Published in final edited form as:

Cancer Lett. 2007 December 18; 258(2): 241–252.

PTHrP increases xenograft growth and promotes integrin $\alpha 6 \beta 4$ expression and Akt activation in colon cancer

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Abstract

Parathyroid hormone-related protein (PTHrP) is expressed by human colon cancer tissue and cell lines. Expression of PTHrP and phosphatidylinositol 3-kinase (PI3-K) pathway components correlates with the severity of colon carcinoma. Here we observed a positive effect of endogenous PTHrP on LoVo (human colon cancer) cell proliferation, migration, invasion, integrin $\alpha 6$ and $\beta 4$ expression, and p-Akt levels. There was a direct correlation between PTHrP expression and anchorage-independent cell growth. PTHrP significantly increased xenograft growth; tumors from PTHrP-overexpressing cells showed increased expression of integrins $\alpha 6$ and $\beta 4$, and PI3-K pathway components. The higher expression of PTHrP in human colon cancer adenocarcinoma vs. normal colonic mucosa was accompanied by increased integrin $\alpha 6$ and $\beta 4$ levels. Elevated PTHrP expression in colon cancer may thus upregulate integrin $\alpha 6 \beta 4$ expression, with consequent PI3-K activation. Targeting PTHrP might result in effective inhibition of tumor growth, migration and invasion.

Keywords

PTHrP; integrin $\alpha 6 \beta 4$; phosphatidylinositol 3-kinase; colon cancer

1. Introduction

Colorectal carcinoma is the second leading cause of cancer-related deaths in the United States [1,2]. Although many patients with stages II, III, and IV colorectal cancer benefit from surgical resection and multimodal therapy, ~ 50% of patients with cancers of the colon and rectum die from this disease [3]. The molecular processes of tumor progression are mediated via both inherent tumor cell characteristics and growth factors, matrix molecules and cytokines in the tumor environment [4–6]. One of these factors is parathyroid hormone-related protein (PTHrP). PTHrP expression correlates with the severity of colon carcinoma — specifically, with cell differentiation, depth of invasion, lymphatic invasion, lymph node and hepatic metastasis, and Dukes' classification [7]. The mechanisms through which PTHrP exerts its effects in colon cancer are not fully understood. Since the gastrointestinal epithelium is prone to cancer development, particularly in the colon, understanding the role of PTHrP in this system may provide important information for the diagnosis and treatment of colon cancer. Here we asked whether endogenous PTHrP affects the proliferation, migration, and invasion of LoVo cells,

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which are derived from a left supraclavicular region metastasis of a Dukes' type C, grade IV colorectal carcinoma [2]. Since dysregulation of cellular proliferation plays a major role in carcinogenesis [8,9], we also assessed the effects of PTHrP on LoVo cell xenograft growth *in vivo*.

Integrins play a major role in tumor invasion and metastasis by activating intracellular signaling pathways that regulate growth, differentiation, apoptosis, cell motility and gene expression. Integrin $\alpha 6 \beta 4$ expression correlates with colon cancer cell invasiveness [10–12]. The extracellular domain of the $\beta 4$ subunit associates exclusively with the $\alpha 6$ subunit to form $\alpha 6 \beta 4$ heterodimers [13]. We therefore asked whether endogenous PTHrP regulates these integrin subunits in LoVo cells.

Phosphatidylinositol 3-kinase (PI3-K), a ubiquitous lipid kinase involved in receptor signaling transduction by tyrosine kinase receptors, plays a major role in colon cancer proliferation and survival [14,15]. Integrin $\alpha 6 \beta 4$ is known to activate the PI3-K pathway [11]. The levels of p-Akt, a downstream effector of PI3-K, are elevated in PTHrP overexpressing cells [16]. Moreover, multiple studies have shown that levels of components of the PI3-K pathway are elevated in colon cancer vs. normal colon mucosa, and that these increases are more pronounced during colon cancer progression [17–21]. We therefore investigated whether there is a link between the levels of PTHrP, the integrin $\alpha 6$ and $\beta 4$ subunits, and Akt in the LoVo cell xenografts and in human colorectal adenocarcinomas, to ask whether elevated levels of PTHrP and integrin $\alpha 6$ and $\beta 4$ accompany the increased expression of PI3-K pathway components.

