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STUDIES OF THE EFFECT OF TRANSFORMING GROWTH FACTOR-BETA ON PTHrP EXPRESSION, mRNA STABILITY, AND SECRETION IN EPITHELIAL CANCERS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

by

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ABSTRACT

Parathyroid hormone related protein (PTHrP) is similar to parathyroid hormone (PTH) and amino-termini of both proteins bind PTH1 receptors (PTH1R) in bone and kidney to increase blood calcium. Epithelial tumors such as squamous cell carcinoma may over-express and secrete PTHrP into the systemic circulation to cause humoral hypercalcemia of malignancy (HHM). Increased levels of PTHrP have also been implicated in prostate cancer bone metastases.

The occurrence of HHM in patients with head and neck squamous cell carcinomas (HNSCC) was examined in 55 HNSCC patients. No patients had increased plasma PTHrP, serum ionized calcium, or altered urine calcium/creatinine. This data indicated that HHM was rare in patients with HNSCC.

TGFβ1 has been shown to increase PTHrP mRNA transcription and mRNA stability. The role of the 3'-untranslated region (3'UTR) of PTHrP (1-141) mRNA stability was examined and demonstrated that coding region sequences were likely important in TGFβ1-induced PTHrP mRNA stability. In addition, TGFβ1 treatment of cells resulted in decreased mRNA-protein binding to the terminal coding region.
PTHrP has a complex gene structure with 3 promoters, P1, P2, and P3, which is alternatively spliced into 3 isoforms encoding 139, 173, and 141 amino acid proteins. PTHrP mRNA (1-139 and 1-141) half-life in canine squamous carcinoma cells (SCC2/88) and human squamous carcinoma cells (HARA) was short (45 min-2 hrs) and that of PTHrP (1-173, not present in dogs) was 2-5-fold that of the other isoforms. TGFβ1 increased the mRNA half-life of only PTHrP (1-141). TGFβ1 treatment. Increased secretion of PTHrP into SCC2/88 conditioned medium but not the HARA cells. TGFβ1 treatment increased the P1 promoter use in both HARA and SCC2/88.

PTHrP and TGFβ1 as well as endothelin-1 (ET-1) have been implicated in the induction of new woven bone formation to form osteoblastic metastases in prostate cancer. TGFβ1 did not increase ET-1 mRNA expression in any prostate cells. TGFβ1 increased PTHrP mRNA expression and protein secretion in prostate epithelial cells (PEC) and in prostate carcinoma cells (PCC), but decreased PTHrP mRNA in prostate stromal cells (PSC).
Dedicated to Nirvana Shaw
ACKNOWLEDGMENTS

Along this journey, I have encountered many people who have helped me progress both in science and veterinary pathology. First, of course, I must thank Thomas Rosol, my advisor, for his support both in my research and pathology training. I would also like to thank Steve Weisbrode and Paul Stromberg for their encouragement and help throughout my pathology training.

People are really the most important part of nearly all experiences in life. I feel fortunate to have had intelligent, funny, and helpful people to work with over the last 5 years: Eric Blomme, Bruce LeRoy, Sarah Tannehill-Gregg, Krista La Perle, Sunee Kunakornsawat, Ramiro Toribio, Virgile Richard, and Deborah Groppe. They made even the hardest days a little easier. Thank you. In addition, I would like to acknowledge people in other labs who were very helpful to me: Melinda Busch from Kathy Boris-Lawrie’s lab; Chris Weghorst and his student, Song; and Karen Duda and Uma Sivaprasad from Charles Brook’s lab.

Finally, I would like to thank members of my committee, Drs. Boris-Lawrie, Weghorst, Capen, and Eaton, for their suggestions and support. I would also like to thank my former mentor, Ronda Moore, for her help, support, and friendship.
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Research Publications

Sellers, R.S., Capen, C.C., and Rosol, T.J.: Messenger RNA stability and mRNA-protein binding of parathyroid hormone related protein by transforming growth factor-β in squamous carcinoma cells. Accepted, Molecular and Cellular Endocrinology.


**FIELDS OF STUDY**

Major Field: Veterinary Biosciences
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CHAPTER 1

INTRODUCTION TO PTHrP

Parathyroid hormone-related protein (PTHrP) was initially identified in 1987 as a tumor-secreted factor that was associated with humoral hypercalcemia of malignancy (HHM)\(^1\). Since that time, PTHrP has been identified in most normal tissues, and likely functions in an autocrine, paracrine, or intracrine fashion\(^4\). PTHrP and parathyroid hormone (PTH) share 8 of the first 13 amino-terminal amino acids, which is sufficient to activate the PTH-1 receptor (PTH1R) in kidney and bone to cause increased serum ionized calcium\(^5\).

The human PTHrP gene is complex, having 3 promoters (P1, P2, and P3) and 9 exons. The majority of the coding region is in exon VI (Fig. 1.1)\(^5\). The first four exons, I, II, III, and IV are noncoding exons and are variably spliced to form different 5'-untranslated regions (5'UTRs). The first coding exon (exon V) encodes the pre-pro region which is cleaved to form the mature peptide. PTHrP is alternatively spliced into 3 isoforms encoding 139, 173, and 141 amino acids, each having in common the first 139 amino acids\(^5\). The mRNAs for PTHrP 139, 173, and 141 isoform have unique 3'-untranslated
regions (3'UTRs) encoded by exons VII, VIII, and IX, respectively. Unlike the human, the dog expresses only PTHrP 139 and 141. Between the dog and human, there is 96% homology of the coding region sequences, 96% homology in exon VII and 98% homology in the exon equivalent to IX in humans. The highly conserved nature of the coding sequences and the 3'UTRs between humans and dogs suggests that they are important not only in the function of the protein, but also in the regulation of the mRNA.

Post-translational processing of PTHrP appears to have physiological roles in both the embryo and the adult. The most likely post-translationally modified forms of PTHrP include the amino-terminal fragment (1-36) with PTH-like activity, mid-region peptides (38-94, 38-95, or 38-101) which are highly conserved across species and important in stimulating calcium transport in the placenta, and the carboxyl-terminal fragment (107-139) which has been demonstrated to inhibit osteoclastic bone resorption (osteostatin). While only a single G protein-coupled 7-transmembrane domain receptor for the amino-terminus of PTHrP has been identified (a shared PTH/PTHrP receptor, PTH1R), it is believed that there are mid-region and carboxyl-region receptors for PTHrP.

PTHrP also has a nuclear localization signal (aa 87-107) and is frequently identified in the nucleus. In addition, non-traditional in frame translation initiation start sites of PTHrP mRNA are suggested to result in a form of PTHrP that functions in an intracrine fashion, translocating to the nucleus without secretion. Its function in the nucleus is uncertain, but it has been
demonstrated to localize to regions of nucleoli. It is theorized to localize with ribonucleoprotein complexes and may be important in inhibiting apoptosis and inhibits rRNA synthesis in chondrocytes.

The regulation and function of PTHrP in normal cells has not been completely elucidated. It is known to be essential for normal development (e.g. placental calcium transport, skeletogenesis, and mammogenesis), and likely plays an important role in growth and differentiation of epithelial cells in the adult. In cancer, PTHrP is thought to have important roles in tumor growth, invasion, and metastasis, as well as with paraneoplastic diseases. The paraneoplastic disease most commonly associated with PTHrP is cancer-associated hypercalcemia due to epithelial cell malignancies. A second syndrome that PTHrP is believed to have an important role is in the development of osteoblastic metastases, in which new bone is induced by prostate carcinoma, almost exclusively, at sites of bone metastasis.

PTHrP is the cause of 80% of the cases of paraneoplastic hypercalcemia. Squamous cell carcinomas (SCC) and other carcinomas express and secrete PTHrP. However, only a small percentage of people develop hypercalcemia, ranging from 0-10%. HHM is associated primarily with epithelial lung tumors, and less frequently with skin and oral squamous cell carcinomas. The reason some people develop hypercalcemia while others do not is uncertain. The cause is likely multifactorial; a combination of increased PTHrP mRNA expression, altered secretion of biologically active PTHrP, and possibly altered degradation of the PTHrP either intra- or extracellularly.
Osteoblastic lesions associated with bone metastasis is a significant cause of morbidity for patients with prostate cancer and breast cancer. Prostate cancer is unique in its metastatic behavior, because it targets bone where it induces new bone formation as well as causing bone lysis. PTHrP can cause bone lysis by activating PTH1 receptors on osteoblasts, which may release osteogenic factors from the bone matrix such as bone morphogenetic proteins (BMPs), insulin-like growth factors (IGFs), and basic fibroblast growth factor (bFGF). Conversely, the carboxyl-terminus of PTHrP (osteostatin) can promote new bone formation by increasing osteoblast differentiation and decreasing osteoclast activation.

Alterations in the regulation of PTHrP mRNA expression, secretion, and degradation by cytokines or other mechanisms are likely important in the development of these paraneoplastic syndromes. PTHrP mRNA expression, stability, and protein secretion has been demonstrated to be increased in both normal and neoplastic cells by transforming growth factor-beta1 (TGFβ1) as well as epidermal growth factor (EGF). It has recently been demonstrated that regulation of mRNA stability is an important mechanism of post-transcriptional gene regulation. PTHrP has a very short half-life, ranging from 30-120 minutes, depending on the cell line. Therefore, changes in PTHrP mRNA stability may be an important mechanism for modulating PTHrP mRNA levels. It is possible that certain paraneoplastic syndromes induced by PTHrP are dependent on TGFβ1 to induce PTHrP gene transcription and regulate PTHrP mRNA stability.
In normal epithelial cells, TGFβ1 functions as a growth inhibitor and tumor suppressor gene\textsuperscript{51}. However, in neoplastic epithelial cells, TGFβ1 expression has long been reported as an important factor in tumor progression, invasion, and metastasis\textsuperscript{51-53}. Interestingly, TGFβ1 has been demonstrated to increase in tumor cells. The work I have accomplished in my doctoral research has focused on the effects of TGFβ1 on the expression and secretion of PTHrP in specific cell types, specifically squamous carcinoma cells and prostate cells. My initial studies examined the incidence of hypercalcemia in human patients with head and neck squamous cell carcinoma at various stages of disease. In these patients, I measured serum ionized calcium, urinary calcium, and plasma PTHrP. In the following studies, I focused on the effect of TGFβ1 on the induction and stability of PTHrP mRNA. These studies included an examination of the specific PTHrP isoforms (PTHrP 1-139, 1-141, or 1-173) upregulated and stabilized by TGFβ1, as well as the determining the role of the 3'UTR on PTHrP mRNA stability. My final research focused on the regulation of PTHrP by TGFβ1 in both normal and neoplastic prostate cells as a potential contributing factor in the development of osteoblastic metastases.

REFERENCES


Figure 1.1 PTHrP gene is transcribed from 3 promoters, P1, P2, and P3 and has 9 exons. Coding region exons have filled boxes, while untranslated regions are delineated as open boxes. The known splicing patterns are shown as solid lines connecting exons. Together, the alternate splicing patterns may generate 15 mature transcripts. Alternate splicing to exons VII, VIII, or IX result in 3 protein isoforms: 139, 173, and 141 amino acids.
CHAPTER 2

HEAD AND NECK SQUAMOUS CELL CARCINOMA: MEASUREMENT OF PLASMA PTHrP AND SERUM AND URINE CALCIUM.

ABSTRACT

Parathyroid hormone-related protein (PTHrP) is expressed by squamous cell carcinomas. Our first objective was to examine the stability of PTHrP in normal human plasma. Our second objective was to determine if plasma PTHrP could be used in patients with head and neck squamous cell carcinoma (HNSCC) as an indicator of tumor burden or relapse. Blood and urine samples from 55 HNSCC patients undergoing tumor resection at The Ohio State University were measured for plasma PTHrP (1-86) concentration, serum ionized calcium concentration, and urine calcium:creatinine ratio. Two of 55 HNSCC patients had detectable levels of plasma PTHrP. Serum ionized calcium concentrations and urinary calcium:creatinine ratios were within normal limits in all patients. Plasma PTHrP was not a valuable indicator of tumor presence or recurrence in our patient population. Plasma PTHrP 1-86 is not a useful marker of tumor presence or recurrence in patients with stage II-IV or recurrent HNSCC.
INTRODUCTION

Parathyroid hormone-related protein (PTHrP) is expressed in a wide variety of normal tissues, including keratinocytes, and acts in an autocrine or paracrine manner to promote differentiation and proliferation \textsuperscript{1-3}. Studies on the role of PTHrP on keratinocytes indicates that it plays an important role in keratinocyte differentiation and hair follicle development \textsuperscript{2}. PTHrP has been shown to be overexpressed in squamous cell carcinoma \textsuperscript{4,5}. In addition, growth factors such as transforming growth factor-\(\beta\) (TGF-\(\beta\)), a cytokine commonly produced by carcinomas, increases PTHrP mRNA expression and stability in keratinocytes \textit{in vitro} \textsuperscript{6,7}.

PTHrP is related to parathyroid hormone (PTH), but is distinct from and more complex than PTH \textsuperscript{8}. PTH and PTHrP share 8 of the first 13 amino acids at the amino terminus, but no homology in the mid- or carboxyl regions \textsuperscript{2,8}. Both PTH and PTHrP (1-36) bind to PTH1 receptors in bone and kidney to increase serum calcium concentration \textsuperscript{1-3,9}. Carcinoma expression of PTHrP has been associated with increased circulating PTHrP concentrations in most patients with the paraneoplastic syndrome humoral hypercalcemia of malignancy (HHM) \textsuperscript{5,10,11}. The pathogenesis of paraneoplastic hypercalcemia in solid malignancies such as squamous cell or mammary carcinoma, and hematologic malignancies such as lymphoma \textsuperscript{9,12}, is primarily associated with secretion of tumor-derived factors which increase renal reabsorption of calcium and increase osteoclastic bone resorption. PTHrP is the most important of these factors \textsuperscript{9,12}. Other factors
act cooperatively or synergistically with PTHrP to induce hypercalcemia and include interleukin-1, 4, and 6, tumor necrosis factor, transforming growth factor-β and prostaglandin E₂. Humoral hypercalcemia of malignancy has been reported in 2-20% cancer patients and may cause fatal renal failure if inadequately controlled. While head and neck cancer accounts for 4% of all reported cancers each year, the incidence of hypercalcemia associated with this form of cancer has been infrequently reported.

There is little data available on plasma PTHrP concentrations in HNSCC or the stability of PTHrP in plasma. The first objective of this study was to measure circulating concentrations of biologically active PTHrP and serum and urine calcium concentrations in patients with head and neck squamous cell carcinoma (HNSCC) undergoing surgical tumor resection. We examined whether measurement of plasma PTHrP concentrations in patients with HNSCC would be a sensitive method of detecting tumor burden, recurrence, or metastasis in the patients. Since PTHrP is a labile protein and rapidly degraded by plasma proteases, our second objective was to investigate the stability of recombinant human PTHrP (1-86) (rhPTHrP) in blood.

**MATERIALS AND METHODS**

*Plasma PTHrP stability:* The degradation rate of recombinant human PTHrP 1-86 (rhPTHrP) in whole blood was measured. Blood from 10 normal adults was collected into either EDTA tubes or Nichols PTHrP Collection Tubes (Nichols...
Institute Diagnostics, San Juan Capistrano, CA) containing heparin, aprotonin (500 KIU/ml), leupeptin (5 ng/ml), pepstatin (5 ng/ml), and EDTA (1 mM). Samples were spiked with rhPTHrP (1-86) (25 pM) and stored at 4°C for up to 24 hours. Aliquots were removed at baseline and at 0, 1, 3, 6, 12, and 24 hours after addition of PTHrP, centrifuged, and the plasma frozen at -20°C. Samples were assayed for PTHrP (1-86) using a two-site immunoradiometric assay (IRMA) for PTHrP by Dr Bruce Leuddecke, Incstar, Inc., Stillwater, MN.

