidly induced apoptosis in cultured CCRC cells. Moreover, the CCRC-derived PTHrP species, which acts as a survival factor, is most likely an NH2-terminal-containing PTHrP fragment, consistent with the major circulating PTHrP species in humoral hypercalcemia of malignancy (4). Additionally, the COOH-terminal PTHrP fragment slightly decreased tumor cell growth in two CCRC cell lines. The inhibitory effect of both the COOH-terminal anti-PTHrP antibody and the exogenously added COOH-terminal PTHrP fragment we observed on CCRC growth are discrepant. However, it should be stressed first that the effect of the COOH-terminal fragment was only observed in two cell lines and at high concentrations, and second, that the COOH-terminal anti-PTHrP antibody used here not only recognizes the COOH-terminal part of the PTHrP molecule but also the full-length PTHrP. Taken together with the results obtained with the PTH1R antagonist, these results suggest that the CCRC-derived PTHrP species, which acts as a survival factor, may contain both NH2- and COOH-terminal determinants, and that this species may be the major CCRC-derived PTHrP fragment. Interestingly, investigating COOH-terminal PTHrP (109–141) expression in human CCRC by immunohistochemistry using a specific antibody, Iwamura et al. (13) have shown that the expression of the COOH-terminal PTHrP (109–141) in CCRC may be associated with lower CCRC recurrence after radical nephrectomy. They hypothesized that the COOH-terminal PTHrP fragment might have an inhibitory effect on CCRC cell growth. Our results are in accordance with these assumptions, suggesting the existence of a receptor recognizing a COOH-terminal PTHrP coupled to CCRC growth inhibition. They suggest, however, that the COOH-terminal fragment, if secreted, is a minor secretory form of PTHrP in human CCRC. The results obtained by Burton et al. (14) in 1990 on one CCRC cell line (SKRC-1 cells), suggesting that PTHrP can function as an autocrine growth factor in human renal cell carcinoma, also support this interpretation. It will be interesting in future studies to specifically identify the PTHrP species secreted by CCRC cells.

In CCRC cells, PTHrP and the PTH1R were localized to both the cytosol and nucleolar compartments. Nuclear/nucleolar localization of PTHrP not only has been described in various cell types including normal and tumoral cells, but also has been associated with cell survival and/or proliferation (6, 23, 25). On the other hand, the molecular targets for PTHrP in the nucleus have not yet been identified. Although the PTH1R has also been seen in the nuclear/nucleolar compartment in a number of tissues (6, 26, 27), its role in the nucleus, as well as its possible interaction with nuclear PTHrP, is incompletely understood. It is tempting to speculate that, upon secretion by CCRC cells, PTHrP binds to PTH1R, followed by internalization of the receptor/ligand complex and nuclear entry, with subsequent inhibition of cell death. However, such a sequence of events has never been described in any cell type. In rat UMR-106.01 osteoblastic cells, PTHrP is targeted to the nucleus through binding to the PTH1R (28). In other cell types, the nuclear presence of PTHrP is a consequence of either alternative translational initiation or non-PTH1R-mediated endocytosis (6, 27). Alternatively, because the PTH1R is coupled to protein kinase A (PKA) and protein kinase C (PKC) pathways in proximal tubular cells, the antiapoptotic effect of PTHrP in CCRC cells might be related to PKA/PKC stimulation (10). However, the question as to whether stimulation of the PKA/PKC pathways by PTHrP might induce proximal tubular cell survival has not yet been clarified. Specific experiments are required to define the cellular pathways responsible for the apoptotic effect of PTHrP/PTH1R interaction.

Another significant finding in the current study was the increased microvessel density in CCRC tumors grown in nude mice in response to PTHrP/PTH1R system inhibition. This latter observation strongly suggests that PTHrP is an angiogenic factor in CCRC. In support of this, PTHrP has been shown to behave as an angiogenic or antiangiogenic factor, depending not only on the cell type but also on whether it acts in an auto/paracrine or intracrine manner (29–31). As a rule, vascularization leads to a phase of rapid growth of a given tumor, a prerequisite for the development of invasive processes. Most, but not all, studies show that CCRC is a highly vascularized tumor (32, 33). Indeed, it has been proposed that in CCRC tumors with poor prognosis, decreased microvessel density is associated with the development of large vessels, which facilitate metastatic spread (33). Thus, the angiogenic effect, i.e., increased microvessel density, in response to the anti-PTHrP/PTH1R maneuvers might be beneficial therapeutically, together with the induction of apoptosis.

pVHL interact with cullin 2, and elongin B and C, forming a
complex with ubiquitin ligase activity, targeting hypoxia-induced factors for degradation by the 26S proteasome (18, 34, 35). Hypoxia-induced factors are transcription factors regulating genes encoding angiogenic factors (e.g., VEGF), growth factors (e.g., PDGF), and vasoactive factors (e.g., endothelin-1; Refs. 18, 34, 35). pVHL also deregulate hypoxia-inducible genes by decreasing mRNA stability, exemplified by VEGF, glucose transporter-1, TGF-α, and TGF-β1 (18, 35–38). Whether this latter effect occurs through the inhibition of hypoxia-induced factor-induced mRNA transcription of hypoxia-induced stabilizing RNA-binding proteins, or through the degradation of these proteins by the VHL ubiquitinating complex, is currently unknown. Finally, pVHL inhibit IGF-1 receptor signaling through their interaction with PKCε (39). As a consequence, pVHL-deficient cells acquire angiogenic and proliferative phenotypes. The critical role of pVHL in CCRC is additionally demonstrated by the fact that 75% of sporadic CCRC have biallelic VHL mutations. With this background, we now report that PTHrP expression is suppressed by pVHL, at both the mRNA and the protein levels. These results not only identify PTHrP as a novel target gene for pVHL, but also suggest a fundamental role for PTHrP in the pathogenesis of human CCRC.
half-life of VEGF, glucose transporter-1, TGF-α, and TGF-β1 transcripts has been proven to be markedly increased in VHL-deficient CCRC cells compared with other cell types (36–38), and it has been proposed that a common mechanism of stabilization of these mRNAs exists in VHL-deficient cells, which takes place at the level of the regulation of specific mRNA-binding stabilizing protein (38). Whether PTHrP belongs to the family of genes directly regulated by such mechanisms remains an open question.

CCRC is highly resistant to all of the currently available therapies. The need for new agents for clinical evaluation remains a high priority in this refractory disease. The current studies argue that agents targeting the PTHrP/PTH1R system in human CCRC may have therapeutic potential.

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