2. Materials and Methods

2.1. Materials

Fetal bovine serum (FBS) and NuSerum were obtained from Atlanta Biologicals (Norcross, GA) and BD Biosciences (San Diego, CA) respectively. The R-PE-conjugated anti- $\alpha 6$ and anti- $\beta 4$ antibodies, and their isotype controls, were obtained from BD PharMingen (San Diego, CA). The Alexa-Fluor 488-conjugated anti-p-Akt antibody (Ser 473) and the isotype control antibody were obtained from Cell Signaling Technologies (Danvers, MA). The R-PE-labeled anti-total Akt antibody and the isotype control antibody were obtained from BD Pharmingen. Antibodies for immunohistochemistry were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Technologies. The FluoroBlok inserts for analysis of migration and invasion were purchased from BD Pharmingen. Matrigel was obtained from BD Biosciences (San Diego, CA), and Calcein-AM was obtained from Molecular Probes (Eugene, OR). The small interfering RNAs (siRNAs) targeting PTHrP, and the corresponding nonspecific (scrambled) siRNA sequences were purchased from Dharmacon (Lafayette, CO).

2.2. Plasmid constructs

A cDNA encoding human PTHrP (from Genentech, Inc., South San Francisco, CA) was cloned into the expression vector pcDNA3.1(+) (Invitrogen, San Diego, CA). Cells transfected with the empty vector were used as control.

The siRNAs targeting the open reading frame of the human PTHrP gene were cloned into the vector pSilencer 2.1 U6 neo (Ambion Inc., Austin, TX). As control, the scrambled siRNA sequences were cloned into the pSilencer 2.1 U6 neo vector.

2.3. Cell culture and transfection

LoVo cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were grown at 37°C in a humidified 95% air/5% CO₂ atmosphere in F12 medium supplemented with 10% FBS and L-glutamine.

The cells were stably transfected by electroporation. Two days after transfection, 600 µg/ml G418 (Geneticin; Life Technologies Inc.) was added, and resistant cells were selected. The establishment and characterization of PTHrP-overexpressing LoVo cell clones has been described [16,22].

PTHrP expression was suppressed using two PTHrP siRNA sequences targeting the open reading frame of the human PTHrP gene (si1, sequence GAGCUGUGUCUG-AACAUCAUU and si2, sequence GAUCGCAGAAAUCCACACAUU), cloned into pSilencer 2.1 U6 neo (Ambion Inc.). Two independent cell lines expressing one of the two PTHrP siRNA sequences were utilized in the experiments described below. Non-specific siRNAs were used as negative controls.

2.4. Analysis of PTHrP, integrin $\alpha 6$, integrin $\beta 4$, and Akt levels

PTHrP, integrin $\alpha 6$, and integrin $\beta 4$ mRNA levels were analyzed by reverse transcription/real-time PCR, as previously described [16]. PTHrP secretion was measured by IRMA (DSL Webster, TX) [22]. Cell-surface protein levels of the integrin $\alpha 6$ and $\beta 4$ subunits were analyzed by FACS, as previously described [22]. p-Akt and total Akt levels were also measured by FACS, as previously described [16], using the following primary antibodies: Alexa-Fluor 488 anti-p-Akt antibody (Ser 473) (Cell Signaling Technologies), R-PE-labeled anti-total Akt (BD Pharmingen) antibody, or the corresponding isotype control antibodies (BD Pharmingen).

2.5. Cell proliferation, migration, and invasion

To measure the effects of suppressed PTHrP expression on cell proliferation, cells were plated in 24-well dishes at 10^4 cells/well in medium containing 10% FBS. After 24 h, the cells were transferred to medium containing 2.5% FBS. Cells were then trypsinized and counted at days 1, 3, 5 and 7 using a Coulter counter (Coulter Electronics Inc., Hialeah, FL). Cell migration and invasion were assessed using the FluoroBlok system (BD Pharmingen), as previously described [16]. Data are presented as the percentage of cells that migrated through the membrane or invaded the Matrigel barrier after 4 h.