Sample procurement: Samples of blood and urine from 55 head and neck squamous cell carcinoma (HNSCC) patients were collected by the Comprehensive Human Tissue Network at the James Cancer Hospital and Research Institute at The Ohio State University during an 8-month period from February 1998 to October 1998. Samples were obtained at the time of surgery for excision or debulking of oral, nasopharyngeal, or laryngeal squamous cell carcinomas. Tumors were staged using the TNM method \(^\text{16}\) prior to surgery unless the patient had recurrent disease. Of the patients presented for initial surgery, none were stage I (primary tumor < 2 cm, no lymph node involvement), 12% were stage II (primary tumor >2 cm but <4 cm, no lymph node involvement), 33% were stage III (tumor > 4 cm, no lymph node involvement or tumor < 4 cm with one lymph node involved), and 55% were stage IV (tumor invaded adjacent structures with or without lymph node involvement or any size tumor with 2-3 lymph nodes involved).
Plasma PTHrP: Blood from patients was collected in EDTA tubes and centrifuged at 4°C. The plasma was removed and stored at -20°C within 6 hours of acquisition. PTHrP concentrations were measured by two-site immunoradiometric assay (IRMA) (Incstar, Inc., St. Paul, MN).

Serum ionized calcium: Blood for measurement of serum ionized calcium concentration was obtained anaerobically in tubes without serum separators, allowed to clot, centrifuged at 4°C, and stored at 4°C until analysis. pH adjusted serum ionized calcium was measured within 20 hours of collection with a Nova8 analyzer (Nova Instruments, Waltham, MA).

Urine calcium: Urine was acidified with 6 N HCl to pH 3-4 and stored at -20°C. Lanthanum chloride was added to samples to a final concentration of 1 mg/ml prior to analysis to reduce the effect of urinary phosphorous on the calcium values. Calcium concentrations were measured by atomic absorption spectrophotometry at 422.7 nm through an air-acetylene flame in a Varian Spectra AA-10/20 (Varian Australia Pty Ltd, Australia). Standard solutions were aliquoted into 0.1 N HCl with 1 mg/ml lanthanum chloride.

Urine creatinine: Urine creatinine was measured by colorimetric assay. Briefly, 0.1 N NaOH and 0.92% picric acid were added to diluted samples. After a 45 minute incubation at room temperature, samples were measured with a microplate reader (Molecular Devices) at a wavelength of 490 nm.
RESULTS

*Stability of plasma PTHrP:* Recombinant human PTHrP (1-86) was stable (>95%) in whole blood at 4°C in both Nichols Institute PTHrP collection tubes and EDTA tubes for at least 6 hours. The percent of PTHrP degraded as well as the standard deviation increased gradually over time. The percent of PTHrP remaining in Nichols and EDTA tubes were: 1 hour, 99.7±1.5 % and 99.5±2.4 %; 3 hours, 98.3±2.8 and 98.9±3.2 %; 6 hours, 98.1±3.7 % and 97.3±2.2 %; 12 hours, 91.3±6.6 % and 94±6 %; 24 hours, 87.2±11.1 % and 88.4±11 %. No subject had detectable levels of PTHrP in their baseline samples (Fig. 2.1).

*Patient population:* The patients in this study comprised both those with recurrent (22/55) or first-time surgery (33/55) for HNSCC. Tumors were staged only for those patients without prior surgeries. Of the patients presenting for first surgeries, 12% were stage II, 33% were stage III, and 55% were stage IV. The average age was 60.1 years and the median age was 62 years. Ages ranged from 17-91 years. The majority of patients were male (69.6%).

*Plasma PTHrP:* The majority of patients had plasma concentrations of PTHrP below the threshold of the IRMA (<0.2 pM). Of the 55 patients, only 2 had concentrations above 0.2 pM, ranging from 0.30-0.34 pM and both patients had normal serum ionized calcium and urinary calcium excretion (Fig. 2.2).
Serum ionized calcium: The average serum ionized calcium was 1.14 mM (normal range=1.13-1.32) with a range of 1.10-1.33 mM. No patient had increased serum ionized calcium concentration (Fig. 2.3).

Urine calcium excretion: All patients had urinary calcium excretion within the normal range. The average calcium (mM)/creatinine (mM) ratio was 0.019 ranging from not detectable to 0.18 (Fig. 2.4). Normal ratios are reported to be less than 0.4, and hypercalcemia is defined as ratios greater than 0.57 19.

DISCUSSION

Parathyroid hormone-related protein secretion by squamous cell carcinoma has been demonstrated to be a primary mediator of humoral hypercalcemia of malignancy 9-12. This study revealed that increased plasma PTHrP concentration and HHM are uncommon in patients at the time of surgery with stage II-IV and recurrent head and neck squamous cell carcinoma. While hypercalcemia has been reported in 2-20% of people with cancer 9,12, this study found no abnormalities in the plasma PTHrP, serum ionized calcium or urine calcium excretion in a population of 55 patients. Most patients had plasma PTHrP levels below the assay's detectable level of 0.2 pM. This indicates that plasma PTHrP is not a reliable marker of the tumor burden, presence, or recurrence in HNSCC. Although our population was too small to define the incidence of HHM in patients with head and neck squamous cell carcinoma, it
was clear that this syndrome is uncommon in our population of cancer patients, which included patients with recurrent neoplasms or advanced disease (stage III to IV).

Although case reports of increased total serum calcium and plasma PTHrP in squamous cell carcinomas are common, few studies have specifically examined these values in patients with head and neck squamous cell carcinoma. In addition, few reports have measured serum ionized calcium concentration. Serum total calcium concentration, in contrast to serum ionized calcium concentration, can be altered by serum phosphate, lactate, citrate, bicarbonate, and albumin concentrations. Serum ionized calcium, measured in this study, is a more accurate representation of the patient’s calcium concentrations compared to total calcium, rendering subtle changes in calcium homeostasis more evident. However, none of the patients examined had increased serum ionized calcium, suggesting that this also is not a useful tool to detect tumor presence or relapse in HNSCC patients.

A previous study of patients with advanced head and neck squamous cell carcinoma reported that hypercalcemia occurred in 4 of 37 patients. These patients had increased serum concentrations of carboxyl-terminal PTHrP (109-141) which correlated linearly to the corrected serum calcium concentration. PTHrP is a prohormone which becomes enzymatically cleaved into amino, mid-region, and carboxyl-terminal peptides. The significance of C-terminal PTHrP concentrations in patients with hypercalcemia is uncertain because the function of C-terminal PTHrP has not entirely been elucidated, but
may have anti-osteoclastic functions. In addition, the circulating half-life of C-terminal PTHrP is unknown, but is likely greater than N-terminal PTHrP (1-86) and may, therefore, be more easily detected in cancer patients. However, intact PTHrP (1-86) is a more specific method of measuring biologically active PTHrP in the plasma of patients with cancer.

While all squamous cell carcinomas, including those of the head and neck, express PTHrP as demonstrated by immunohistochemistry and in situ hybridization, the reason why some patients have detectable levels of plasma PTHrP and/or develop hypercalcemia and others do not has not been determined. The presence of PTHrP in the plasma and the development of hypercalcemia could be related to the 1) level of secretion of biologically active PTHrP 2) tumor or stromal cell expression of PTHrP-inducing cytokines such as TGF-β 3) size of the tumor (tumor burden) 4) methylation status of the PTHrP gene 5) location of the tumor 6) capacity to degrade PTHrP locally and systemically and 7) ability of a patient to maintain calcium homeostasis. Future work to understand which of these factors impact the development of abnormalities in calcium homeostasis in patients is essential to fully understanding the pathophysiology of this paraneoplastic syndrome and to determine which patients are most at risk for the development of HHM.
REFERENCES


Ref Type: Report


Fig. 2.1. Stability of rhPTHrP (1-86, 25 pM) in plasma at 4°C. There was no difference in PTHrP degradation in EDTA and Nichols Institute PTHrP collection tubes. No subject had detectable levels of PTHrP in their baseline sample.
Fig. 2.2. Plasma PTHrP: Plasma PTHrP concentrations were less than 0.2 pM in 53/55 patients. Two patients had PTHrP levels of 0.3 and 0.34 pM.
Fig. 2.3 Serum ionized calcium: Serum ionized calcium concentrations of 55/55 patients ranged from 1.10 to 1.33 mM and were within the range of normal (1.13-1.32 mM).
Fig. 2.4. Urine calcium:creatinine ratios: Urine calcium (mM):creatinine (mM) ranged from not detectable to 0.18 in 55/55 patients and was within the normal range (<0.4).
CHAPTER 3

MESSENGER RNA STABILITY OF PARATHYROID HORMONE-RELATED PROTEIN REGULATED BY TRANSFORMING GROWTH FACTOR-β1

ABSTRACT

Humoral hypercalcemia of malignancy (HHM), a paraneoplastic syndrome associated with epithelial cancers, including squamous cell carcinoma, is due to expression and secretion of parathyroid hormone-related protein (PTHrP). Transforming growth factor-β1 (TGFβ1), expressed by many tumors, has been demonstrated in vitro to increase the half-life of PTHrP mRNA. In this study, oral squamous carcinoma cells (SCC2/88) had a 2-fold increase in PTHrP mRNA stability (from 45 to 90 minutes) in response to treatment with TGFβ1. In order to examine the mechanism of TGFβ1-mediated PTHrP mRNA stability, a cell-free assay of mRNA degradation was utilized in which the degradation of in vitro-transcribed mRNA incubated with cytoplasmic protein extracts from SCC2/88 treated with vehicle or TGFβ1 was measured. In this assay, full-length PTHrP mRNA was not significantly stabilized in TGFβ1-treated samples when compared to vehicle treated samples. However, there was a striking (>5-fold)
increase in PTHrP mRNA half-life in TGFβ1-treated samples when PTHrP mRNA lacked the 3'-untranslated region (3'-UTR). In contrast, the degradation of 3'-UTR-truncated PTHrP mRNA using the cell-free assay was not altered in vehicle-treated samples. UV cross-linking of PTHrP mRNA and cytoplasmic proteins from cells treated with either vehicle or TGFβ1 revealed numerous mRNA-binding proteins. TGFβ1 treatment resulting in decreased binding of 33, 31, 27, 20 and 18 kD binding proteins to the terminal coding region. These studies revealed that TGFβ1-induced PTHrP mRNA stability might be, in part, the result of cis-acting sequences within the coding region of the PTHrP mRNA.

INTRODUCTION

Production and secretion of parathyroid hormone-related protein (PTHrP) by certain cancers can result in increased plasma PTHrP concentration and potentially life threatening hypercalcemia (humoral hypercalcemia of malignancy, HHM) \(^1\text{–}^3\). Parathyroid hormone related protein (PTHrP), a protein similar to, but distinct from, parathyroid hormone (PTH), acts in an autocrine, paracrine, or intracrine fashion. The protein is essential for normal fetal development, placental calcium transport, and fetal calcium homeostasis \(^4\text{–}^8\). Its exact function in adults has not been completely elucidated, but is believed to be important in growth and differentiation as well as cell proliferation and apoptosis \(^9\). High concentrations of PTHrP and its mRNA are produced in normal epithelial cells, including keratinocytes \(^10\text{–}^{11}\). Cancers that have been
associated with PTHrP overexpression include epithelial tumors (such as squamous cell carcinoma, SCC) and lymphoma. HHM has been reported in 0 to 20% of cancer patients and may cause fatal renal failure if inadequately controlled. While all SCC produce PTHrP, an explanation for the development of HHM in only a subset of patients has not been elucidated.

Many cancers, including carcinomas, express transforming growth factor-β1 (TGFβ1) and epidermal growth factor (EGF) which can increase PTHrP mRNA transcription and stability and protein secretion in vitro. These factors may act in an autocrine or paracrine manner in neoplasms to increase PTHrP mRNA and protein expression and contribute to the development of HHM.

PTHrP (1-141) has a very short mRNA half-life, with estimates ranging from 30-120 min. The short half-life of PTHrP mRNA suggests that strict control of PTHrP gene expression is important biologically. Regulation of mRNA turnover is an important mechanism of post-transcriptional control of gene expression. The half life of PTHrP mRNA can be increased in vitro 2-4 fold by cytokines such as TGFβ1 and EGF. The mechanism of TGFβ1-mediated PTHrP mRNA stabilization has not been extensively examined. Conserved sequences in the 3'-untranslated region (3'UTR) of PTHrP suggest that there may be regulatory elements that function with TGFβ1 to alter the rate of decay of PTHrP mRNA. In this investigation, we demonstrated that loss of the 3'UTR resulted in marked stabilization of the PTHrP mRNA in TGFβ1-treated samples. In addition, we demonstrated that loss of the 3'UTR had no...
effect on PTHrP mRNA stability in vitro without exposure to TGFβ1. We suggest that the TGFβ1-induced increase in PTHrP mRNA stability is the result of interactions of trans-acting factors with cis-acting sequences in the terminal coding region rather than the 3'UTR. This stabilizing effect, however, is effectively reduced in the presence of the 3'UTR. Tumor expression of TGFβ1, by increasing PTHrP mRNA stability, may be important in the pathogenesis of HHM in patients with squamous cell carcinoma.

MATERIALS AND METHODS

Cell Lines and Culture Conditions: Squamous carcinoma cells (SCC2/88), originally isolated from a canine oral carcinoma, were grown on 10 cm tissue culture plates in William's E medium (Life Technologies, Inc., Gaithersburg, MD) with 2 mM l-glutamine (Life Technologies), 50 μg/ml gentamicin, 10% fetal calf serum, 1 ng/ml epidermal growth factor (EGF) (Life Technologies), and 10 μM cholera toxin (Calbiochem, Inc., San Diego, CA). These cells have been characterized by our laboratory and secrete PTHrP. Both PTHrP mRNA expression and protein secretion were increased by TGFβ1. At 60-80% confluence the cells were placed in William's E basal medium (2 mM l-glutamine and 50 μg/ml gentamicin) for 16-24 hrs. Cells were then treated with 3 ng/ml recombinant human TGFβ1 (generously donated by Genetech, Inc., San Francisco, CA) or the equivalent volume of vehicle (4 mM HCl, 0.1% bovine serum albumin) for 2 hrs.
**PTHrP mRNA stability in intact cells:** The transcription inhibitor, 5,6-dichloro-1-beta-ribofuranosyl benzimidazole (DRB) was added to the cell cultures 2 hrs after incubation with vehicle or TGFβ1. Total RNA was extracted from the cells at 0, 0.5, 1, 2, and 4 hrs post-treatment with DRB in Trizol reagent (Life Technologies) followed by chloroform extraction and ethanol precipitation. Total RNA (20 μg) was separated on a 1% formaldehyde-agarose gel and transferred to a nylon membrane (Duralon, Life Technologies) and crosslinked with UV light (Stratalinker, Stratagene, La Jolla, CA). Membranes were hybridized with [³²P]-labeled cDNA for PTHrP (#661, PTHrP 1-141, obtained from Genetech), and 18S rRNA (Invitrogen) in QuikHyb (Stratagene). Blots were developed by autoradiography and quantitated using a Phosphorimager (Molecular Dynamics, 425F-120, Sunnyvale, CA).

