

Influence of parathyroid hormone-related peptide gene in colon cancer cell line growth

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ABSTRACT

Objective: Parathyroid hormone-related peptide (PTHrP) has been found to be expressed in different human tumors including colon. We used a human colon cell line (Lovo) as a model to study the mechanism for the proliferation effects of PTHrP.

Methods: This study was conducted in the Department of Pharmacology at University of Texas Medical Branch, Galveston, Texas, United States of America and Department of Biochemistry at College of Science King Saud University, Riyadh, Kingdom of Saudi Arabia from June 2001 until September 2003. The PTHrP constructs expressing in sense or antisense orientation were constructed by cloning human PTHrP cDNA and transfected into Lovo cells. Single clone of stably

transfected cells was isolated and grown for 2, 4 and 7 days.

Results: The proliferation rate of Lovo clones with sense overexpressing PTHrP was less rapid than that of antisense or empty vector transfected cells; however, at day 7 the cell number of the antisense PTHrP overexpressing clones was more than 2-folds compared with the empty vector transfected clones.

Conclusion: The sense PTHrP gene blocks translation decrease the cell growth, which might be significant implications for the role of PTHrP in colon cancer.

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Parathyroid hormone-related peptide (PTHrP) was originally known to be the major factor causing the humoral hypercalcemia of malignancy (HHM),¹ and bone metastases development.² It has a significant amino-terminal sequence homology with the biologically active domain of parathyroid hormone (PTH).³ This homology is sufficient for PTHrP-1-34 to confer similar biological action in classical PTH target tissues such as bone and kidney via a common PTH/PTHrP receptor (PTH1R) and elicits PTH-like activity that gives rise to HHM.^{4,5} The PTH/PTHrP receptor is a 7 transmembrane glycoprotein that has been cloned from kidney and osteoblast cell lines,^{6,7} and coupled through G-proteins to a both adenylate cyclase and phospholipase C pathways.^{8,9} Malignancy-associated hypercalcemia is a very common complication in number of cancer patients, since these cancers

frequently secrete high levels of PTHrP¹⁰⁻¹² in both cytoplasm and nucleus of paraffin-embedded tumor specimens.¹³ Although, initially discovered in malignancies, PTHrP known to be produced by most cells and tissues in the body.¹⁴⁻¹⁶ Unlike PTH, PTHrP does not circulate in appreciable amounts in normal subjects but it is thought to exert its effects in an autocrine/paracrine manner.¹⁰ As cancer is predominantly a disease of disordered balance between proliferation, differentiation, and apoptosis, disruptions in the function of the calcium-sensing receptor (CaR) could contribute to the progression of neoplastic disease, and the activation of the CaR has also been linked to increased expression and secretion of PTHrP.¹⁷ In addition to its roles in calcium homeostasis, unrelated functions have been ascribed, including cellular differentiation and modulation of cell growth in normal and

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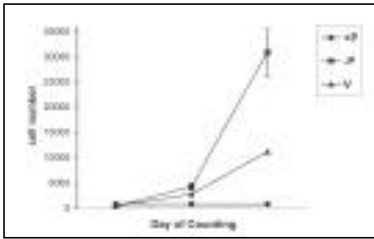


Figure 1 - Lovo cell line proliferation after expressing of PTHrP gene in sense or antisense orientation. Single clone was isolated, plated at 10^4 cells/well then the cells were trypsinized and counted at day 2, 4 and 7. Each point is the mean \pm SEM of 3 independent experiments. PTHrP - parathyroid hormone-related peptide, +P - means PTHrP construct in sense orientation, -P - means PTHrP construct in antisense orientation, V - means empty vector.