2.6. Monolayer scratch assay

Cells were grown to confluence in 6-well dishes in medium containing 5% NuSerum. NuSerum was used to decrease the exposure of cells to the extracellular matrix (ECM) proteins present in FBS. The confluent monolayer was scraped with a P200 pipette tip, and then rinsed with PBS to dislodge cellular debris. The cells were then further incubated under standard conditions for 5 days. Pictures were taken at time zero, and at 3, 4, and 5 days after wounding, using a Nikon phase contrast microscope. The extent of migration was analyzed using the NIH image software (<http://rsb.info.nih.gov/nih-image/Default.html>).

2.7. Soft agar growth

Colony formation in soft agar was assayed as described [23]. Cells (1×10^4 cells) were suspended in 1 ml of 2x medium/20% FBS kept at 37 °C, and then mixed with 0.6% agar (BMA, Rockland, ME) at 45 °C, such that the final concentration of agar was 0.3%. The cells were then layered over 1.5 ml of solidified 0.4% agar in 6-well dishes. After the top agar had solidified, 1 ml of medium containing 10% FBS was added. Dishes were incubated at 37°C in a humidified 95% air/5% CO₂ atmosphere. Two days after plating, 5 fields/well were counted to ensure that the plating efficiencies of the different clones were similar. The medium was replaced every 3 days. After 2 weeks, photographs were taken at high magnification to measure the clone size, using the imaging software ImageJ (NIH). Photographs were also taken at low magnification to measure clone frequency. Five fields per well were photographed, and all

clones in focus > 50 μm in size were measured. At least two independent experiments were performed in triplicate.

2.8. Nude mouse tumor studies

LoVo cells were cultured in medium containing 10% FBS. At 70% confluency, the cells were trypsinized and the cell pellet was washed once with FBS-containing medium and three times with PBS. The cells were then resuspended in PBS at 3×10^6 cells/100 μl .

Male athymic nude mice, ~6 weeks of age (Harlan Sprague Dawley, Indianapolis, IN), received subcutaneous injections on the dorsal surface with 100 μl of the cell suspension. The following LoVo cell clones were used (6 mice/group): four independent PTHrP-overexpressing clones, three independent empty vector-transfected clones (control), and parental cells. Tumor volumes were monitored twice weekly [24]. Mice were sacrificed on day 35 after injection, and the tumors were excised and weighed. All animal experiments were carried out under an Institutional Animal Care and Use Committee-approved protocol.

2.9. Tissue procurement

Primary colorectal adenocarcinoma and adjacent mucosa (approximately 5–10 cm from the cancer) were obtained from patients undergoing elective surgical resection over a 4-year period from 2001 to 2005 at the University of Texas Medical Branch (UTMB), Galveston, TX [21]. Tumor stage (TNM classification, [21]) and differentiation grade were assessed. Immediately after collection, samples were placed in liquid nitrogen and stored at -80°C . The samples were then removed from -80°C and placed into 10% neutral buffered formalin overnight at room temperature. Tissue acquisition and subsequent use were approved by the Institutional Review Board at UTMB. These samples have been previously used to analyze expression of PI3-K pathway components [21].

2.10. Immunohistochemistry

Portions of the dissected mouse tumors were fixed immediately in 10% neutral buffered formalin for 24 h at room temperature after harvesting, and then placed in 70% ethanol. Formalin-fixed human and mouse tissues were embedded in paraffin, and sections (5 μm) were cut from the paraffin blocks. The sections were deparaffinized in xylene, and rehydrated in descending ethanol series. Protein staining was performed using the DAKO EnVision Kit (Dako Corporation, Carpinteria, CA). Briefly, sections were incubated overnight at 4°C with monoclonal antibodies (diluted in 0.05 mol/L Tris-HCl + 1% BSA) against PTHrP, the integrin $\alpha 6$ and $\beta 4$ subunits, total Akt, p85 α (Santa Cruz Biotechnology, Inc.), p-Akt (Ser 473), and p110 α (Cell Signaling). After 3 washes with TBST, the sections were incubated for 30 min with secondary antibody labeled with peroxidase, then washed 3 times with TBST. Lastly, peroxidase substrate DAB was added for staining. All sections were counterstained with haematoxylin and observed by light microscopy. For negative controls, sections were incubated with rabbit IgG (Santa Cruz Biotechnology) in place of primary antibody. Images were recorded using a Nikon microscope at $40\times$ magnification.