**PTHrP Plasmids and 3'-UTR-Deleted Templates:** pGEM (Promega) containing PTHrP 1-141 (exons VI and IX) (PRP-1) was kindly supplied by William Philbrick, PhD, Yale University, New Haven, CT. The construct contained 10 bases of the 5'-untranslated region (5'-UTR), all of the coding sequence, and the entire 3'-untranslated region (3'-UTR) followed by 30 adenylates (polyA tail). The plasmid was linearized with either Kpn I to generate a full-length mRNA or with Sty I to generate a mRNA devoid of the majority of the 3'-UTR including all four AUUUA sequences (truncated) for use as templates to generate cDNAs by PCR. These were purified from a 1.5% agarose gel (Qiagen Quick Gel Purification
Kit, Qiagen, Valencia, CA). Forward primers included a T7 polymerase sequence (underlined)

5'-TAATACGACTCACTATAGGGAGAACGATGCAGCGGAGACTGGT. The reverse primer to generate template for the full-length PTHrP mRNA was 5'-T30-TGTCCTTGGGAAGGTCTCTGC, and for the truncated PTHrP mRNA was 5'-T30-ATGGACAAAATAAAATTATGG. PCR was conducted utilizing a PCR kit (Life Technologies) using 1X PCR buffer, 2.5 mM MgCl2, 0.2 mM dNTPs, 5' and 3' primers (0.2 μM), and 1U Platinum Taq polymerase (Life Technologies) at 95°C for 2 min followed by 35 cycles of 95°C, 55°C, and 72°C of 1 min each. The size of PCR-amplified cDNA sizes (1100 nt for the full-length and 595 nt for truncated PTHrP) were confirmed using a 1.5% agarose gel. Samples were treated with 200 ug/ml proteinase K (Sigma, St. Louis, MO), extracted with 25:24:1 phenol:chloroform:isoamyl alcohol (Sigma), and the cDNA precipitated in 70% ethanol.

Generation of [32P]-labeled PTHrP mRNA in vitro: The DNA templates were transcribed with the Maxi-Script kit (Ambion, Austin, TX). Briefly, 1 μg template DNA was mixed with 1 μl of 100 μM CTP and ATP, 2 μl 10X transcription buffer, 1 μl of either 1:1 ratio of 7-methyl GTP:GTP (100 μM) (for mRNA) or 1 μl of 100 μM GTP (for RNA), 3 μl of 2 μM UTP, 5 μl of 3000Ci/mmol α-[32P]-UTP, 10 U RNase inhibitor (Life Technologies), and 10 U T7 polymerase (Ambion). Samples were incubated at 37°C for 1 hr, heated to 95°C for 3 min, and placed on ice. The DNA template was degraded with 2 U of RNase-free DNase I.
(Ambion) at 37°C for 15 min. Messenger RNA samples for the cell-free mRNA decay assay were purified using a 4% Urea-PAGE gel to ensure that full-length polyadenylated mRNA was obtained. To the mRNA, 20 μl of RNA loading buffer (Ambion) was added, and the samples were vortexed, heated to 95°C for 3 min, chilled on ice, vortexed, pulse centrifuged, and loaded onto a 0.75 mm 5 M urea 8% acrylamide (17:1 bis-acrylamide). The gel was run at 250 volts for 1.5 hrs, then exposed to x-ray film to reveal the mRNA, and the bands excised. The gel pieces were minced and placed in 400 μl of high salt gel extraction buffer (25 mM Tris-Cl, pH 7.6, 0.1% SDS, 400 mM NaCl) plus 20 μg/ml proteinase K overnight at room temperature, as described by Ford and Wilusz. Samples were extracted with acid phenol:chloroform (Ambion), precipitated in isopropanol, and washed with 70% ethanol. RNA for the UV-cross-linking assay was extracted with acid phenol:chloroform (Ambion), precipitated with isopropanol, and washed with 70% ethanol. The mRNA was resuspended in 20-50 μL DEPC-treated water (Sigma) and counted in a scintillation spectrophotometer.

S100 cytoplasmic protein extraction: SCC 2/88 cells were washed twice with ice cold phosphate-buffered saline (PBS) and scraped into PBS. Cells were pelleted at 500 xg at 4°C, resuspended in homogenization buffer (10 mM TRIS pH 7.4, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT) plus 1.5 mM NaOrthovanadate (Sigma), 50 mM NaF (Sigma), 10 μg/ml pepstatin (Roche, Indianapolis, IN), 33 μg/ml Aprotinin pepstatin (Roche), and 0.3 mM 32
phenylmethanesulphonyl fluoride (PMSF) (Sigma) and allowed to swell on ice for 20 min. Samples were homogenized with 20 slow strokes of a Dounce homogenizer and membranes and nuclei were pelleted at 12,000 xg for 2 min at 4°C. Supernatants were measured and transferred to a 5 ml ultrrafuge tube and 0.11 volumes of 10X extraction buffer (100 mM TRIS pH 7.4, 15 mM MgCl₂, 1.5 M KCl, 5 mM DTT) were added. The extract was centrifuged at 100,000 xg at 4°C for 1 hour and the supernatant (S100) frozen in liquid nitrogen. Proteins were quantitated in triplicate utilizing the Bradford Method in a 96-well ELISA plate with bovine serum albumin (BSA) standards.

Cell-free mRNA Degradation Assay: The assay was performed as described by Ford and Wilusz. [³²P]-labeled mRNA (1.5 x 10⁵ cpm) was mixed with 40 μg of S100 extract from TGFβ1- or vehicle-treated SCC 2/88 cells, 500 μg polyA (Amersham Pharmacia Biotech, Uppsala, Sweden), 0.7 mM ATP (Sigma), 17.5 mM phosphocreatinine (Sigma), 15 U RNAsin (Life Technologies), and brought to equal volumes. Samples were removed in triplicate at 0, 15, 30, 45, 75, and 120 min and placed into stop buffer (final concentration of 50 mM NH₄OAc, 10 mM EDTA). The mRNA was extracted with an equal volume of acid phenol:chloroform followed by isopropanol precipitation with 20 μg tRNA (Life Technologies) and a 70% ethanol wash. Samples were resuspended in 10 μl DEPC-treated water and 10 μl of RNA loading buffer (Ambion), heated to 55°C for 15 min, chilled on ice, vortexed, pulse centrifuged, and loaded onto a 1% agarose-formaldehyde gel. The gel was run at 60 V for 30 min, transferred to a
nylon membrane (Duralon, Life Technologies) and UV cross-linked (Stratalinker, Stratagene). Radioactivity was quantitated by Phosphorimager analysis (Molecular Dynamics).

**Generation of cDNA templates for UV cross-linking Assay:** The PTHrP cDNA (exons VI and IX, PTHrP 1-141) was excised from PRP-1 by HindIII and EcoRI and purified from a 1.5% agarose gel. Five portions of the coding region and 3'UTR were amplified by PCR using 5 sets of primers (for regions 1-5). All forward primers were tagged with a 5'-T7 promoter (TAATACGACTCACTATAGGGA) for in vitro transcription, followed by the sequence of interest (Table 3.1). Region 1 extended from 3 nt upstream of the start codon (-3) to +131 into the coding region; region 2 extended from +113 to +448; region 3 extended from +345 to +508; region 4 extended from +424 to +560; region 5 encompassed the entire 3'UTR (+533 to end). PCR was conducted as described above. Samples were treated with 200 μg/ml proteinase K (Sigma), extracted with phenol:chloroform:isoamyl alcohol (Sigma), and the cDNA precipitated with 70% ethanol.

**UV Cross-Linking Assay:** S100 extract (10 μg) was incubated with 200,000 cpm of mRNA. To this reaction was added 0.2 μg/ml BSA (Amresco, Solon, OH), 8 U RNase inhibitor (Life Technologies), and 40 μg/ml tRNA (Life Technologies). Samples were incubated on ice for 20 min, and crosslinked with UV light (0.25J total energy, Stratalinker, Stratagene) on ice. Samples were treated with 1 μg/μl
RNAse A (Sigma) at 37°C for 30 min. Loading buffer (0.35 M Tris-Cl pH 6.8, 30% (v/v) glycerol, 12% (w/v) SDS, 0.6 M DTT, and 0.18 M bromphenol blue) was added, samples were heated to 95°C for 3 min, and separated on a 12% SDS-PAGE gel with 37:1 acrylamide:bis-acrylamide (Ambion). The gel was run at 4°C for 11 hrs at 30 mA. To ensure that the bands on the gel represented RNA:protein interactions and not RNA hybrids, 10- and 100-fold excess unlabeled mRNA was added to subsequent samples. Molecular weight standards were used to estimate the size of RNA-binding proteins (Rainbow Molecular Weight Markers, Amersham Pharmacia Biotech, Piscataway, NJ). Differences between RNA-protein binding between vehicle and TGFB1-treated samples were quantitated by phosphorimager with background subtracted from band values.

Statistical Analysis: Data from the mRNA stability assays were analyzed using a single factor ANOVA analysis to determine the significance of differences between mean values. Probability (p) values less than 0.05 were considered statistically significant. Graphical representation of data includes the mean ± the standard error.

RESULTS

PTHrP mRNA stability was increased by TGFB1 treatment in squamous carcinoma cells: PTHrP mRNA stability in the squamous carcinoma cells (SCC 35
2/88) was measured after treatment with TGFβ1 and the transcription inhibitor, DRB. Northern blot analysis of the samples (Fig. 3.1A) demonstrated that the half-life of PTHrP mRNA in SCC2/88 cells ranged from 30-45 min. Treatment of SCC2/88 cells with TGFβ1 for two hr prior to the addition of DRB resulted in a 2-fold increase in PTHrP mRNA stability with the half-life increasing to 90 min (Fig. 3.1B). Comparison of mRNA between TGFβ1-treated cells and controls demonstrated that PTHrP mRNA stability with TGFβ1 was significantly increased from controls (p<0.05) at 30 and 60 min (ANOVA).

*PTHrP mRNA turnover was recapitulated in cell free assay:* The use of a cell-free assay permitted rapid and highly reproducible examination of PTHrP mRNA stability. The assay has been shown to be highly reproducible in other studies of mRNA stability. The cell-free assay, however, does not evaluate the effects of translation or nuclear proteins on mRNA stability. To determine if the cell-free assay recapitulated mRNA stability in cells, stability of full-length 32P-labeled PTHrP mRNA was measured using S100 cytoplasmic protein extracts. Full-length PTHrP mRNA stability was similar to that in cell culture, with a half-life of approximately 38 min. The effect of TGFβ1 treatment on PTHrP mRNA stability in SCC2/88 cells was compared to that of vehicle-treated samples at each time point, 0, 15, 30, 45, 75, and 120 min (Fig. 3.2A). TGFβ1 increased PTHrP mRNA stability at 15 min (not statistically significant), but there was no evident difference at later time points (Fig. 3.2B). The difference in TGFβ1-induced PTHrP mRNA stability between the cell-free assay compared
to intact cells may be due to characteristics of the cell-free assay conditions, lack of transcription/translation, or absence of important nuclear factors.

Stability of PTHrP mRNA without the 3'UTR was increased in response to TGFβ1 in the cell-free assay: Because the 3'UTR of PTHrP mRNA contains highly conserved and AU-rich sequences, we and others have hypothesized that the 3'UTR is important not only in TGFβ1-mediated stability, but also mRNA turnover. In order to measure the effect of the 3'UTR on TGFβ1-enhanced PTHrP mRNA stability, the cell-free assay was performed with a truncated form of the PTHrP mRNA in which no 3'UTR was present (Fig. 3.3A). Truncation of PTHrP mRNA had no effect on PTHrP mRNA stability with cytoplasmic extracts from vehicle-treated SCC 2/88 cells compared to the full-length PTHrP mRNA (t1/2 ~ 30 min) (Fig. 3.3B). This demonstrated that the rapid turnover of PTHrP mRNA in vitro was not dependent on AU-rich sequences in the 3'UTR. However, TGFβ1 dramatically increased PTHrP mRNA half-life 5-fold (t1/2>120 min) (p<0.05, ANOVA) when the truncated form of PTHrP mRNA was compared to full-length PTHrP mRNA (Fig. 3.3B and C).

Effects of TGFβ1 on cellular protein binding to PTHrP mRNA in the coding region and 3'UTR: Messenger RNA-protein interactions are central to the regulation of mRNA decay. Proteins that bind to mRNA can alter mRNA stability by blocking endonuclease sites, promoting ubiquitination and decay, and promoting endonuclease binding and mRNA cleavage. In order
to identify binding sites and *trans*-acting factors important in PTHrP mRNA stability, mRNA-protein UV-cross-linking studies were conducted using 5 overlapping regions in the coding and 3′UTR of PTHrP mRNA (Fig. 3.4A). [32P]-PTHrP mRNA corresponding to each of the overlapping regions (1-5) was mixed with S100 extracts from vehicle- and TGFβ1-treated squamous carcinoma cells (Fig. 3.4B). Coding region RNAs (regions 1-4) had multiple binding proteins. Differences in binding between treated and untreated samples (minus background) were measured by phosphorimager. Bands in control lanes were considered non-specific, and may represent RNA with secondary structure which was not digested by RNAse A or RNA that was non-specifically binding albumin, and were negated as bands from vehicle and TGFβ1 treated samples. Region 1 (early coding region) had proteins present at 29 and 31 kD (other bands were duplicated in the control lane and were considered non-specific). Region 2 (coding region) had a single protein at 50 kD. Region 3 (coding region) had multiple RNA binding proteins that were decreased with TGFβ1 treatment (33, 31, 27, 20, and 18 kD). Region 4 shared the 33, 31, and 27 kD binding proteins with region 3, which were also reduced by TGFβ1 treatment. Additional mRNA binding proteins of 24 and 16 kD were present in region 4, but were unchanged by TGFβ1 treatment. Binding of proteins to region 4 RNA was 4.5-fold reduced (33 kD protein), 3.1-fold reduced (31 kD protein), and 3.3-fold reduced (27 kD protein). Similar reductions in protein binding were evident in region 3. In addition, the 20 kD and 18 kD binding proteins in region 3 were reduced 2.0- and 2.3-fold, respectively, with TGFβ1 treatment. Region 5
(3'UTR) had binding proteins ranging from 33 to 60 kD, none of which had alterations in RNA binding with TGFβ1 treatment. All bands were normalized to background.

We investigated the specificity of protein binding in regions 3 and 4, based on the presence of numerous similar bands. First, the specificity of protein binding to region 4 RNA was examined by the addition of 10 and 100-fold excess unlabeled region 4 RNA (Fig. 3.5A), which effectively reduced or eliminated gel bands. We also demonstrated that the 33, 31, and 27 proteins were shared between region 4 and 3 RNA by adding 10 and 100-fold excess unlabeled region 4 RNA to region 3 samples (Fig. 3.5B). Regions 3 and 4 RNA overlap by 91 nt in the terminal coding region.