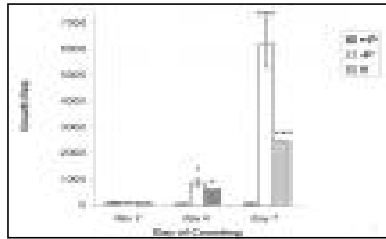


Figure 2 - Rate values of Lovo cell line proliferation expressing PTHrP in the sense or antisense orientation. Each point is the mean \pm SEM of 3 independent experiments. PTHrP - parathyroid hormone-related peptide, +P - means PTHrP construct in sense orientation, -P - means PTHrP construct in antisense orientation, V - means empty vector.

transformed cells such as prostate cancer and renal carcinoma cells.^{18,21} The purpose of this study, therefore, is to investigate the association of the sense and antisense PTHrP gene with colon cancer cell line growth.

Methods. Cell culture. Human epithelial colon tumor cell line (Lovo) were obtained from the European Type Culture Collection (ECCAC). The cells were grown to 80% confluence in defined media (F-12) (Gibco, UK) at 37°C in a humidified 95% O₂, 5% CO₂ atmosphere with 10% fetal bovine serum (FBS), L-glutamine, and 1% penicillin, streptomycin and amphotericin (PSA) (Gibco, UK). The cells were subcultured into 24 well-plates following cell dispersion with 10% trypsin/ethylenediaminetetraacetic acid (EDTA) (Gibco, UK) solution in F-12 medium.

Plasmids. The PTHrP constructs expressing PTHrP in the sense or antisense orientation were constructed by cloning hPTHrP complementary DNA (cDNA) (a generous gift from Dr. Miriam Falzon, Department of Pharmacology, University of Texas Medical Branch, Galveston, USA) coding for amino acids -5 to +139 in the expression vectors pCDNA3.1 (+) or (-) (Invitrogen, UK), respectively [(+) and (-) refer to the orientation of the multiple cloning site within the vector, relative to the direction of transcription from the T7 promoter]. The T7 primer is the upstream primer (5' TAATACGACTCACTATAGGG 3'), and downstream, the pCDNA3.1 reverse primer (5' TAGAAGGCACAGTCGAGG 3'). These primers recognize sequences within the pCDNA3.1 vector.

Transfection design and cell proliferation. The expression vectors pCDNA3.1 (+) or (-) or (v) were

stably transfected into Lovo cell lines with FuGENE 6 (Roche, Germany) transfection according to the manufacturer's specifications. Two days after transfection, 100 µg/ml G418 (Gibco, UK) was added, and resistant clones were selected. Single clones of stably transfected cells was isolated by limiting dilution and plated in 24 well plates at 10⁴ cells/well then transferred to 6 well plates and cultured in medium containing 100µg/ml G418. The cells were then trypsinized and counted at day 2, 4 and 7 in Coulter counter (Coulter Electronics Inc., USA).

Statistical analysis. All data are representative of 3 independent experiments. The statistical significance was defined using t tests, with thresholds of $p < 0.05$, $p < 0.001$.

Results. The proliferation rate of Lovo clones (+P) overexpressing PTHrP was less rapid than that of empty vector; however, after 7 days incubation the cell number of the PTHrP overexpressing clones (-P) was more than 2-folds compared with the empty vector transfected clones (V). The growth rate of antisense PTHrP transfected clones was significantly less than that of the empty vector-transfected clones (Figure 1). The percentage differences in the rate of proliferation of the sense and antisense transfected cells compared with empty vector transfected cells was shown in Figure 2. At day 4, the percentage rate of proliferation of empty vector transfected cells was almost the same as that of the sense vector transfected cells. However, at day 7 in culture the percentage of sense vector transfected cells were significantly high ($p < 0.001$) than the antisense and empty vector transfected cells.