2.11. Statistics

Numerical data are presented as the mean $\pm$ SEM. The data were analyzed by ANOVA, followed by a Bonferroni post-test to determine the statistical significance of differences. All statistical analyses were performed using InStat Software (GraphPad Software, Inc., San Diego, CA). $P < 0.05$ was considered significant.

The expression patterns detected by IHC staining were assessed using a four-tier scoring system of 0–3, with staining intensity graded as: 0 = negative, 1 = weak, 2 = moderate, and 3 = strong

[25]. Low expression was graded as 0–1, and high expression was graded as 2–3. When heterogeneity in staining was observed, the proportion of stained cells was evaluated as 1 (< 10% of cells stained), 2 (< 30% of cells stained), 3 (< 70% of cells stained), or 4 (> 70% of cells stained). Results of staining intensity and heterogeneity were combined and graded between 0 and 3 [25]. The summarized values presented were obtained after evaluation of each slide by three observers.

3. Results

3.1. Direct correlation between PTHrP and integrin $\alpha 6 \beta 4$ expression, and p-Akt levels

Two siRNA sequences (si1 and si2) cloned in the vector pSilencer 2.1 U6 neo (Ambion Inc.) were used to generate two independent cell lines with suppressed PTHrP expression. Transfection with these siRNAs produced an ~ 80% decrease in PTHrP mRNA levels and secreted protein levels (Fig. 1, A and B), when compared to cells transfected with the scrambled siRNA-expressing construct. Suppression of PTHrP expression was accompanied by a significant decrease in integrin $\alpha 6$ and $\beta 4$ expression at the mRNA and cell-surface protein (Fig. 1, A, C and D) levels.

Integrin $\alpha 6 \beta 4$ expression is linked to PI3-K activation [11]. p-Akt levels were significantly lower in PTHrP siRNA transfectants (Fig. 1E); total Akt levels were unchanged (data not shown). The scrambled siRNA had no effect on the levels of PTHrP, the integrin $\alpha 6$ and $\beta 4$ subunits, p-Akt or total Akt, when compared to parental cells (data not shown). Similar effects were obtained using the two independent siRNA sequences, thereby eliminating a site-specific effect of the PTHrP siRNA.

3.2. Endogenous PTHrP increases cell proliferation, migration, and invasion

Cells transfected with the PTHrP siRNA-expressing constructs had a decreased rate of proliferation; the cell number of the PTHrP siRNA transfectants was ~ 50% that of the scrambled siRNA controls after 7 days in culture (Fig. 2A). The decrease in cell proliferation of the PTHrP siRNA transfectants was already evident after 3 days in culture. The two independent PTHrP siRNA-transfected cell lines had comparable rates of proliferation, again ruling out a site-specific effect of the PTHrP siRNA.

PTHrP increased the migration of LoVo cells, as measured using the FluoroBlok system. The cells were plated onto the inserts in the absence of FBS, to avoid any contributions to migration by ECM proteins present in serum. Approximately 22% of PTHrP siRNA transfectants migrated through the FluoroBlok membrane, vs. ~ 30% of scrambled siRNA transfectants (an ~ 30% decrease in migration; Fig. 2B). Suppressing PTHrP expression also decreased LoVo cell invasion; ~ 15% of PTHrP siRNA-transfectants invaded the Matrigel barrier, vs. ~ 20% of scrambled siRNA transfectants (an ~ 30% decrease in invasion; Fig. 2C). The two PTHrP siRNA sequences produced comparable effects (Fig. 2, B and C), and there was no difference in the migration and invasion of scrambled siRNA transfectants vs. that of parental cells (data not shown).

Cell migration was also assessed using the monolayer scratch assay, which measures the ability of cells to migrate and bridge a gap induced in a cell monolayer by scratching. Repair of the cell monolayer was significantly slower for cells transfected with the PTHrP siRNA than for the control cells (Fig. 2D). Similar results were obtained with the second independent PTHrP siRNA-transfected clone (data not shown). These PTHrP siRNA-transfected cells continued to proliferate, as evidenced by the confluence of the monolayer, with multilayers in certain areas, that surrounds the wounded area. Since the proliferation of PTHrP siRNA transfectants is slower than that of the scrambled siRNA-transfected controls (Fig. 2A), the observed

difference in monolayer repair by PTHrP siRNA- vs. scrambled siRNA-transfected cells may be attributed to a difference in cell migration as well as cell proliferation. There was no significant difference in cell monolayer repair between parental cells and scrambled siRNA transfectants (data not shown). Significant differences in cell migration between PTHrP siRNA-transfected and control cells were also observed 3 days and 4 days after wounding the cell monolayer (Fig. 2E).