DISCUSSION

Our results provide insight into the mechanism by which TGFβ1 controls PTHrP mRNA stability in cancer cells, and may have revealed a novel mechanism of growth factor-associated regulation of mRNA stability. Our studies confirmed, in squamous carcinoma cells, previous reports that TGFβ1 increased PTHrP mRNA stability, as reported in non-neoplastic keratinocytes, such as normal keratinocytes and HaCaT cells. Of particular interest was the effect of TGFβ1 on stabilization of the truncated form of PTHrP mRNA (no 3'UTR) in the cell-free mRNA stability assay. While loss of the 3'UTR of PTHrP

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had no direct effect on the intrinsic mRNA stability, incubation of truncated PTHrP mRNA with S100 cytoplasmic proteins from squamous carcinoma cells treated with TGFβ1 resulted in greater than a 5-fold increase in PTHrP mRNA stabilization. Interestingly, we also found that EGF, which stabilizes PTHrP mRNA\(^{17,31}\), had a similar effect to TGFβ1 in the cell-free mRNA decay assay (data not shown). The effect of TGFβ1 on mRNA stability of truncated PTHrP mRNA was greater than the 2-fold increase in mRNA stability that occurred in intact cells, and was in marked contrast to the minimal effect of TGFβ1 on the rate of full-length PTHrP mRNA decay in the cell-free assay. These data suggest that there are coding region sequences (cis-acting elements) that are important in PTHrP mRNA stability. These sequences may represent mRNA cleavage or ubiquitination sites.

In addition, the marked increase in mRNA stability of truncated PTHrP mRNA by TGFβ1 suggests an inhibitory role of the 3'UTR on the effect of TGFβ1 on PTHrP mRNA stability. Therefore, the 3'UTR of PTHrP may play an important role in modulating the stability of its mRNA in the presence of factors such as TGFβ1 or EGF. We demonstrated several proteins, 33, 31, 27, 20, and 18 kD, that have decreased binding to terminal coding region RNAs. Three of these proteins, the 33, 31, and 27 kD proteins, may represent variations in protein binding interactions to a single region of RNA which is within a 84 nt region of the PTHrP coding region RNA (overlap between 3 and 4). Decreases in the binding in the terminal coding region with TGFβ1-treatment of SCC indicates that there are important interactions between sequences and proteins.
associated with the coding region of PTHrP. These may serve to modulate the overall stability of PTHrP mRNA and may represent a regulatory control mechanism between the coding region and 3'UTR to fine-tune the levels of PTHrP mRNA in the cell in response to various growth factors. Changes in the cellular milieu (e.g. cytokines, growth factors, cell confluency) may have a significant effect on the repressive role of the 3'UTR of PTHrP mRNA on TGFβ-mediated mRNA stability.

Messenger RNA sequences in the coding region that are important for regulating mRNA stability have been reported in c-fos, c-myc, and β-tubulin. The c-fos instability element appears to be coupled to translation because it requires the attachment of ribosomes, and the element is sequence and not transcript-based. A 75 kD protein has been demonstrated that binds the coding region of c-myc (a destabilizing sequence) and is thought to protect the mRNA from a ribosome-associated endoribonuclease. However, in the case of β-tubulin, the transcript is important in the induction of mRNA decay. In the presence of excess tubulin protein monomers, the nascent β-tubulin chain mediates the rapid decay of the mRNA. In addition, viruses such as human papillomavirus, have elements in their coding region which are important for the stability of the mRNA. Our results demonstrated that there is an element in the coding region of PTHrP mRNA which is important to TGFβ1-mediated stabilization of PTHrP mRNA, and may be interacting with the 33, 31, 27, 20 or 18 kD mRNA binding proteins identified in these studies.
In studies on mRNA stability, increased mRNA stability has been associated with loss or gain of a mRNA-protein binding. Messenger RNA-binding proteins for short-lived mRNAs (e.g. c-fos, c-jun, GM-CSF, IL-2) include HuR/HuA and AUF1/hnRNAP D families, which bind AU-rich elements (AREs) in the 3'UTR. These proteins function to both stabilize and destabilize mRNA. The most well described of these proteins is the AUF1 family of mRNA-binding proteins, which destabilize mRNA by binding to AREs. This family of proteins consists of four isoforms, p37, p40, p42, and p45. Two forms, p37 and p40, have the highest ARE-binding affinity. Recent studies have shown that PTH, which is related to PTHrP, has an ARE in its mRNA which is bound by AUF1 and mediates its turnover. It would seem likely that PTHrP mRNA would have similar mechanisms of post-translational control, despite a dissimilarity between the 3'UTRs of PTH and PTHrP.

Two species of PTHrP mRNA-binding proteins identified by UV-crosslinking assays were in the size range of the AUF1 family (33 and 30 kD). Both proteins decreased after treatment with TGFβ1, suggesting that they may play a role in mRNA stability similar to that of AUF1. Preliminary supershift studies utilizing AUF1 antibody (obtained from Gary Brewer), however, have been negative (data not shown). Localization of these proteins to the terminal coding region of PTHrP mRNA was significant since coding region sequences were demonstrated to be important for TGFβ-mediated PTHrP mRNA stabilization.
The rapid turnover of PTHrP is similar to immediate early genes (i.e. c-fos, GM-CSF, IL-2), whose turnover has been demonstrated to be due to AU-rich instability elements (AREs) in the 3'UTR. AREs are generally characterized by AU-rich regions with or without multiples of individual or overlapping AUUUA motifs. However, AREs are highly variable in their size, number of AUUUA motifs, and AU content. The lack of sequence homology in these motifs makes them difficult to predict. Similar to these mRNAs, PTHrP has conserved AU-rich sequences in the 3'UTR. The AU-rich motifs in the 3'UTR of PTHrP (1-141) are highly conserved between species which is consistent with an important role in the turnover of PTHrP mRNA. Therefore, it seemed likely that the turnover of PTHrP, unrelated to TGFβ1, might be regulated by a 3'UTR ARE. Theoretically, if an ARE mediated the rapid turnover of PTHrP mRNA, loss of the 3'UTR would result in mRNA stabilization. In our studies on the regulation of PTHrP mRNA stability by TGFβ1, we discovered that the rate of PTHrP mRNA decay was not different between mRNA with or without the 3'UTR (truncated form) in SCC2/88 cells. Studies in HeLa cells have demonstrated that PTHrP mRNA was not effectively stabilized by the β-globin 3'UTR (a highly stable message), nor did the 3'UTR of PTHrP significantly destabilize PTH mRNA (W. Philbrick, Yale University, personal communication). These findings corroborate data from our studies that indicate that the 3'UTR of PTHrP is not solely responsible for the rapid turnover of the PTHrP mRNA, despite the highly conserved nature of the AU-rich motifs.
Our studies demonstrated that the mechanism by which TGFβ1 increased PTHrP mRNA stability is unique. Regulation is controlled, at least in part, by cis-acting sequences in the terminal coding region, and this effect does not require the translational machinery. Alterations in the stability of PTHrP mRNA were mediated by interactions between trans-acting factors binding to the terminal region of the coding sequence of PTHrP mRNA. Proteins that bind to the terminal coding region may be important in TGFβ1-mediated PTHrP mRNA stability. Additionally, the 3'UTR of PTHrP, perhaps through protein-protein interactions, appears to have a negative regulatory effect on the stabilization of PTHrP mRNA by TGFβ1. Changes in mRNA stability is an important mechanism of altering gene expression post-transcriptionally. Therefore, the effect of TGFβ1 on the stability of PTHrP mRNA likely plays an important role in normal physiology as well as the development of humoral hypercalcemia of malignancy. Further investigations into the nature of this interaction and the associated trans-acting factors induced/activated by TGFβ1 are essential for understanding the development of HHM as well as the regulation of PTHrP in normal keratinocytes.
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Fig. 3.1. PTHrP mRNA stability was increased in SCC 2/88 cells by TGFβ1 (3 ng/ml). After 2 hours of TGFβ1 (3 ng/ml) (T) or vehicle (V) treatment, 0.25 ng/ml of the transcription inhibitor, DRB, was added to cell cultures and RNA was extracted at 0, 0.5, 1, 2, and 4 hrs. (A) Total RNA (20 μg) isolated from treated SCC 2/88 was analyzed by Northern blot. (B) Image analysis quantitation representative of all data. Data are expressed as the mean ± standard error of 3 separate experiments. * p<0.05, significantly different from controls at same time point.
Fig 3.2. $^{32}$P-labeled PTHrP (1-141) mRNA decay in cell-free assay using S100 proteins from SCC2/88 cells. (A) Schematic drawing of the human PTHrP cDNA and the full-length capped and polyadenylated PTHrP mRNA constructs used in cell-free mRNA stability assay. (B) $^{32}$P-labeled PTHrP (1-141) mRNA decay: $^{32}$P-labeled PTHrP (1-141) mRNA was incubated with 40 μg of either cytoplasmic protein extracts (S100) from SCC 2/88 cells treated with either vehicle (V) or 3 ng/ml TGFβ1 (T) and extracted at 0, 15, 30, 45, 75, and 120 minutes. (C) Image analysis quantitation representative of all data. Data are expressed as the mean ± standard error of 2 separate experiments (each experiment done in duplicate or triplicate).
Fig 3.3. [$^{32}$P]-labeled truncated PTHrP mRNA (no 3'UTR) decay in cell-free assay using S100 proteins from SCC2/88 cells. (A) Schematic drawing of the human PTHrP cDNA and the truncated capped and polyadenylated PTHrP mRNA constructs used in cell-free mRNA stability assay. (B) [$^{32}$P]-labeled truncated PTHrP mRNA decay: [$^{32}$P]-labeled truncated PTHrP mRNA was incubated with 40 μg of either cytoplasmic protein extracts (S100) from SCC 2/88 cells treated either with vehicle (V) or 3 ng/ml TGFβ1(T) and extracted at 0, 15, 30, 45, 75, and 120 minutes. (C) Image analysis quantitation representative of all data. Data are expressed as the mean ± standard error of 4 separate experiments (each experiment done in duplicate or triplicate). * p<0.05, statistically significant difference induced by TGFβ1.
Fig. 3.4. UV-crosslinking of overlapping regions of PTHrP mRNA demonstrate mRNA binding proteins. (A) Schematic diagram of overlapping regions of the PTHrP 3'UTR and coding region used for UV-crosslinking. (B) Cytoplasmic proteins crosslinked to overlapping regions of [\(^{32}P\)]-labeled PTHrP 1-5 RNA separated on a 12% SDS-PAGE gel. Reduced RNA-protein interactions with TGF\(\beta1\) treatment (T) as compared to vehicle treatment (V) of SCC2/88 cells are associated primarily with region 3 and 4 at 33, 30, and 27 kD (brackets) binding proteins. There was also reduced binding of the 18 and 20 kD binding proteins in region 3. Lanes without protein served as control (C). UV-crosslinking of regions 3, 4, and 5 were examined in 3-4 separate experiments, regions 1 and 2 were examined in 2 separate experiments.
Fig. 3.5. Regions 3 and 4 of the PTHrP coding region share a 27, 30, and 33 kD RNA binding protein, which may represent a protein complex binding to a single region of the RNA. (A) To confirm that these bands represented specific protein-mRNA binding, the binding to region 4 was competed with 10 and 100-fold excess unlabeled region 4 RNA (specific) as well as 10 and 100-fold excess cold region 1 RNA (non-specific). (B) Binding to region 3 was competed with 10 and 100-fold excess unlabeled region excess region 4. No bands were present in the control (C) lane (no protein) in either experiment. Experiments were repeated 2-3 times.
<table>
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<th>Sequence 5'-3' (reverse primer)</th>
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<td>Region 5</td>
<td>T7-AATTTTCAGCAGAGACCIT</td>
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Table 3.1. DNA primers for PCR amplification of PTHrP coding region and 3'UTR for RNA-protein UV-crosslinking. The 5' primer of each primer pair had a T7 promoter sequence for *in vitro* RNA transcription.
CHAPTER 4

MESSENGER RNA EXPRESSION AND STABILITY OF PARATHYROID HORMONE-RELATED PROTEIN ISOFORMS 1-139, 1-141, AND 1-173 REGULATED BY TRANSFORMING GROWTH FACTOR-β1

ABSTRACT

Parathyroid hormone-related protein (PTHrP) is a multifunctional protein that was initially identified as a tumor-secreted factor causing humoral hypercalcemia of malignancy (HHM). The human PTHrP gene is complex, comprised of 9 exons and 3 promoters, P1, P2, and P3, and is alternatively spliced into 3 isoforms encoding 139, 173, and 141 amino acid proteins, each with a unique 3'-untranslated region (3'UTR). Transforming growth factor-β1 (TGFβ1), has been demonstrated to increase the half-life of PTHrP mRNA in vitro. The effect of TGFβ1 on PTHrP promoter usage, PTHrP mRNA isoform stability, PTHrP secretion, and the presence of active TGFβ1 in conditioned medium using human lung squamous carcinoma cells (HARA) and canine oral squamous carcinoma cells (SCC2/88) were examined. Northern blot analysis of these cells using specific cDNA probes for exons VII, VIII, and IX revealed differences in mRNA half-life between the various isoforms. The half-life of PTHrP mRNA transcripts containing exon VII (PTHrP 1-139) was 120 and 90
min in human HARA cells and in canine SCC2/88, respectively. TGFβ1 increased steady state PTHrP (1-139) mRNA levels 1.7- and 1.5-fold over control in HARA and SCC2/88 cells, respectively, but had no effect on PTHrP (1-139) mRNA stability. The half-life of PTHrP mRNA with exon IX (PTHrP 1-141) was 90 and 45 min in HARA and SCC2/88 cells, respectively. TGFβ1 treatment increased PTHrP (1-141) mRNA half-life 2-fold in SCC2/88 cells and 3-fold in HARA cells, and mildly increased mRNA expression levels in HARA cells (1.3-fold). The half-life of PTHrP mRNA containing exon VIII (PTHrP 1-173, not present in dogs) in HARA cells was 4 hours. Expression levels and mRNA stability did not significantly increase after 6 hours of TGFβ1 treatment. SCC2/88 secreted 10-fold more PTHrP (1-86) (7.5 ± 1.2 pM/ug DNA) into conditioned medium compared to HARA cells (0.55 ± 0.01 pM/ug DNA), and TGFβ1 significantly increased PTHrP secretion into the conditioned medium of SCC2/88 but not HARA cells at all concentrations (p<0.01) (24 ± 7.0, 29 ± 6.2, and 35 ± 3.2 pM/ug DNA at 0.3, 1.0, and 3.0 ng/ml TGFβ1, respectively). SCC2/88 cells used the P1 promoter, and expression increased with TGFβ1 treatment. HARA cells used predominately the P2 and P3 promoters, with P1 usage evident after TGFβ1 treatment. Active TGFβ1 in conditioned medium was measured by ELISA and was very low in both SCC2/88 and HARA cells, (3.8 pg/ug DNA ± 0.013 and 8.2 pg/ug DNA ± 0.018, respectively).
INTRODUCTION

Parathyroid hormone related-protein (PTHrP) was originally cloned in 1987 and identified as the tumor-secreted factor responsible for humoral hypercalcemia of malignancy (HHM) \(^1\text{-}^3\). PTHrP shares amino-terminal homology to parathyroid hormone (PTH), and in patients with HHM, PTHrP is secreted into the circulation in a concentration sufficient to stimulate parathyroid hormone receptors in kidney and bone, causing increased serum calcium \(^4\). However, the endocrine effect of PTHrP in HHM is not reflective of its normal paracrine, autocrine, and intracrine effects in many tissues of the body \(^5\). Its exact function in adults has not been completely elucidated, but is believed to be important in growth and differentiation as well as cell proliferation and apoptosis \(^6\). Normal epithelial cells express and secrete PTHrP \(^7\text{-}^8\), and cancers that have been associated with PTHrP overexpression include epithelial tumors (such as squamous cell carcinoma; SCC) \(^9\text{-}^9\) and lymphoma \(^10\). HHM has been reported in 0 to 20% of cancer patients \(^11\text{-}^13\). While all SCC produce PTHrP, an explanation for the development of HHM in only a subset of patients has not been elucidated.

