Parathyroid Hormone-related Protein as a Growth Regulator of Prostate Carcinoma


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ABSTRACT

Parathyroid hormone-related protein (PTHrP) is produced by prostate carcinoma cells and tumors, but little is known of its role in prostate carcinogenesis. The goal of this study was to evaluate PTHrP expression in the regulation of prostate carcinoma growth using human and animal models. PTHrP expression was assessed in prostate cancer cell lines in vitro. Seven of nine cell lines produced PTHrP, and increased expression was seen during cell proliferation. The MatLyLu rat prostate carcinoma model was used to determine the effects of PTHrP overexpression on prostate tumor growth. PTHrP overexpression did not alter proliferation of cells in vitro. However, when PTHrP-overexpressing cells were injected into rat hind limbs, primary tumor growth and tumor size were significantly enhanced as compared with control cells. To evaluate PTHrP in human prostate carcinoma patients, immunohistochemistry was performed on metastatic bone lesions. Immunolocalization of PTHrP protein was found in the cytoplasm and nucleus of cancer cells in the bone microenvironment. Because nuclear localization of PTHrP has been associated with an inhibition of apoptosis, the ability of full-length PTHrP to protect prostate cancer cells from apoptotic stimuli was examined. Cells transfected with full-length PTHrP showed significantly increased cell survival after exposure to apoptotic agents as compared with cells producing no PTHrP (plasmid control) or cells transfected with PTHrP lacking its nuclear localization signal. To determine the mechanism of action of PTHrP in prostate cancer cells, the parathyroid hormone/PTHrP receptor status of the cells was determined. These cell lines did not demonstrate parathyroid hormone/PTHrP receptor-mediated binding of iodinated PTHrP or steady-state receptor message by Northern blot analysis, but they did have a detectable receptor message by reverse transcription-PCR analysis. In summary, PTHrP is expressed in many prostate carcinoma cell lines and in metastatic bone lesions in vivo. PTHrP expression positively influences primary tumor size in vivo and protects cells from apoptotic stimuli. These data suggest that PTHrP plays an important role in the promotion of prostate tumor establishment and/or progression.

INTRODUCTION

Prostate cancer is currently the second leading cause of cancer-related death in males in the United States (1). One of the major causes of morbidity and mortality in prostate cancer is metastatic bone disease. The most frequent sites of metastasis are the bones of the pelvis and the spine, and involvement of the lumbar and dorsal vertebrae is more common than that of the thoracic vertebrae and the sacrum (2, 3).

Often, bone metastases grow at a more rapid rate than primary tumors or other metastatic lesions. This has been attributed to inter-
1. Background

- **Prostate cancer** is a significant health issue, with a high incidence in developed countries. The incidence has been rising, especially in men over the age of 50.

2. Methods

- **Preclinical Models**
  - **LNCaP** (Human androgen-dependent prostate cancer cell line)
  - **MLL** (Murine osteosarcoma cell line)

- **Cell Culture**
  - Cells were cultivated in **RPMI 1640** supplemented with 10% **fetal bovine serum**.
  - **Penicillin** and **streptomycin** were added to the medium at concentrations of 100 units/ml and 100 μg/ml, respectively.

- **Transfection**
  - Cells were transfected with plasmids using Lipofectamine 2000.

3. Results

- **PTHRp** Immunohistochemistry
  - Immunohistochemical staining was performed using the **ABC** method.

- **Proliferation and Apoptosis Assays**
  - **MTT** assay was used for proliferation assays.

4. Conclusion

- The study highlights the importance of **PTHRp** in prostate cancer progression and the potential therapeutic implications of targeting this receptor.

### Table 1: Cell lines used in this study

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Species</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC-3</td>
<td>Human</td>
<td>Androgen-independent, initiated from a 62-year-old male with grade IV prostate adenocarcinoma</td>
</tr>
<tr>
<td>PC-3 MB</td>
<td>Human</td>
<td>Subline of PC-3 that metastasizes to bone</td>
</tr>
<tr>
<td>LNCaP</td>
<td>Human</td>
<td>Androgen-dependent, originally isolated from a lymph node needle aspiration biopsy from a 50-year-old male with metastatic prostate cancer</td>
</tr>
<tr>
<td>DU-145</td>
<td>Human</td>
<td>Androgen-independent, isolated from a brain lesion in a patient with metastatic prostate cancer (prostate and lymphocytic leukemia)</td>
</tr>
<tr>
<td>AT-2.1</td>
<td>Rat</td>
<td>Derived from Dunning rat prostate tumor, low to moderate metastatic potential</td>
</tr>
<tr>
<td>MLL</td>
<td>Rat</td>
<td>Derived from Dunning rat prostate tumor, high metastatic potential to lymph node and lung</td>
</tr>
<tr>
<td>AT-3.1</td>
<td>Rat</td>
<td>Derived from Dunning rat prostate tumor, high metastatic potential to lymph node and lung</td>
</tr>
<tr>
<td>MAT-Lu (ML)</td>
<td>Rat</td>
<td>Derived from Dunning rat prostate tumor, high metastatic potential to lung</td>
</tr>
<tr>
<td>GP9F3</td>
<td>Rat</td>
<td>Derived from Dunning rat prostate tumor, high metastatic potential</td>
</tr>
<tr>
<td>Control cell lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROS17/2.8</td>
<td>Rat</td>
<td>Osteosarcoma, positive control for PTHrP receptor</td>
</tr>
<tr>
<td>MC3T3-E1</td>
<td>Murine</td>
<td>Immortalized calvarial cell, positive control for PTHrP receptor</td>
</tr>
</tbody>
</table>

### Notes

- **PTHRp** and **prostate carcinoma** are studied extensively to understand the role of PTHRp in prostate cancer progression and potential therapeutic targets.