3.3. PTHrP supports anchorage-independent cell growth

To investigate the effect of PTHrP on anchorage-independent cell growth, we used PTHrP-overexpressing cells [16,22] and cells with suppressed PTHrP expression. These cells were grown in soft agar for 15 days. PTHrP facilitated soft agar clone formation. Thus, overexpressing PTHrP increased both the size (Fig. 3A) and number (Fig. 3B) of colonies in soft agar; conversely, transfection with the PTHrP siRNA significantly decreased both the size (Fig. 3A) and number (Fig. 3B) of colonies. Increasing the incubation time from 15 to 21 days did not increase the number of colonies formed by any of the clones (data not shown).

3.4. PTHrP increases LoVo xenograft growth

To measure the effects of PTHrP on tumor xenograft growth, PTHrP-overexpressing and control (empty vector-transfected and parental) LoVo cells were injected subcutaneously into the dorsal surface of athymic nude mice. Both control and PTHrP-overexpressing cells produced tumors; the same incidence of tumor formation was observed at the later time points (> 24 days). However, PTHrP increased both the rate of xenograft growth and the size of the tumors. At day 9, tumors from PTHrP-overexpressing cells and control cells had a relative tumor volume of ~ 4 mm³. Starting at day 15, the relative tumor volume of tumors produced by PTHrP-overexpressing cells was significantly greater than that produced by the control cells (Fig. 4B). At the time of sacrifice, tumors derived from PTHrP-overexpressing cells were on average ~ 5-fold heavier than tumors resulting from the control cells (Fig. 4, A and C). There were no significant differences in any of the parameters measured between the tumors produced by parental cells and those produced by empty vector transfectants (Fig. 4, A–C).

3.5. Increased expression of the $\alpha 6$ and $\beta 4$ subunits and PI3-K pathway components in tumors from PTHrP-overexpressing cells

Staining patterns for PTHrP, integrins $\alpha 6$ and $\beta 4$, and PI3-K pathway components are shown in Fig. 5. The staining intensity score was obtained after grading a minimum of five sections each from four independent PTHrP-overexpressing clones and three empty vector-transfected clones. As expected, PTHrP expression was higher in xenografts from mice injected with PTHrP-overexpressing cells than in corresponding xenografts from control cells (Fig. 5). Increased PTHrP expression was accompanied by corresponding strong increases in staining for integrins $\alpha 6$ and $\beta 4$ (Fig. 5).

Xenografts from PTHrP-overexpressing LoVo cells also showed increased staining for p-Akt and total Akt, and for the PI3-K subunits p85 α and p110 α (Fig. 5). Staining for p110 α was low in xenografts from control cells (Fig. 5). The difference in staining between sections from PTHrP-overexpressing and control cells was less pronounced for p110 α than for p85 α (Fig. 5). No staining was observed when IgG was used as the primary antibody (Fig. 5). There were no differences in staining between sections obtained from the tumors derived from parental cells and empty vector transfectants (data not shown).

3.6. Expression of PTHrP, integrin $\alpha 6$, and integrin $\beta 4$ in human colorectal cancers and the corresponding normal mucosa

Colorectal cancers (Stage II), adenomas (Stage 0) and adjacent normal mucosa from 20 patients [21] were analyzed for expression of PTHrP and the integrin $\alpha 6$ and $\beta 4$ subunits. The patient characteristics, tumor location, and TNM staging of these tumors have been described [21]. Representative staining patterns are shown in Fig. 6A, and semi-quantitative staining analysis is summarized in Fig. 6B. Expression of PTHrP and the integrin $\alpha 6$ and $\beta 4$ subunits was low in normal mucosa, with staining for the two integrin subunits evident on the surface epithelium of the cells making up the acinar structures. Staining was significantly stronger in the sections derived from the tubulovillous adenomas and was evident within the acini; the highest expression was evident for integrin $\beta 4$ (Fig. 6). The tissue derived from Stage II cancer, which showed partial or total loss of the acinar structure, also showed strong staining for all three proteins (Fig. 6). Again, the difference in staining between cancer and normal tissue was most pronounced for the integrin $\beta 4$ subunit (Fig. 6).