PTHrP has a complex gene structure, comprised of 9 exons and 3 promoters, P1, P2, and P3. PTHrP is alternatively spliced into 3 isoforms encoding 139, 173, and 141 amino acid proteins, each with a unique 3'-untranslated region (3'UTR), exon VII, VIII, and IX, respectively \(^4\text{-}^14\). All three isoforms share coding exons V and VI. Certain factors, such as epidermal growth factor (EGF) and transforming growth factor-beta1 (TGFβ1), have been
reported to increase both the transcription and mRNA stability of PTHrP \(^{15-20}\). EGF and TGF\(\beta\)1 have also been demonstrated to increase P3 promoter use in human breast cancer cell lines \(^{21,22}\), and P1, P2, and P3 in HaCaT (immortalized human keratinocytes) cells \(^{18}\). The estimates of PTHrP mRNA half-life using a common coding region probe vary widely, ranging from 30 minutes to 3 hours \(^{15,18,23}\). The half-life of PTHrP mRNA can be increased 2-10 fold \textit{in vitro} by cytokines such as TGF\(\beta\)1 \(^{15,23,24}\) and EGF \(^{18}\).

Many cancers, including carcinomas, express transforming growth factor-\(\beta\)1 (TGF\(\beta\)1), which may have an important role in the overexpression of PTHrP by certain cancers \(^{25,26}\), with subsequent development of HHM. Although there are reports on the effect of TGF\(\beta\)1 on PTHrP mRNA transcription and stability \(^{15,18}\), promoter usage and changes in mRNA stability of specific isoforms of PTHrP have not been extensively examined. It is likely that differences in PTHrP half-life between cell lines may be the direct result of variations in isoform expression. In this report, we have examined the effect of TGF\(\beta\)1 on the PTHrP promoter usage and PTHrP mRNA stability of the three isoforms (1-139, 1-173, and 1-141) in a human and canine squamous carcinoma cell line.
MATERIALS AND METHODS

Cell Lines and Culture Conditions: Cell lines were originally isolated from a canine oral squamous cell carcinoma from a normocalcemic patient (SCC2/88) and a human lung squamous cell carcinoma (HARA) from a hypercalcemic patient. SCC2/88 cells were cultured in William's E medium (Invitrogen, Carlsbad, CA) with 2 mM L-glutamine (Invitrogen), 50 μg/ml gentamicin, 10% fetal calf serum (FCS), 1 ng/ml epidermal growth factor (EGF) (Invitrogen), and 10 μM cholera toxin (Calbiochem, Inc., San Diego, CA). HARA cells were cultured in RPMI with 10% FCS, 2 mM L-glutamine, and 50 μg/ml gentamicin. Both cell lines have been characterized to express and secrete PTHrP. At 60-80% confluence the cells were placed in basal medium (William's E or RPMI with 2 mM L-glutamine and 50 μg/ml gentamicin) for 16-24 hrs prior to treatment with TGFβ1.

Generation of PTHrP Isoform Specific cDNA Probes: Isoform-specific cDNAs for PTHrP were generated by PCR. Primers were designed from exons VII, VIII, and IX to be specific to PTHrP 139, 173 and 141, respectively. The forward and reverse primers specific for PTHrP 139 (403 nt) were 5'-TTCTGCCTTGCTTGACAA-3' and 5'-ATGATGCTGTCTGCCAGTG-3', respectively; PTHrP173 (447 nt) were 5'-CAGCACCCTCTGTTGGGGTTG-3' and 5'-GGCAATAAGTAGGGGTCCCTT-3', respectively; and PTHrP 141 (335 nt) were 5'-GGACATATTGCAGGTCTCCTG-3' and 5'-
CAACCACAAATAGAGACAC-3'. PCR was conducted utilizing a Platinum Taq PCR amplification kit (Invitrogen) using 1X PCR buffer, 2.5 mM MgCl₂, 0.2 mM dNTPs, 5' and 3' primers (0.2 uM), and 1U Platinum Taq polymerase (Invitrogen) at 95°C for 2 min followed by 35 cycles of 95°C, 55°C, and 72°C of 45 sec each using a Bio-Rad iCycler (BioRad Laboratories, Hercules, CA). The PCR-amplified cDNA sizes were confirmed using a 1.5% agarose gel and purified with the Qiaquick PCR purification kit (Qiagen, Valencia, CA). ³²P-labeled cDNA was generated using the StripEZ (Ambion, Austin, TX) random primer cDNA labeling kit and unincorporated nucleotides were removed by the Qiaquick nucleotide removal kit (Qiagen).

Northern Blot Analysis: PTHrP mRNA steady state and stability were measured in squamous carcinoma cells after treatment with 3 ng/ml TGFβ1 (R & D Systems, Minneapolis, MN) or equal volume of vehicle (0.1% BSA in phosphate-buffered saline, PBS) for 6 hrs. After 6 hrs of treatment with TGFβ1 or vehicle, cells were treated with 25 μg/ml of the transcription inhibitor, 5,6-dichloro-1-beta-ribofuranosyl benzimidazole (DRB, Calbiochem). At 0, 1, 2, and 4 hrs after addition of DRB, total RNA was extracted from the cells with Trizol reagent (Invitrogen) followed by chloroform extraction and ethanol precipitation. Total RNA (20 μg) was separated on a 1% formaldehyde-agarose gel and transferred to a nylon membrane (Duralon, Invitrogen), crosslinked with UV light (Stratalinker, Stratagene, La Jolla, CA), and hybridized with ³²P-labeled cDNA for PTHrP exons VII, VIII, or IX (as previously described) and GAPDH (780 bp PstI/XbaI fragment of human GAPDH) in Ultrahyb (Ambion) at 42°C for 16 hrs.
Membranes were washed twice for 5 min at 42°C in 2X SSC and 0.1% sodium doedecyl sulfate (SDS) and once for 30 min at 42°C in 0.1X SSC and 0.1% SDS. Blots were developed by autoradiography and quantitated using a PhosphorImager (425F-120, Molecular Dynamics, Sunnyvale, CA).

**PTHrP Immunoradiometric Assay (IRMA):** Secretion of PTHrP (1-84) by squamous carcinoma cells was measured by IRMA (Diasorin, Stillwater, MN). Each experiment was conducted three times in triplicate. Cells were treated with either TGFβ1 (0.1, 1 or 3 ng/ml) or vehicle in 12-well plates. After 24 hrs of treatment, conditioned medium was collected and frozen at −20°C until analysis.

**DNA Quantitation:** DNA from squamous carcinoma cells was measured to normalize conditioned medium PTHrP concentrations between culture wells. After removal of the medium for the IRMA, cells were lysed with 5M guanidine isothiocyanate (GITC) containing 0.5% sodium lauryl sarcosine and 20 mM sodium citrate, and the lysate was stored at −20°C. DNA was measured using Hoescht 23345 dye (Sigma Biochem, St. Louis, MO) in a 96-well black U-bottom Microfluor plate (Dynatech Labs, Ashford, Middlesex, UK). Samples were aliquoted into 100 µl TNE buffer (10mM Tris pH 8.0, 0.5 mM EDTA, 0.25 M NaCl). The standard curve was generated with calf thymus DNA in TNE with an equivalent amount of GITC as used in the sample wells. 23345 Hoescht dye (100 µl of 0.1 mg/ml) was added to each well and samples were measured at 450 nm on an IDEXX fluorimeter (IDEXX, Westbrook, ME).
**TGFβ1 ELISA:** To examine the endogenous secretion of TGFβ1 into conditioned medium by the two squamous carcinoma cell lines, cells were grown to 70% confluence in 12 well plates. Cells were then placed in basal medium with 200 μg/ml bovine serum albumin (BSA). The basal medium was changed 4 times over 24 hours and then placed in basal medium without BSA for 24 hours. Conditioned medium was removed, frozen to -80°C until analysis by ELISA. ELISA plates were generated using R & D System’s DuoKit ELISA for active TGFβ1. The total amount of TGFβ1 in the conditioned medium was measured after acidification of the conditioned medium of 0.5 ml with 0.1 ml 1 N HCl (to pH 4) for 10 min to convert inactive TGFβ1 to active form medium to a pH of 4.0 for 10 min. After 10 min, the medium was neutralized by the addition of 0.1 ml of 1.2 N NaOH.

**PTHrP Promoter usage:** Utilizing a technique described by Southby et al, changes in PTHrP promoter usage induced by TGFβ1 were measured by rtPCR. Total RNA was isolated from squamous carcinoma cells treated with vehicle or 3 ng/ml TGFβ1 for 6 hrs as previously described. RNA was treated with 20 U DNAse I (Roche) for 15 min at room temperature. EDTA (25 μM) was added to stop the reaction and samples were heated to 65°C for 15 min to inactivate the DNAse I and then chilled on ice. RNA was re-extracted by the Trizol method (Invitrogen), and the RNA quantitated. Reverse transcription was performed using 750 ng total mRNA with the SuperscriptRT kit from Invitrogen.
using oligo-dT first strand synthesis primers. PCR was performed for 30-35 cycles using promoter-specific primers. Forward primer specific to promoter P1 was 5'-AGGTACCTGCTTTCTAATA-3'; to promoter P2 was TTCTCCGGCAGGGTTTG-3'; to promoter P3 was 5'-GTTGGAGTAGCCGGTTGCTA. The reverse primer was common for all three PCR reactions: 5'-TGCGATCAGATGGTGAAGGA-3'.

**Statistical Analysis:** Data from the mRNA stability assays were analyzed using a single factor ANOVA analysis to determine the significance of differences between mean values. Probability (p) values less than 0.05 were considered statistically significant. Graphical representation of data includes the mean ± the standard error.

**RESULTS**

*PTHrP mRNA (all transcripts) Stability was Increased by TGFβ1 in SCC2/88 but not HARA Cells:* PTHrP mRNA stability in the squamous carcinoma cells (SCC 2/88 and HARA) was measured after treatment with either vehicle or TGFβ1 for 6 hrs, followed by treatment with the transcription inhibitor, DRB. Analysis of PTHrP mRNA half-life using a cDNA probe to the coding region of PTHrP (common to all three isoforms) revealed an average mRNA half-life of 45 min in SCC2/88 cells (Fig. 1A and B) and 240 min in HARA cells (Fig. 1C and D). TGFβ1 increased the half-life of PTHrP in SCC2/88 cells 2-fold to 90 min.
Comparison of mRNA between TGFβ1-treated SCC2/88 and controls demonstrated that PTHrP mRNA stability with TGFβ1 was significantly increased from controls (p<0.05) at 30 and 60 min. TGFβ1 treatment of HARA cells had no effect on overall PTHrP mRNA stability.

**PTHrP mRNA Stability of Isoform 1-141 but not 1-139 or 1-173 was increased by TGFβ1 in SCC2/88 and HARA cells:** Northern blot analysis of HARA cells using specific cDNA probes for exons VII, VIII, and IX revealed differences in mRNA half-life between the various isoforms. The half-life of PTHrP mRNA transcripts containing exon VII (PTHrP 1-139) was similar between the canine SCC2/88 cells (Fig. 4.2A and B) and human HARA cells (Fig. 4.2C and D) at 120 and 90 min, respectively. Treatment of both cell lines for 6 hours with TGFβ1 did not alter the mRNA stability of transcripts containing exon VII. However, TGFβ1 did increase steady-state mRNA expression 1.5 and 1.7-fold (p<0.05) compared to vehicle-treatment in SCC2/88 and HARA cells, respectively. The half-life of mRNA containing exon VIII in HARA cells (PTHrP 1-173, not present in dogs) was 4 hrs and did not significantly increase after TGFβ1 treatment (Fig. 4.3A and B). Steady-state levels of PTHrP (1-173) increased 1.5-fold after treatment with TGFβ1 for 6 hours, however this was not statistically significant. The half-life of PTHrP mRNA with exon IX (PTHrP 1-141) in HARA cells (Fig. 4.4A and B) was 90 min and was 45 min in SCC2/88 cells (Fig. 4.4C and D). TGFβ1 treatment increased the mRNA half-life of exon IX-containing transcripts 2-fold in SCC2/88 cells (p<0.05 at 1 hr) and 3-fold in
HARA cells (p<0.05 at 1 and 4 hrs). TGFβ1 also mildly, but significantly (p<0.05) increased the mRNA expression of PTHrP (1-141) 1.3-fold (p<0.05) in HARA cells, but only 1.1-fold in SCC2/88.

**Secretion of PTHrP (1-84) into Conditioned Medium after Treatment with TGFβ1:** Conditioned medium from 24-hour cultures of cells treated with 0, 0.3, 1.0, or 3.0 ng/ml TGFβ1 demonstrated significant differences between both baseline and TGFβ1-induced concentrations of PTHrP in SCC2/88 and HARA cells (Fig. 4.5). All values were normalized to total DNA. SCC2/88 secreted 10-fold more PTHrP into conditioned medium compared to HARA cells. The conditioned medium of HARA cells had 0.55 ± 0.01 pM/ug DNA in control samples, and SCC2/88 cells had 7.5 ± 1.2 pM/ug DNA. HARA cells did not respond to TGFβ1 with increased PTHrP secretion. TGFβ1 significantly increased PTHrP in the conditioned medium of SCC2/88 at all concentrations (p≤0.01), increasing to 24 ± 7.0, 29 ± 6.2, and 35 ± 3.2 pM/ug DNA at 0.3, 1.0, and 3.0 ng/ml TGFβ1, respectively. Experiments were performed 3 times in triplicate.

**PTHrP Promoter Usage in SCC2/88 and HARA Cells in Response to TGFβ1:** rtPCR to identify specific promoter usage in the SCC2/88 and HARA cells as well as changes in promoter usage with TGFβ1 treatment was examined (Fig. 4.6). The SCC2/88 cells used only the P1 promoter, and expression increased
with TGFβ1 treatment. The size of the PCR fragment for P1 in the dog was smaller than that of the human, and indicated that exon 1 is likely spliced directly to exon V in the SCC2/88 cells. Basal expression of PTHrP in HARA cells was predominately from the P2 and P3 promoters, without evidence of P1 promoter use. However, with TGFβ1 treatment, P1 promoter usage was evident and P2 promoter use was increased. There was no change in P3 promoter use in HARA cells with TGFβ1-treatment.

**Secretion of active TGFβ1 by SCC2/88 and HARA cells:** Active TGFβ1 was measured by ELISA in conditioned medium from SCC2/88 and HARA cells and normalized to DNA (Fig. 4.7). The conditioned medium was acidified to measure total TGFβ1. The amount of active TGFβ1 in the conditioned medium was very low in both SCC2/88 and HARA cells, at 3.8 pg/ug DNA ± 0.01 and 8.2 pg/ug DNA ± 0.02, respectively. After acidification of the medium, the total TGFβ1 in the conditioned medium of SCC2/88 and HARA cells was 14.0 ± 0.5 and 8.4 ± 0.4, respectively. SCC2/88 cells had significantly more active and total TGFβ1 (p<0.03, ANOVA)

**DISCUSSION**

Most human cells express all three isoforms of PTHrP. The importance of the alternative splicing of PTHrP to encode 3 proteins differing only in their carboxyl-termini is uncertain. Studies of breast cancer suggest that
increased expression of PTHrP mRNA (1-139) correlates with increased risk of bone metastasis. Therefore, the expression of specific isoforms could be important in our understanding of cancer, including paraneoplastic syndromes such as HHM. The role of growth factors on changes in PTHrP mRNA transcription and alternative splicing events has had limited examination.