DISCUSSION. In cancer patients, the effects of PTHrP on calcium levels are mediated via PTHrP entering the circulation and activating the PTH1R in classic PTH organs (such as bone and kidney), resulting in HHM.^{22,23} Parathyroid hormone-related peptide modulates growth in prostate and breast cancer cells,^{20,24,25} Leydig tumor cells,²⁶ renal carcinoma cells,²¹ osteoblasts²⁷ and pancreas.²⁸ Proliferative and antiproliferative effects seem to be dependent on the cell type. Parathyroid hormone-related peptide has joined the ever-increasing list of peptide hormones and growth factors that elicit their biological responses in a dual manner, through interaction with cell surface receptors linked to signal transduction cascades and through intranuclear localization.²⁹ Parathyroid hormone-related peptide contains a signal sequence that directs the nascent peptide to the secretory pathway, must avoid the endoplasmic reticulum and remain in the cytoplasmic compartment before its nuclear translocation. Various potential mechanisms have been proposed, which include the use of alternative, non-adenine, uracil, guanine (AUG) translational start sites, internalization of the ligand-receptor complex by endocytosis, and reverse translocation or dislocation from the endoplasmic reticulum lumen to the cytoplasm.^{29,30}

Our results show that the inhibition of PTHrP synthesis using an antisense oligodeoxynucleotide blocks translation of the peptide and decrease the Lovo cell proliferation. The effect of autocrine/paracrine and intracrine effects of PTHrP in Lovo cells overproducing the peptide is accelerated cell growth. Many studies have shown that PTHrP also acts in an intracrine fashion after translocation to the nucleus or nucleolus.^{19,31-33} The PTHrP action through the autocrine/paracrine pathway exerts an antiproliferative effect. These results differ from those of Birch et al.,³⁴ who found a mitogenic response to PTHrP in breast cell line. In our current study, we show that overexpression of PTHrP in Lovo cells results in a dramatic stimulatory effect on cell proliferation (**Figure 1**). Massfelder et al.¹⁹ reported a similar effect in vascular smooth muscle cells, and Henderson et al.³² have shown that nuclear PTHrP is associated with an inhibitory effect on apoptosis. Although PTHrP is clearly targeted to the nucleus in a number of systems such as vascular smooth muscle cells,¹⁹ and a nucleolar localization in chondrocytes and osteoblasts,³² and in a human keratinocyte cell line.³³ In addition, cell type specific expression of PTHrP mRNA isoforms occurred between the various cell line.³⁵ At present, it is unknown whether this difference is a reflection of the different cell types under study. It is unknown at this time the mechanism by which nuclear accumulation of PTHrP activates the cell cycle, thereby increasing

proliferation in number of cell types.^{19,24,32,33} The peptide may interact with key proteins involved in cell cycle regulation or with nucleic acids via an intracrine pathway independent of its classical nuclear localization sequence.³⁶ This novel pathway could mediate the effects of PTHrP on tumor progression. Recent study has shown that the peptide can interact with both homopolymeric and total cellular ribonucleic acid (RNA).³⁷ As the nucleolus is the major site for biogenesis of ribosomes, nucleolar PTHrP may influence cellular functions by modulating ribosomal RNA synthesis, either by affecting RNA polymerase I activity or by altering ribosome assembly or function, or both. In summary, this study shows that PTHrP produces opposing mitogenic and antimitogenic actions in the colon cancer Lovo cell line. Using PTHrP sense oligodeoxynucleotide, our data confirm that the PTHrP decreases cell proliferation when acting via the autocrine/paracrine pathway, mediated through the cell surface PTH1R.

In conclusion, when acting through the intracrine pathway, the peptide dramatically stimulates cell growth. The proliferative effects of overexpressed PTHrP seem to predominate, in that overexpression of the peptide results in accelerated cell growth. Inhibition of PTHrP synthesis using a sense oligodeoxynucleotide resulted in a net decrease in Lovo cell proliferation. The net effect of autocrine/paracrine and intracrine effects of PTHrP in Lovo cells overproducing the peptide is accelerated cell growth. As PTHrP imparts a growth advantage to colon cancer cells, we suggested the findings may lead to the development of future PTHrP therapeutic strategies for colon cancer treatment.

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