4. Discussion

Multiple studies demonstrate that PTHrP plays a major role in tumors that metastasize to the bone, such as breast and prostate cancer [26,27]. However, there is increasing evidence that PTHrP also plays a role in cancers that metastasize to other regions of the body, such as colon tumors, which show a preference for the liver [2]. In this study, we show that endogenous PTHrP increases the *in vitro* proliferation, migration and invasion of the human colon cancer cell line LoVo. PTHrP also enhances the adhesion of the human colon cancer cell line HT-29 to collagen type I and increases the proliferation of the rat intestinal crypt cell line IEC-6 [28,29]. We also show that PTHrP increases the transformation potential of LoVo cells, as assessed by measuring anchorage-independent growth in soft agar, and xenograft growth in a nude mouse model. Taken together, these data indicate that PTHrP positively regulates cell transformation.

We also show that endogenous PTHrP regulates the *in vitro* expression of the integrin $\alpha 6$ and $\beta 4$ subunits at the mRNA and cell-surface protein levels. In line with these findings, we observe an increase in the levels of these integrin subunits in xenografts derived from PTHrP-overexpressing cells vs. those from the corresponding control cells. Higher levels of these integrins were also observed in human colon adenocarcinoma samples, compared to matched samples from normal mucosa. These colon cancers also showed higher staining for PTHrP. The pro-invasive integrin $\alpha 6\beta 4$ plays a pivotal role in the biology of invasive carcinoma [30, 31], and is expressed in many tumor cells that exhibit a motile phenotype characteristic of invasion and metastasis [30]. Integrin $\alpha 6\beta 4$ expression has been linked to tumor invasiveness of colorectal, breast, thyroid, bladder, and gastric tumors, among others [31]. Stable expression of integrin $\alpha 6\beta 4$ in $\beta 4$ -deficient colon cancer cells results in a significant increase in cell invasiveness [10,11]. Regulation of integrin $\alpha 6\beta 4$ expression by PTHrP may thus contribute to the observed pro-migratory and pro-invasive effects of PTHrP in colon cancer. Integrin $\beta 4$ signaling also promotes ErbB2-mediated cell proliferation in a mammary tumor model [32]. Since ErbB2 exerts pro-survival effects in colon carcinoma cell lines [33,34], the effects of PTHrP on colon cancer cell proliferation *in vitro* and on xenograft growth *in vivo* may be mediated via the integrin $\beta 4$ /ErbB2 pathway.

PTHrP upregulates integrin $\alpha 6$ and $\beta 4$ expression at the mRNA level, indicating a transcriptional and/or post-transcriptional mechanism of action. The protein may either be functioning via an intracrine pathway to influence integrin $\alpha 6$ and $\beta 4$ gene expression directly and/or indirectly, or may function via an autocrine/paracrine pathway to ultimately regulate the activity of nuclear factors involved in the expression of these integrin subunits. The increase in cell surface protein expression of the integrin $\alpha 6$ and $\beta 4$ subunits in PTHrP-overexpressing

cells may be secondary to the PTHrP-mediated increase in the mRNA levels of these integrin subunits. However, PTHrP may also exert its effects on cell surface integrin $\alpha 6$ and $\beta 4$ levels via a direct effect on protein synthesis/degradation or protein mobilization.

Class IA PI3-Ks are strongly expressed in colonic epithelial carcinoma cells lines [17]. There is increasing evidence that the activation of PI3-K and its downstream effector Akt is associated with colorectal carcinoma, and can convert differentiated human gastric or colonic mucosa to a less differentiated, more malignant phenotype [18]. Both the regulatory (p85 α) and catalytic (p110 α) subunits of PI3-K play a role in colorectal cancers [19,20], and that there is a direct correlation between p85 α and p110 α staining intensity and the clinical stage of colon cancer [20,21]. Similar findings were reported in breast cancer [35]. In this study, we show higher staining for both the p85 α and the p110 α subunits in xenografts from PTHrP-overexpressing cells than in those from control cells, indicating a link between PTHrP and PI3-K in the growth and invasiveness of colon cancer cells.