The half-life of PTHrP using a probe to the conserved coding region have been reported in numerous normal and neoplastic keratinocytes, with wide variation ranging from 30 min to 3 hours. Previous studies have demonstrated that PTHrP mRNA transcripts containing exon VIII (PTHrP 1-173) are more stable than the other two transcripts. The data in our studies corroborate these findings. The half-life of PTHrP mRNA transcripts containing exon VIII (PTHrP 1-173) was 2-5-fold longer than transcripts containing exon VII (PTHrP 1-139) and IX (PTHrP 1-141). Therefore, variations in the expression levels of transcripts containing exon VIII (PTHrP 1-173) may account for the marked differences in PTHrP mRNA stability in various cell lines. Cells that express high levels of PTHrP 1-173 will have a much longer PTHrP mRNA half-life than cells which express little PTHrP 1-173. An interesting corollary to this finding is that humoral hypercalcemia of malignancy is most common in human beings, and humans are also the only known species to express PTHrP 1-173. Overexpression of PTHrP 1-173 may therefore be important in the pathogenesis of HHM, and further studies on this would be important in elucidating the mechanism of this paraneoplastic syndrome.
Increases in PTHrP mRNA stability with EGF and TGFβ1 treatment using probes to the conserved coding region have been reported in normal and neoplastic keratinocytes. Few studies have examined the effect of these growth factors on the mRNA stability of the individual isoforms containing each of the 3'-untranslated exons, VII, VIII, and IX. Studies on the effect of EGF on PTHrP mRNA stability in HaCaT cells revealed stabilization only with PTHrP mRNA containing exons VII (PTHrP 1-139) and VIII (PTHrP 1-173) but not IX (PTHrP 1-141). Contrary to these findings, our studies revealed that in the canine SCC2/88 and the human HARA cells, PTHrP transcripts containing exon VII (1-139) and exon VIII (PTHrP 1-173) were not stabilized with TGFβ1 treatment, whereas the mRNA stability of PTHrP transcripts containing exon IX (PTHrP 1-141) were increased. These findings suggest that although TGFβ1 and EGF both increase PTHrP mRNA stability, the mechanism for induction of stability likely differs between these growth factors. Interestingly, mRNA expression of PTHrP (1-139) increased the most dramatically of the three isoforms, despite lack of increased mRNA stability. This indicates that TGFβ1 may have its most important effect on expression of PTHrP through induction of PTHrP 1-139 in SCC. Although endogenous secretion of TGFβ1 was low in our cell cultures, endogenous expression and secretion of TGFβ1 by tumor cells and their associated stromal cells may contribute to the mRNA expression of PTHrP, and subsequent development of HHM, through induction of PTHrP (1-139) mRNA and stabilization of PTHrP (1-141) mRNA.
Many factors have been found to upregulate PTHrP mRNA expression, such as TGFβ, EGF, angiotensin II, estrogen, and colchicine $^{18-20;34;34-36}$. These factors may contribute to differential control of the alternately-spliced PTHrP mRNAs. In COLO16 cells, derived from a cutaneous SCC, EGF and TGFβ1 increased the expression of all three PTHrP isoforms, with the least effect on PTHrP 1-141 $^{31}$. Both TGFβ1 and EGF had a greater effect on promoters P1 and P2 in these cells, with a lesser effect on P3 $^{31}$, which is similar to our findings. In HaCaT cells (immortalized human keratinocytes), EGF increased P1 promoter usage 16-fold and P2 and P3 promoter usage 3- and 2.4-fold, respectively $^{18}$. Both studies reported that TGFβ1 and EGF increased the use of P1 and P2 promoters in transformed keratinocytes. Conversely, in MDA-MB-231 breast cancer cells, TGFβ1 increased usage of the P3 promoter in a SMAD3/Ets1-dependent fashion $^{21}$. In our studies, TGFβ1 increased use of the P1 promoter most significantly, similar to the COLO16 cells. Increases in the use of P3 with TGFβ1-treatment in our study were not evident by rtPCR.

The mechanism by which TGFβ1 alters PTHrP mRNA stability is uncertain. AU-rich elements (AREs) within the 3'UTR of rapidly turned over messages have been demonstrated (i.e. c-fos, GM-CSF, IL-2) to mediate the rapid degradation of these mRNAs $^{37}$. AREs are generally characterized by AU-rich regions with or without multiples of individual or overlapping AUUUA motifs $^{38;39}$. However, AREs are highly variable in their size, number of AUUUA motifs, and AU content $^{38;40}$. The lack of sequence homology in these motifs makes them difficult to predict. Similar to these mRNAs, PTHrP has conserved AU-rich
sequences in its 3'UTRs. The AU-rich motifs in the 3'UTR of PTHrP, specifically 4 AUUUA motifs in both exons VII and IX and 2 in exon VIII, are highly conserved in the 3'UTR between species \(^{41}\), which is consistent with an important role in the turnover of PTHrP mRNA \(^{42}\). Because PTHrP mRNA transcripts containing exon VIII have a half-life of 4 hours suggests that the AU-rich motifs in exon VIII do not represent an ARE. In addition, studies performed in our laboratory and others suggest that the 3'UTR of PTHrP does not play an important role in the rapid turnover of PTHrP mRNA (1-141) or the stabilizing effect of TGFβ1 on PTHrP mRNA \(^{24}\). Instead, these studies on PTHrP mRNA (1-141) suggest that the effect of TGFβ1 on PTHrP mRNA stability is in the terminal coding region of PTHrP (1-141).

The human patient from whom the HARA squamous cells originated had hypercalcemia, but the dog from which the SCC2/88 cells originated was normocalcemic. The dichotomy of the secretion levels of PTHrP in culture by these cell lines (HARA cells had low PTHrP concentrations in conditioned medium whereas SCC2/88 cells had 10-fold higher concentrations) is interesting. Studies in our laboratory suggest that high expression of PTHrP mRNA does not always correlate to high concentrations of PTHrP in conditioned medium, and cells that have large of quantities of PTHrP in the conditioned medium may be from patients which did not have HHM (data not shown). This may be an artifact of the in vitro system or may reflect the method of measuring PTHrP by a two-site IRMA. PTHrP is post-translationally modified and cleaved to form amino (1-36), mid-region (38-94,38-95,or 38-101) \(^{43-45}\), and
carboxyl (101-139) peptides\textsuperscript{46,47}. The two-site IRMA used to measure PTHrP (1-84) in conditioned medium is more sensitive than the one-site radioimmunometric assay (RIA). However, if PTHrP were cleaved to the calcitropic peptide (1-36) and secreted, it would not be detected by IRMA.

The complexity of regulation of PTHrP mRNA splicing, post-translational control, and secretion make understanding of the mechanisms of HHM and the role of PTHrP in metastasis difficult. It is therefore important in the study of PTHrP to take into consideration not only PTHrP mRNA isoform variation (including variations in the 5'UTR), but also variations in post-translational modifications and intracrine functions of the protein. Differences in these aspects of PTHrP may help to explain why the function and mechanisms of action of PTHrP have been difficult to elucidate and at times appear contradictory.

Our results provide insight into the regulation of PTHrP mRNA transcription and stability by TGFβ1 in squamous carcinoma cells. There have been few studies on differences in PTHrP mRNA isoform expression in various cancers and the effect of growth factors. Understanding the regulation of PTHrP mRNA isoform expression and mRNA stability has potential prognostic importance in cancer, such as predicting the occurrence of HHM, cancer invasion and metastasis, and response to medical intervention.
REFERENCES


Fig. 4.1. PTHrP mRNA stability was increased in SCC2/88 cells but not HARA cells by TGFβ1 (3 ng/ml). After 6 hours of TGFβ1 (3 ng/ml) or vehicle treatment, 0.25 ng/ml of the transcription inhibitor, DRB, was added to cell cultures and RNA was extracted at 0, 1, 2, and 4 hrs. (A) Total RNA (20 µg) isolated from treated SCC2/88 was analyzed by Northern blot. (B) Image analysis quantitation representative of all data. Data are expressed as the mean ± standard error of 3 separate experiments. (C) Total RNA (20 µg) isolated from treated HARA cells was analyzed by Northern blot. (D) Image analysis quantitation representative of all data. Data are expressed as the mean ± standard error of 3 separate experiments. * p<0.05, significantly different from controls at same time point.
Fig 4.2. PTHrP (1-139) mRNA stability not increased in HARA or SCC2/88 cells by TGFβ1 (3 ng/ml). After 6 hours of TGFβ1 (3 ng/ml) or vehicle treatment, 0.25 ng/ml of the transcription inhibitor, DRB, was added to cell cultures and RNA was extracted at 0, 1, 2, and 4 hrs. (A) Total RNA (20 ng) isolated from treated SCC2/88 cells was analyzed by Northern blot. (B) Image analysis quantitation representative of all data. Data are expressed as the mean ± standard error of 3 separate experiments. (C) Total RNA (20 µg) isolated from treated HARA cells was analyzed by Northern blot. (D) Image analysis quantitation representative of all data. Data are expressed as the mean ± standard error of 3 separate experiments.
Fig 4.3. PTHrP (1-173) mRNA stability increased in HARA cells by TGFβ1 (3 ng/ml). After 6 hours of TGFβ1 (3 ng/ml) or vehicle treatment, 0.25 ng/ml of the transcription inhibitor, DRB, was added to cell cultures and RNA was extracted at 0, 1, 2, and 4 hrs. (A) Total RNA (20 μg) isolated from treated HARA cells was analyzed by Northern blot. (B) Image analysis quantitation representative of all data. Data are expressed as the mean ± standard error of 3 separate experiments.
Fig. 4. PTHrP (1-141) mRNA stability increased in both SCC2/88 or HARA cells by TGFβ1 (3 ng/ml). After 6 hours of TGFβ1 (3 ng/ml) or vehicle treatment, 0.25 ng/ml of the transcription inhibitor, DRB, was added to cell cultures and RNA was extracted at 0, 1, 2, and 4 hrs. (A) Total RNA (20 µg) isolated from treated SCC2/88 was analyzed by Northern blot. (B) Image analysis quantitation representative of all data. Data are expressed as the mean ± standard error of 3 separate experiments. (C) Total RNA (20 µg) isolated from treated HARA cells was analyzed by Northern blot. (D) Image analysis quantitation representative of all data. Data are expressed as the mean ± standard error of 3 separate experiments. * p<0.05, statistically significant difference induced by TGFβ1.
Fig. 4.5. SCC2/88 respond to TGFβ1 with increased PTHrP into conditioned medium and have 10-fold higher PTHrP in conditioned medium compared to HARA cells. After 24 hours of 0, 0.3, 1, and 3 ng/ml TGFβ1 treatment, conditioned medium was removed and PTHrP was quantitated by IRMA and normalized to the total DNA. * p<0.05, statistically significant difference induced by TGFβ1.
Fig. 4.6. SCC2/88 and HARA cells have low concentrations of active (CM) and total (aCM) TGFβ1 in conditioned medium. After 24 hours in basal medium, conditioned medium was removed and frozen to -80°C. Active and total (after acidification) TGFβ1 was measured by ELISA. Data are expressed as the mean ± standard error of 3 separate experiments done 6 times.
<table>
<thead>
<tr>
<th>Treatment</th>
<th>SCC2/88</th>
<th>HARA</th>
<th>HaCAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT Veh</td>
<td>+</td>
<td>Veh</td>
<td>+</td>
</tr>
<tr>
<td>TGFB1</td>
<td>+</td>
<td>TGFB1</td>
<td>-</td>
</tr>
<tr>
<td>Control</td>
<td>+</td>
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</tr>
</tbody>
</table>

- **P1 Transcripts**:
  - SCC2/88: 739 bp
  - HARA: 406 bp
  - HaCAT: -

- **P2 Transcripts**:
  - SCC2/88: -
  - HARA: 468 bp
  - HaCAT: -

- **P3 Transcripts**:
  - SCC2/88: -
  - HARA: 242 bp
  - HaCAT: -

- **All Transcripts**:
  - SCC2/88: -
  - HARA: 161 bp
  - HaCAT: -

- **GAPDH**:
  - SCC2/88: -
  - HARA: -
  - HaCAT: 414 bp

Fig. 4.7. Promoter use in SCC2/88 and HARA cells after vehicle or TGFB1 treatment. After 6 hours of TGFB1 (3 ng/ml) or vehicle (Veh) treatment, the RNA was extracted, DNAse I treated, re-extracted, and reverse transcribed followed by PCR. The SCC2/88 cells used only the P1 promoter, and expression increased with TGFB1 treatment. TGFB1 induced P1 promoter usage in HARA cells and increased P2 promoter use. The size of the PCR fragment for P1 (406 bp) in the dog is smaller than that of the human (739 bp). HARA cells used predominately the P2 and P3 promoters. HaCat cells served as a positive control.
CHAPTER 5

TRANSFORMING GROWTH FACTOR-β INCREASES PARATHYROID HORMONE-RELATED PROTEIN BUT NOT ENDOTHELIN-1 EXPRESSION IN CANINE PROSTATE EPITHELIAL AND CARCINOMA CELLS

ABSTRACT

Bone metastases of prostate carcinoma are associated with new woven bone formation to form osteoblastic metastases. Tumor-derived factors, such as parathyroid hormone-related protein (PTHrP) and endothelin-1 (ET-1), may promote the development of osteoblastic metastases. We examined the effect of transforming growth factor-β1 (TGFβ1), present in bone matrix and secreted by tumor cells, on PTHrP and ET-1 mRNA expression and PTHrP secretion in normal canine prostate epithelial and stromal cells, and in canine prostate carcinoma cells. Primary cultures of prostate epithelial (PEC), stromal (PSC), and carcinoma (PCC) cells were produced. The effect of TGFβ1 on PTHrP and ET-1 mRNA expression was measured by Northern blots, and secretion of PTHrP into culture medium was measured by immunoradiometric assay (IRMA). Degradation of recombinant-human PTHrP (rhPTHrP) (1-84) inoculated
in PEC cultures was measured over 24 hours. Arginine esterase activity in tissue and conditioned medium were also examined. TGFβ1 increased PTHrP but not ET-1 mRNA expression in a time- and dose-dependent manner in PEC and in PCC. TGFβ1 decreased PTHrP mRNA in PSC. PSC produced low levels of ET-1. TGFβ1 significantly increased PTHrP secretion (p<0.05) into PEC but not PSC conditioned medium. rhPTHrP was significantly (p<0.05) degraded in PEC conditioned medium as compared to PSC conditioned medium. Arginine esterase activity was present in prostate and prostate carcinoma tissue and low in conditioned medium from PEC. TGFβ1 increased PTHrP mRNA expression in canine PEC and PCC, and decreased expression in PSC. ET-1 expression was not altered by TGFβ1. This regulatory pathway may be important in the formation of osteoblastic...
INTRODUCTION

Prostate cancer is the most common form of cancer among men in the United States. Prostate carcinomas frequently metastasize in later stages to regional lymph nodes and bone, particularly lumbar vertebrae and pelvic bones, causing significant pain. In contrast to other tumor types, bone metastases of prostatic carcinoma are associated with excessive new woven bone formation (osteoblastic metastases) and varying degrees of bone resorption. Explanations for induction of osteoblastic lesions as well as the targeting of prostate carcinomas to bone have been the subject of extensive research and speculation.

The bone changes at the sites of prostate metastasis are thought to be the result of prostate and bone matrix-derived factors (growth factors, cytokines, inflammatory mediators, etc) that modulate the activity of osteoblasts and osteoclasts locally. Three of the factors that have been implicated in osteoblastic metastasis are transforming growth factor-β1 (TGFβ1), parathyroid hormone related protein (PTHrP), and endothelin-1 (ET-1). All three factors can promote both bone formation and carcinoma survival, and are expressed by prostate tissue and/or bone. TGFβ1 expression is high in both prostate carcinomas as well as in bone matrix, and may promote osteogenesis. PTHrP is secreted by prostate epithelial and stromal cells, and can potentially increase bone formation by the C-terminal peptide (107-111, osteostatin), as well as promote osteoclastic bone resorption by N-terminal binding to the PTH1 receptor on osteoblasts.
expression and secretion in response to TGFβ1 has been demonstrated in multiple epithelial cell types. Although increased PTHrP expression has been demonstrated in prostate carcinomas, the potential role of TGFβ1 on PTHrP upregulation in normal and neoplastic prostate cells has not been extensively examined. Endothelin-1, which is a potent vasoconstrictor, is produced by prostate epithelial cells and promotes new bone formation.

TGFβ1 has been shown to increase ET-1 protein secretion in PC3, a human prostate carcinoma cell line. Because TGFβ1 is secreted by prostate epithelial cells and is present in high concentrations in bone matrix, it may be important in the pathogenesis of osteoblastic metastasis in prostate cancer due to its effects on PTHrP and ET-1 expression by tumor cells.

Rodent models of prostate carcinoma development and progression do not recapitulate the human disease, and may lack prostatic intraepithelial neoplasia (PIN). In contrast, dogs develop prostate carcinoma, benign prostatic hyperplasia, and PIN, and may be a better model for human prostate carcinoma. In addition, normal canine prostate epithelial and stromal cells have been reported to express PTHrP and ET-1 similar to humans. In the present study, we demonstrated that TGFβ1 upregulates PTHrP but not ET-1 mRNA expression by both normal and neoplastic canine prostate epithelial cells. These results further implicate TGFβ1 as an important mediator in the induction of osteoblastic metastases.
MATERIALS AND METHODS

Canine Prostate Tissue Preparation: Canine prostate tissue with no macroscopic or microscopic evidence of neoplasia was obtained from adult male mongrel dogs immediately following euthanasia. The bladder was exteriorized, clamped, and the prostate excised from the urethra. The prostate was removed en bloc, and stored in ice-cold Dulbecco's Modified Eagle/F12 medium (DMEM/F12) (Life Technologies, Inc., Gaithersburg, MD) containing 10 μg/ml gentamicin sulfate. Prostate tissues were washed three times in medium. Sharp dissection was used to remove periprostatic connective tissue, the fibrous capsule of the prostate, and the prostatic urethra. The prostate tissue was minced into 1-mm³ pieces and incubated in type I collagenase in basal DMEM/F12 media (200 mg/ml, Worthington Biochemical Corp., Lakewood, NJ) for 3-4 hrs at 37°C. The digested prostate tissue was washed once with DMEM/F12 with 5% fetal bovine serum (FBS), and three times with DMEM/F12. Prostate cell digests were mixed by repeat pipeting, pelleting at 1500 xg at 4°C for 5 min, resuspension in DMEM/F12 with 10% FBS, and plating in T-75 tissue culture flasks. Cells were incubated for 24 hrs at 37°C and 5% CO₂ to allow cell attachment. After attachment, medium was changed to Keratinocyte Serum-Free Medium (KSFM) (Life Technologies) to selectively enrich the epithelial cell population. Stromal cells were cultured in DMEM/F12 with 5% FBS.
A prostate carcinoma from 9 year old Great Dane (castrated as an adult) was collected and neoplasia was confirmed histologically. Tissue was prepared for cell culture in the same manner as that for the prostate epithelial cells and maintained for 2 passages in vitro.

**Immunohistochemistry:** Immunohistochemistry was performed on canine prostate cells as previously described. Canine prostate epithelial cells were grown to 70-80% confluence and trypsinized (0.05% Trypsin-EDTA, Life Technologies) for 5 min. Trypsinization was stopped by addition of serum. Cells were washed twice with medium and pelleted by centrifugation at 5,000 xg for 5 min. The cell pellet was resuspended in 200 µl of bovine plasma. Fibrin polymerization was initiated with 10 units human thrombin (Sigma, St. Louis, MO). Fibrin-clotted cells were fixed in cold 10% neutral-buffered formalin for 24 hrs, routinely processed and paraffin-embedded. Sections (4µm) were stained either with hematoxylin and eosin (HE) or examined immunohistochemically for pan-cytokeratin, vimentin, α-smooth muscle actin, and desmin. Briefly, paraffin-embedded sections were dewaxed in xylene and hydrated with decreasing concentrations of ethanol and water. Endogenous peroxidase activity was inhibited with 0.3% hydrogen peroxide for 15 min. Primary antibodies for vimentin (1:400; Biogenex, San Ramon, CA), pan-cytokeratin (1:100; Boehringer Mannheim, Indianapolis, IN), α-smooth muscle actin (1:100; Boehringer Mannheim), and desmin (1:100; Biogenex) were diluted in phosphate-buffered saline (PBS). All samples were labeled with a biotin-conjugated universal secondary antibody (Research Genetics, Huntsville, AL). Slides were incubated with avidin-biotin-horseradish peroxidase complex (ImmunoPure ultra-Sensitive ABC Staining Kit, Pierce Chemical Company,
Rockford, IL), and antigens were detected by incubation in diaminobenzadine for 2 minutes (Research Genetics). Negative control slides were treated with the biotin-conjugated secondary antibody and no primary antibody.

**Northern Blot Analysis:** Steady state PTHrP mRNA was measured in prostate stromal and epithelial cells after treatment with TGFβ1 (R & D Systems, Minneapolis, MN) for 6 h (0.1, 1 or 3 ng/ml) or equal volume of vehicle (0.1% BSA in 4 mM HCl). PTHrP mRNA expression was also measured in prostate epithelial cells over 24 hours (1, 3, 9, 12, and 24 hrs) in response to 3 ng/ml TGFβ1. Prostate carcinoma cells were available in limited number and were treated with 0 and 1 ng/ml TGFβ1 for 6 h. Total RNA was extracted from the cells with Trizol reagent (Life Technologies) followed by chloroform extraction and ethanol precipitation. Total RNA (20 µg) was separated on a 1% formaldehyde-agarose gel and transferred to a nylon membrane (Duralon, Life Technologies), crosslinked with UV light (Stratalinker, Stratagene, La Jolla, CA), and hybridized with ³²P-labeled cDNA for PTHrP (537 bp Acc1/Sst1 fragment of human PTHrP, Genentech, Inc, San Francisco, CA.), endothelin-1 (pSCR1-1, 507 bp fragment of human ET-1, Abbott Labs, Inc. Abbott Park, IL), and GAPDH (780 bp Pst1/Xba1 fragment of human GAPDH) in QuikHyb (Stratagene). Blots were developed by autoradiography and quantitated using a Phosphorimager (425F-120, Molecular Dynamics, Sunnyvale, CA).
Immunoradiometric Assay (IRMA): Secretion of PTHrP (1-84) by prostate stromal and epithelial cells was measured by IRMA (Diasorin, Stillwater, MN). Cells were treated with either TGFβ1 (0.1, 1 or 3 ng/ml) or vehicle in 12-well plates. After 24 h of treatment, conditioned medium was collected and frozen at −20°C until analysis.

DNA Quantitation: DNA from prostate epithelial and stromal cells was measured to normalize conditioned medium PTHrP concentrations between wells. After removal of the medium for the IRMA, cells were lysed with 5M guanidine isothiocyanate (GITC) containing 0.5% sodium lauryl sarcosine and 20 mM sodium citrate. Lysate was stored at −20°C. DNA was measured using Hoescht 23345 dye (Sigma Biochem) in a 96-well black U-bottom Microfluor plate (Dynatech Labs, Ashford, Middlesex, UK). Samples were aliquoted into 100 µl TNE buffer (10mM Tris pH 8.0, 0.5 mM EDTA, 0.25 M NaCl). The standard curve was generated with calf thymus DNA in TNE with an equivalent amount of GITC as used in the sample wells. 23345 Hoescht dye (100 µl of 0.1 mg/ml) was added to each well and samples were measured at 450 nm on an IDEXX fluorimeter (IDEXX, Westbrook, ME).

PTHrP Degradation in Vitro: To assess if prostate cells degraded PTHrP in vitro, we added recombinant-human PTHrP (rhPTHrP) (1-84) (Incstar, Inc, Stillwater, MN) to cultures of prostate epithelial or stromal cells. Experiments were performed twice with each sample examined in duplicate. Canine prostate epithelial and stromal cells were
plated at 1 x 10^4 cells per well into 24-well culture dishes and grown to 80% confluence, at which time they were placed in either KSFM (prostate epithelial cells) or DMEM/F12 (prostate stromal cells) without supplements except for 50 μg/ml gentamicin. After 16 hours, the medium was removed and replaced with fresh basal medium containing 100 pM rhPTHrP (1-84). Conditioned medium was removed at 0, 2, 4, 8, and 24 hrs and frozen at −20°C. PTHrP concentrations from the conditioned medium were measured by IRMA (Diasorin).

Arginine Esterase Assay: Arginine esterase activity in canine prostate, prostate carcinoma and conditioned medium from prostate epithelial cells was assayed as described 60. Briefly, 25-50 mg pieces of tissue were homogenized for 60 sec (Tissue-Tearor, Biospec Products, Bartlesville, OK) in 2.0 ml cold PBS containing Complete™ Mini protease inhibitor cocktail (Boehringer-Mannheim) and 0.01% Triton X-100 (Sigma). Total protein in the homogenates was measured by the BCA assay (Pierce, Rockford, IL) 61. Conditioned medium was collected from cells that were 60-70% confluent, 12 and 24 hours after the addition of fresh basal medium. Homogenates and conditioned medium were stored at -80°C until analysis. A 1 mM solution of arginine esterase substrate (benzoyl arginine ethyl ester, BAEE, Sigma) was prepared in 10 mM Tris-HCl, pH 8.0. The assay was performed at 37°C in a 1ml quartz cuvette, and the absorbance of the BAEE cleavage product was measured spectrophotometrically at 253 nm following the addition of 10-100 μg homogenate or conditioned medium protein.
Data Analysis: Results were analyzed by single factor ANOVA to determine the significance of differences between means. Probability values under 5% (p<0.05) were interpreted to be statistically significant. Data is presented as the mean ± the standard error (SE). Experiments were performed multiple times (as noted in results), each time with cells from separate dogs.

RESULTS

Immunohistochemistry of Primary Cultures of Canine Epithelial and Stromal Prostatic Cells: Immunohistochemistry of isolated canine prostate cells has been published previously by our laboratory and data is not shown. Greater than 95% of the epithelial cells and carcinoma in culture were pan-cytokeratin positive and none expressed vimentin. Stromal cells were 98% positive for vimentin and α-smooth muscle actin and none expressed pan-cytokeratin.

PTHrP mRNA Expression and Protein Secretion by Canine Prostate Cells after Treatment with TGFβ1: PTHrP mRNA expression was abundant in epithelial cells by northern blot analysis. Treatment of canine prostate epithelial cells with increasing concentrations of TGFβ1 (0, 0.1, 1, and 3 ng/ml) for 6 hours resulted in increased PTHrP mRNA expression. Maximal expression of PTHrP mRNA (2 to 4-fold increase over control) occurred at concentrations of 1 and 3 ng/ml of TGFβ1 for 6 hours (Fig. 5.1A and B). The experiment was performed 3 times.

PTHRP mRNA expression was abundant in prostate stromal cells by northern blot analysis. Treatment of canine prostate stromal cells with
increasing concentrations of TGFβ1 (0, 0.1, 1, and 3 ng/ml) for 6 hours resulted in mildly decreased PTHrP mRNA expression (Fig. 5.1C). At 1 and 3 ng/ml TGFβ1, PTHrP mRNA levels were 80 and 60% of control cells, respectively (Fig. 5.1D). Experiments were performed 3 times.

PTHrP mRNA was expressed in canine prostate carcinoma cells by northern blot analysis. Treatment of canine prostate carcinoma cells with TGFβ1 increased PTHrP 4-fold at 1 ng/ml TGFβ1 (Fig. 5.1E and F). The experiment was performed once since the cells failed to thrive in culture.

Expression of PTHrP mRNA over time in prostate epithelial cells after treatment with 3 ng/ml TGFβ1 for 0, 3, 9, 12, and 24 hours resulted in progressive increases in PTHrP mRNA expression, reaching maximum mRNA expression after 9 hours (Fig. 5.2A). This represented a 2-fold increase over baseline PTHrP expression, and a 3.5-fold increase in PTHrP mRNA expression over vehicle-treated cells at the same time point (Fig. 5.2B). The experiment was performed 3 times.

**PTHrP Secretion by Canine Prostate Cells after Treatment with TGFβ1:** PTHrP secretion from prostate epithelial and stromal cells into the culture medium was measured by IRMA after 24 hours of TGFβ1 treatment (0, 0.1, 1, and 3 ng/ml). There was moderate variability in PTHrP secretion by prostate epithelial cells obtained from different dogs. The average concentrations ranged from 0.5 to 1.3 pM/μg DNA at 0 ng/ml TGFβ1, 2.0 to 4.0 pM/μg DNA at 0.1 ng/ml TGFβ1, 1.5 to 3.9 pM/μg DNA at 1 ng/ml TGFβ1, and 1.5 to 3.8 pM/μg DNA at 3 ng/ml.
TGFβ1 (Fig. 5.3). There was a significant (p<0.05) increase in PTHrP secretion both within and between samples after treatment with 0.1, 1, and 3 ng/ml TGFβ1 as compared to control epithelial cells. The increase in PTHrP secretion as compared to baseline was similar between dogs. PTHrP secretion by prostate epithelial cells increased an average of 2.5-fold at 0.1 ng/ml TGFβ1, 3.1-fold at 1 ng/ml TGFβ1, and 3.6-fold at 3 ng/ml compared to baseline levels. Experiments were performed 3 times, each time in triplicate.

Basal secretion of PTHrP into culture medium was higher in prostate stromal cells compared to epithelial cells (~6-fold), ranging from 2.4 to 8.2 pM PTHrP/µg DNA (Fig. 5.3). Dogs with epithelial cells that secreted lower concentrations of PTHrP also had stromal cells that secreted lower concentrations of PTHrP into conditioned medium. Treatment of prostate stromal cells with TGFβ1 for 24 hours resulted in no significant change in PTHrP secretion. Experiments were performed 3 times, each time in triplicate.

**ET-1 mRNA Expression by Canine Prostate Cells After Treatment with TGFβ1:**

ET-1 mRNA was moderately expressed in canine prostate epithelial cells (Fig. 5.4A), and TGFβ1 did not significantly alter ET-1 expression after 6 hours of treatment with 3 ng/ml TGFβ (Fig. 5.4B). ET-1 mRNA expression was present in prostate stromal cells, but was less abundant than in epithelial cells (Fig. 5.4C). Treatment of prostate stromal cells had no significant effect on ET-1 expression (Fig. 5.4D). Endothelin-1 expression by prostate carcinoma cells did not significantly change in response to 1 ng/ml TGFβ1 after 6 hours of
treatment (Fig. 5.4E and F). The experiments were performed 3 times with epithelial cells, twice with stromal cells, and once with carcinoma cells.

Treatment of prostate epithelial cells with 3 ng/ml TGFβ1 for 0, 3, 9, 12, and 24 hours did not alter ET-1 mRNA expression. ET-1 mRNA expression increased slightly at 3 hours in both vehicle- and TGFβ1-treated prostate epithelial cells (Fig. 5.5A and B). Experiments were performed twice.