Given our findings and those of previous investigators, and since integrin $\alpha 6\beta 4$ is known to activate the PI3-K pathway [11], we infer that the effects of PTHrP on colon cancer cell proliferation, migration and invasion may be mediated via upregulation of integrin $\alpha 6\beta 4$ expression, with consequent activation of the PI3-K pathway. We report a correlation between expression of PTHrP and that of integrins $\alpha 6$ and $\beta 4$ (this study) and PI3-K pathway components [21] in human colon adenocarcinoma samples. However, the effects of integrin $\alpha 6\beta 4$ may be mediated via other distinct pathways. For example, integrin $\alpha 6\beta 4$ may promote cell cycle progression via the Ras-MAPK cascade [36,37]. Here we have concentrated on the PI3-K/Akt pathway, since expression of its components has been well characterized in early and late stage colon cancer, and in matched normal mucosa [21].

In this and a previous study [16], we showed that PTHrP expression correlates with the levels of phosphorylated (active) Akt in LoVo cells. The effects of PI3-K on tumor growth and progression are thought to be mediated by Akt, which is overexpressed in several cancers, including those of the colon, pancreas, ovary, and breast [38]. Moreover, Akt phosphorylation in human colon carcinomas correlates with cell proliferation and inhibition of apoptosis, as well as with different clinicopathological parameters such as invasive grade, vessel infiltration, lymph node metastasis, and tumor stage [39,40]. Both Akt1 and Akt2 have been implicated in colon cancer progression [39–41]. Here we use an antibody that recognizes all three Akt isoforms to show that both total and p-Akt levels are elevated in tumors from PTHrP-overexpressing cells compared to those from the control cells. Thus it appears that PTHrP activates Akt in these colon cancer xenografts.

In conclusion, multiple studies have established a correlation between expression of PI3-K components and colon cancer progression [17–21]. Increased PTHrP expression both in tumor xenografts and in human colon cancer samples is accompanied by activation of this pathway. Thus, these studies indicate that elevated PTHrP expression in colon cancer may result in upregulation of integrin $\alpha 6\beta 4$ expression, with consequent activation of the PI3-K pathway. Therefore, targeting PTHrP expression might result in effective inhibition of tumor growth, migration and invasion.

Acknowledgements

We thank Anusha Srinivasan for technical assistance and Dr. David Konkel for critically reading the manuscript. This work was supported by NIH grants DK035608 and CA104748.

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Figure 1. Expression of the integrin $\alpha 6$ and $\beta 4$ subunits and p-Akt in LoVo cells with suppressed PTHrP expression

(A) mRNA levels of PTHrP and the integrin $\alpha 6$ and $\beta 4$ subunits were measured by reverse transcription/real-time PCR. (B) PTHrP secretion was measured using an immunoradiometric assay. (C and D) Cells were stained with phycoerythrin-conjugated antibodies against the integrin 6 or 4 subunits, and analyzed by FACS. (E) Cells were stained with an Alexa Fluor 488-conjugated anti-p-Akt antibody and analyzed by FACS. In (C-E), positive cells are those whose log fluorescence intensity (MFI) is greater than that of the isotype control antibody-stained cells. Values are expressed relative to the control (scrambled siRNA) value, set at 100%. sc = scrambled siRNA control; si1 and si2 = two independent cell lines derived by transfection with independent PTHrP siRNAs. In (A-E), each bar is the mean $\pm$ SEM of three independent experiments. * = Significantly different from the control value ($P < 0.001$).

Figure 2. Effect of endogenous PTHrP on LoVo cell proliferation, migration and invasion

(A) Proliferation. Cells were plated in 96-well dishes at 10^4 cells/well. Cell number was determined at the indicated time intervals. For si and sc, each point is the mean $\pm$ SEM of three independent experiments each for si1 and si2 or the respective scrambled (sc) siRNA control. (B and C) Migration and invasion. Cells (0.5×10^6) were loaded with Calcein-AM and plated onto FluoroBlok inserts in the absence of FBS. To measure invasion, the inserts were overlaid with Matrigel. FBS (10%) was used as the chemoattractant. Migration and invasion were measured after 4 h. In (B and C), 1 and 2 refer to two independent PTHrP siRNA-transfected cell lines. Each bar is the mean $\pm$ SEM of three independent experiments. (D) Migration was analyzed using a monolayer scratch assay. Images at 0 and 5 days after wounding are shown. The arrowed lines mark the edges of the monolayer. Magnification, $\times 10$. (E) Quantitation of cell migration 3, 4, and 5 days after wounding the cell monolayer. si is the mean $\pm$ SEM of three independent experiments each for si1 and si2. * = Significantly different from the control value ($P < 0.001$).