**PTHRP (1-84) is Degraded in Canine Prostate Epithelial but not Stromal Cell Conditioned Medium:** The rate of degradation of rhPTHrP (1-84) in cell culture medium was measured by IRMA. rhPTHrP concentrations from prostate epithelial cell conditioned medium decreased over 24 hours. Recombinant-human PTHrP was degraded 72% (± SE 8%) at 24 hours in the medium of prostate epithelial cells, with a loss of 32% (± SE 10%) within the first 2 hours in culture (Fig. 5.6). In contrast, degradation of rhPTHrP in stromal cell conditioned medium was lower after 24 hours in culture (26% ± SE 8%). There was a significant difference (p < 0.05) between the degradation of rhPTHrP in medium from prostate epithelial cells compared to prostate stromal cells. Experiments were performed 3 times (in duplicate) with epithelial cells and twice (in duplicate) with stromal cells.

**Arginine Esterase Activity Low in Conditioned Medium of Prostate Epithelial Cells:** In an effort to identify if arginine esterase, an enzyme expressed by
canine prostate epithelial but not stromal cells, could be responsible for the
degradation of rhPTHrP in conditioned medium, arginine esterase activity was
measured in prostate epithelial cell culture medium, as well as prostate stromal
cell culture medium. Protein extracts from prostate and carcinoma tissues were
positive for arginine esterase activity (Fig. 5.7). Very low AE activity was
present in conditioned medium from prostate epithelial cell cultures (Fig. 5.7),
and no AE activity was present in conditioned medium in prostate stromal cell
cultures. This indicates that canine prostate cells did not continue to secrete
high levels of arginine esterase in vitro.

DISCUSSION

Understanding the mechanism of targeting of prostate carcinoma to bone
and the development of osteoblastic metastasis is essential in order to develop
therapeutic methods of prevention. Increased expression of TGFβ1, ET-1, and
PTHrP has been associated with increased invasiveness and metastasis of
prostate carcinoma. Localization of prostate carcinoma cells to bone is likely multifactorial, involving integrins, carcinoma-secreted matrix
factors, and carcinoma-secreted and bone matrix-associated cytokines.

Osteoblastic metastases histologically appear as new woven bone which
is formed on trabeculae of mature bone within the medullary cavity. This new
bone formation must be distinguished from endosteal or periosteal new bone
that may develop at metastatic sites due to bone instability or microfractures. In
this study, we demonstrated that production of TGFβ1 could result in increased expression of PTHrP but not ET-1, in normal canine prostate cells and canine prostate carcinoma cells. TGFβ1-regulated expression of PTHrP suggests a significant role for TGFβ1 in the pathophysiology of the new bone formation observed at metastatic sites. PTHrP can cause both bone formation and bone resorption. The N-terminus of PTHrP (1-36) binds to PTH1 receptors on osteoblasts and can induce bone resorption by activation of osteoclasts. Bone lysis may result in the release of bone matrix-associated growth factors, such as TGFβ1 and IGFs. These factors could increase bone formation through stimulation of osteoblasts, and contribute to the osteoblastic appearance of prostate cancer bone metastases.

The lytic effects of PTHrP in bone may be partly inhibited by prostate specific antigen (PSA) in the human, or arginine esterase (AE) in the dog. AE is a glandular kallikrein and is expressed both by canine prostate epithelial cells and prostate carcinoma cells but not prostate stromal cells. PSA, but not AE, has been shown to cleave the N-terminus of PTHrP in vitro to an inactive form. Cleavage is proposed to destroy the PTH-like effect of the N-terminus. The C-terminal peptide (107-111; osteostatin) of PTHrP has been demonstrated to decrease osteoclastic bone resorption as well as to increase osteoblastic bone formation. These factors could contribute to greater osteoblast stimulation at the metastatic site and explain the unique ability of prostate carcinomas to induce osteoblastic rather than osteolytic metastases. Therefore, both tumor-associated and bone matrix-associated TGFβ1 could
result in a marked increase in PTHrP secretion by metastatic tumor cells in the bone microenvironment, promoting bone formation with lesser amounts of bone lysis at metastatic sites.

The role of AE in the development of osteoblastic metastasis in dogs is uncertain. In our system, rhPTHrP was cleaved rapidly in the canine prostate epithelial cell conditioned medium. AE activity was detected at very levels in the conditioned medium from canine prostate epithelial cells, but no activity was present in stromal cell conditioned medium. This may explain why PTHrP was rapidly degraded in conditioned medium of prostate epithelial cells but not prostate stromal cells. It is possible that even low AE activity was capable of degrading the relatively low level (pM concentrations) of secreted PTHrP by the epithelial cells. However, the role of other degradative enzymes secreted by prostate epithelial cells cannot be ruled out. The experiments demonstrating the degradation of rhPTHrP in prostate epithelial cell culture medium may explain the relatively low concentration of PTHrP in prostate epithelial cell conditioned medium as compared to stromal cell conditioned medium: PTHrP may degraded soon after secretion from the epithelial cells.

While PTHrP is likely important in both bone formation and bone resorption at sites of prostate cancer metastases, ET-1 could promote bone formation. ET-1 is mitogenic for osteoblasts and inhibitory to osteoclasts. In addition, PSA has been demonstrated to enhance the effects of ET-1 on bone formation. AE or other enzymes secreted by the canine prostate may serve this function in canine bone metastasis. Treatment of canine
prostate epithelial cells with TGFβ1 had no effect on ET-1 expression in either prostate epithelial, carcinoma, or stromal cells. TGFβ1 has been demonstrated to increase ET-1 expression in PC3 cells, a human prostate carcinoma cell line. Because we were able to examine only one canine prostate carcinoma in cell culture and only for a limited number of passages, it is possible that neoplastic transformation is important for increased ET-1 expression by TGFβ1.

However, equally essential to the development of osteoblastic metastasis in prostate carcinoma is the survival of the cells once they are localized in the bone microenvironment. The three factors examined in these studies, TGFβ1, PTHrP, and ET-1, have all been demonstrated to be important in the survival and proliferation of prostate carcinoma cells.

Expression of TGFβ1 markedly increases when prostate epithelial cells undergo neoplastic transformation. TGFβ1 is normally a negative regulator of cell growth and controls apoptosis in normal prostate epithelial cells. However, neoplastic cells become unresponsive to the growth inhibitory effects of TGFβ1, and it acts as a stimulatory factor in prostate carcinoma invasion and metastasis. TGFβ1 from carcinoma cells and bone matrix may also contribute to tumor survival by promoting angiogenesis and decreasing the local immune surveillance.

Increased expression of PTHrP by TGFβ1 may be important in promoting prostate carcinoma survival and growth in the bone microenvironment. PTHrP has been shown to be a growth regulator of prostate carcinoma using the MatLyLu rat prostate carcinoma model. In this system,
cancer cells with greater PTHrP expression were injected into rats, and resulted in greater primary tumor growth compared to control cells. Metastatic bone lesions had increased nuclear staining of PTHrP, which has been suggested to promote cell survival after exposure to apoptotic signals. ET-1 is an important proliferation and survival factor for prostate carcinoma cells. It is mitogenic for prostate epithelial cells, often acting in combination with other growth factors such as insulin like growth-factor-I (IGF-I), insulin-like growth-factor-II (IGF-II), platelet-derived growth factor (PDGF), and epidermal growth factor (EGF). ET-1 also inhibits prostate epithelial cell apoptosis.

Because TGFβ1 expression and secretion increases in prostate carcinoma and is present in high concentrations in the bone microenvironment, it likely regulates expression of important factors (e.g. PTHrP and ET-1) in both the development of osteoblastic prostate metastasis and prostate carcinoma growth and survival in bone. Advantages of using canine prostate cells for the study of the pathogenesis of osteoblastic metastasis are multiple. The canine prostate is simple to obtain, relatively large, recapitulates the morphologic progression of prostate carcinoma in humans, and has been demonstrated in our laboratory to induce new bone formation when implanted over the calvaria of nude mice.
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Fig. 5.1. Northern blot and graphical representation of PTHrP expression in canine prostate cells in response to TGFβ1. GAPDH mRNA was used as a loading control. A and B. Prostate epithelial cells increase PTHrP mRNA expression at 0.1, 1.0, and 3.0 ng/ml TGFβ1. Maximal expression was seen at 3 ng/ml with a 4-fold increase in PTHrP mRNA expression. Three separate experiments had similar results. C and D. Prostate stromal cells had mildly reduced mRNA expression of PTHrP at 1.0 and 3.0 ng/ml TGFβ1, to 80 and 60% of controls respectively. Three separate experiments had similar results. E and F. Prostate carcinoma cells treated with 1.0 ng/ml TGFβ1 increased PTHrP mRNA expression by 4-fold. Single experiment was performed.
Fig. 5.2 Northern blot of PTHrP mRNA expression in canine prostate epithelial cells in response to 3 ng/ml TGFβ1 for 0-24 hours. GAPDH mRNA was used as a loading control. PTHrP mRNA progressively increases with time and there is maximal expression at 9 hours after treatment with TGFβ1. At 9 hours, PTHrP mRNA expression increased 2-fold increase over baseline and 3.5-fold over vehicle-treated cells at the same time point. Three separate experiments had similar results.
Fig. 5.3. Secretion of PTHrP (1-84) into conditioned medium from prostate epithelial and stromal cells in response to TGFβ1 as measured by a 2-site IRMA. PTHrP secretion into culture medium increased significantly with 0.1, 1.0, and 3 ng/ml TGFβ1 treatment (*p<0.05) relative to vehicle-treated cells. Prostate stromal cell conditioned medium had greater concentrations of PTHrP than prostate epithelial cell conditioned medium (6-fold), secretion was not significantly increased with TGFβ1 treatment. Three separate experiments (in triplicate) had similar results.
Fig. 5.4. Northern blot and graphical representation of ET-1 mRNA expression in canine prostate cells in response to TGFβ1. GAPDH mRNA was used as a loading control. A and B. Prostate epithelial cells moderately express ET-1, and there was no significant change in expression in response to TGFβ1 treatment. Three separate experiments had similar results. C and D. Prostate stromal cells expressed low levels of ET-1 and did change expression in response to TGFβ1. Two separate experiments had similar results. E and F. Prostate carcinoma cells expressed moderate amounts of ET-1 mRNA at 1 ng/ml TGFβ1. A single experiment was performed.
Fig. 5.6 Northern blot ET-1 expression in canine prostate epithelial cells in response to 3 ng/ml TGFβ1 for 0-24 hours. GAPDH mRNA was used as a loading control. ET-1 mRNA expression increased after 3 hours in both vehicle and TGFβ1-treated samples. There was no significant change in ET-1 expression either over time or with TGFβ1 treatment. Two separate experiments were performed with similar results.
Fig. 5.6. Degradation of rhPTHrP inoculated into conditioned medium from prostate epithelial and stromal cells. The rate of degradation over 24 hours of 100 pM rhPTHrP inoculated into cell culture medium measured by IRMA. rhPTHrP concentrations from prostate epithelial cell conditioned culture medium decreased to 28 ± S.E. 8% (*p < 0.05) of original concentrations over 24 hours. rhPTHrP was minimally degraded in prostate stromal cell culture medium to 74% ± S.E. 4% over 24 hours. Degradation of rhPTHrP in prostate epithelial cell conditioned medium was significantly different at 8 and 24 hours (**p<0.05) from that in prostate stromal cell conditioned medium. Data represents 3 separate experiments.
Fig. 5.7 Arginine esterase activity measured spectrophotometrically (5 minutes after the addition of BAEE). Protein extracts from both prostate epithelial and carcinoma cells had high AE activity. 24-hour conditioned medium from prostate epithelial but not stromal cells had low levels of AE. Data represents 2 separate experiments.
PERSPECTIVES

Tumor and tumor-associated stromal cell secretion of TGFβ1 may act in an autocrine or paracrine fashion to increase tumor production of PTHrP and this may have an important role in the pathogenesis of HHM and tumor invasion and metastasis. The significant aim of the first chapter was to identify patients with HNSCC who also had HHM. The goal was to try to identify differences in PTHrP mRNA expression and secretion between tumors from patients with and without HHM as well differences in the expression of TGFβ1 and its effect on PTHrP mRNA expression. However, we were unable to address the role of TGFβ1 in the development of HHM in these patients because we discovered that HHM was very rare in HNSCC. Interestingly, all tumors examined produced PTHrP mRNA and protein as evidenced by Northern blot and immunohistochemistry.
These findings bring up an interesting question regarding the mechanism of development of HHM: why is HHM more common in lung squamous cell carcinoma (SCC) than in HNSCC when the tumors are morphologically identical? Differences may be associated with differences in translation, secretion, or degradation in these cells. These differences are may be important to better understanding the pathogenesis of this paraneoplastic disease.

The second and third chapters addressed the mechanisms by which TGFR1 increases PTHrP mRNA stability. There is very little known on the mechanisms by which TGFB1 alters PTHrP mRNA stability. The presence of multiple alternatively spliced forms of PTHrP makes examination of changes in PTHrP mRNA stability very complex. In chapter 2, I examined the role of cis-acting sequences in the mRNA of PTHrP (1-141) in TGFB1-induced mRNA stability. These studies suggested that coding region sequences, possibly in the terminal coding region, might be important to the effect of TGFB1-treatment on the stabilization of PTHrP mRNA (1-141).

However, these studies only examined a single isoform (1-141) of PTHrP. All three isoforms of PTHrP (1-139, 1-173, and 1-141) have the same mRNA sequence encoding up to amino acid 139. Because each has the amino terminus, each is equally capable of inducing HHM. However, little is known about the differences between processing and expression of these isoforms in various tissues. Chapter 4 is an effort to better understand the importance of TGFB1 on the expression and stabilization of the individual PTHrP mRNA isoforms, each of which has a unique 3'UTRs. This data revealed that there are
differences not only in the mRNA half-life between these isoforms, but also
differences in the effect of TGFβ1 on the mRNA expression. For example,
PTHrP mRNA (1-139) has a short half-life and expression is increased with
TGFβ1 treatment. However, its mRNA half-life is unaltered by TGFβ1
treatment. In contrast, PTHrP mRNA (1-173) has a very long half-life and
TGFβ1 treatment stabilizes its mRNA. Differences in the expression of these
various isoforms may be important to better understand the pathogenesis of
HHM as well as important in predicting prognosis or response to therapy.

Finally, in the last chapter, I studied the effect of TGFβ1 on a different
epithelial cancer: prostate carcinoma. While prostate cancer has not been
extensively associated with HHM, it is associated with osteoblastic metastases
in bone. PTHrP has been suggested as an important factor in both the induction
of osteoblastic metastasis and as a survival factor in prostate tumor cells.
Because both prostate tumor and bone cells produce large amounts of TGFβ1,
we felt that the same paracrine/autocrine mechanisms affecting PTHrP
expression in SCC might be important in promoting PTHrP expression and
secretion in prostate cancer. Increased PTHrP expression by the prostate
carcinoma could promote tumor progression and the development of
osteoblastic metastasis. The data from this chapter indicates that TGFβ1
increased PTHrP expression in prostate cancer, and therefore might be an
important contributing factor in prostate cancer invasion, metastasis, and
osteoblastic metastases.
Better understanding of the regulation of the expression of the mRNA encoding the 3 isoforms of PTHrP as well as the mechanisms regulating secretion are essential to the development of methods of prevention and treatment of HHM and may be important for preventing prostate cancer invasion and metastasis. TGFβ1 is only one of potentially many factors that may play an important regulatory role in this protein, and further investigations to elucidate these regulatory mechanisms would be of great interest.


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Ref Type: Abstract