Figure 3. Effects of PTHrP on anchorage-independent cell growth

Assays to determine colony formation in soft agar were performed in 60 mm dishes containing a bottom layer consisting of 1.5 ml culture medium containing 0.4% (w/v) agar. Cells (1×10^4) were plated in a top layer of 0.3% agar. (A) After 2 weeks in culture, the plates were photographed at $40 \times$ magnification, and clone size was measured using the ImageJ software (NIH). (B) Photographs were also taken at $10 \times$ magnification to measure clone frequency. All clones in focus $> 50 \mu\text{m}$ in size were measured. (C) Colony size. Each bar is the mean $\pm$ SEM of 20 colonies for each of three independent clones overexpressing PTHrP (+), three independent empty vector-transfected clones (V), two independent cell lines (si1 and si2) generated by transfection with independent PTHrP siRNAs (si), or the respective scrambled siRNA sequences (sc), or parental cells (P). (D) Average number of colonies. Each bar is the mean $\pm$ SEM of three fields per plate for each of the cell lines described in (C). (C and D) * = Significantly different from the control value ($P < 0.001$).

Figure 4. Effects of PTHrP on LoVo cell xenograft growth in nude mice

Athymic nude mice were injected with LoVo cells overexpressing PTHrP, or with control cells. (A) Representative tumors from four independent PTHrP-overexpressing clones [+ (1) to + (4)], three independent empty vector-transfected clones (V1 to V3), and parental cells (P). (B) Mean volumes of tumors produced by PTHrP-overexpressing cells ($\circ$), empty vector transfectants ($\blacktriangledown$), and parental cells ($\bullet$). (C) Tumor weight at time of sacrifice. In (B) and (C), each point or bar is the mean $\pm$ SEM of tumor volumes (B) or weight (C) from four independent PTHrP-overexpressing clones, three independent empty vector clones and parental cells (6 animals/clone).

Figure 5. Immunohistochemical analysis of xenografts from PTHrP-overexpressing and control LoVo cells

(A) Staining for PTHrP, integrin $\alpha 6$, integrin $\beta 4$, and components of the PI3-K pathway is shown. H&E = sections stained with haematoxylin and eosin. IgG = sections stained with an anti-rabbit IgG antibody (negative control). + = xenograft sections from PTHrP-overexpressing cells; V = xenograft sections from control (empty-vector-transfected) cells. Each panel is representative of five sections for each of six tumors from mice injected with one of four independent PTHrP-overexpressing clones or three empty vector-transfected clones. Magnification, $\times 40$. The staining intensity for each protein was graded between 0–3, and was obtained by comparing the level of staining present and the proportion of stained cells in sections from PTHrP-overexpressing cells vs. that of control cells. (B) The pie charts summarize the expression patterns obtained from 120 sections for PTHrP-overexpressing cells (5 slides for each of 4 independent clones; 6 animals/clone) and 90 sections for the empty vector transfectants (5 slides for each of 3 independent clones; 6 animals/clone).

Figure 6. Immunohistochemical analysis for PTHrP and the integrin $\alpha 6$ and $\beta 4$ subunits in human colorectal cancer and patient-matched normal mucosa

(A) Representative expression patterns are shown for tubulovillous adenoma (Stage 0) and colorectal carcinoma (Stage II), each with patient-matched normal mucosa. Each panel is representative of a minimum of five slides for each of 20 patients. Magnification, $\times 40$. The staining intensity was graded between 0 and 3, as described in Fig. 5. (B) The pie charts summarize the expression patterns obtained from sections from 20 patients (5 slides/patient). The chart for normal sections summarizes two sets of data (normal control data from Stage 0 and Stage II patients).