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*Source: Data from the study by Dr. John Isaacs and colleagues.*

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*Image source: Used for illustrative purposes only.*
shaking. The unbound peptides were washed off the cell monolayer twice with HBSS, the cells were lysed with 0.5 m NaOH for 30 min, and the resultant suspension was counted in a scintillation counter.

RT-PCR. RT-PCR of the PTH/PTHrP receptor was performed using the Gene Amp PCR kit (Roche Molecular Systems, Branchburg, NJ). Total RNA from ROS-17/2.8 or LNCaP cells was isolated using the guanidinium isothiocyanate method (see above). The RT-PCR reaction was performed as described in the manufacturer’s protocols with 1 µg of total RNA, using random hexamers for priming. A negative control reaction, without the addition of reverse transcriptase, was performed for each RNA sample. The PCR reaction was carried out using one-half of the reverse transcriptase product. The primers used for amplification of the receptor were 5'-ACCAATGAGACTCGTGAACGG-3' and 5'-AAGGA-CAGGAACAGGTGTCATG-3', with an amplification product of 167 bp. Annealing was done at 60°C, and the reaction was carried out for 35 cycles. RT-PCR products were analyzed by ethidium bromide staining.

Animal Studies. Male Copenhagen rats (200–250 g) were obtained from Harlan Sprague Dawley (Indianapolis, IN). Fig. 1 outlines the three experiments performed. To determine the effects of MLL cell transfectants on tumor growth rate and metastasis (LCM study), male Copenhagen rats were injected s.c. in the upper leg with 1 x 10^5 of the transfected MLL cells described above (10 rats/group). Two-dimensional measurements of the primary tumors were taken daily using calipers. Tumors were removed when they reached a size of 1 cm^3. Competitive binding assays were performed with all indicated cell lines and electrophoresed in a 12% gel (20 mg/lane), and Northern blot analysis was performed using radiolabeled rat PTHrP cDNA as a probe. Blots were stripped and rehybridized with an 18S rRNA probe.

RESULTS

Prostate Cancer Cell Lines Express and Secrete PTHrP in Vitro. Seven of nine prostate cancer cell lines surveyed, including MAT-Lu, MLL, AT-2.1, AT-3.1, PC-3, PC-3 MB, and GP9F3, expressed high levels of steady-state PTHrP mRNA (Fig. 2). These cells represent cell lines of human and rat origin and have varying degrees of metastatic potential and androgen dependency, as indicated in Table 1. PTHrP mRNA could not be detected by Northern blot analysis in two human prostate carcinoma cell lines, namely, LNCaP and DU-145.

Given the high level of PTHrP mRNA expression, we selected representative human (PC-3) and rat (MLL) cell lines to examine for production and secretion of PTHrP into the tissue culture medium using an immunoradiometric assay. The amount of secreted protein was normalized relative to the cell number as determined by total DNA content. Full-length PTHrP secretion increased as the cells proliferated in culture until they reached confluence and decreased thereafter (Fig. 3).

Fig. 3. PTHrP production by prostate cancer cell lines during proliferation. MLL or PC-3 cells were plated as described in “Materials and Methods.” Cell supernatants were collected every 24 h, and PTHrP levels were measured by RIA. Data are expressed as the mean ± SE of triplicate samples. The asterisk denotes when the cells became confluent.

Fig. 1. Experimental design of three in vivo studies. LCT and HCT studies evaluated the ability of MLL transfectants to alter primary tumor size at a fixed time. LCM and HCT studies evaluated the ability of MLL transfectants to alter primary tumor growth rate and subsequent metastasis to lung.
cell lines listed in Table 1 and with the three LNCaP-transfected cell lines used for apoptosis studies. Specific binding of PTHrP was not found in the prostate carcinoma cell lines. To determine whether there were low levels of PTH/PTHrP receptor expression in the LNCaP cell line, RT-PCR was performed using receptor-specific primers. Using RT-PCR, a positive signal for PTH/PTHrP receptor mRNA was detected (Fig. 4).

Characterization of MLL Sublines. As indicated above, the MLL subline of the R3327 prostate adenocarcinoma expresses PTHrP. The MLL cell line (47) was derived from the Dunning (R3327) rat prostate adenocarcinoma (48). Injection of MLL sublines into male Copenhagen rats is a well-established model for studying the metastatic potential of various cell lines and the effects of treatments (43, 49). To investigate whether overexpression of PTHrP would affect parameters of tumor growth and the metastatic potential of the cells, stable transfectants of the MLL cells were generated with either a plasmid expressing full-length rat PTHrP cDNA (PTHrP-MLL) or a control plasmid (pcDNA-MLL). Northern blot analysis of the cell lines showed a marked increase in PTHrP mRNA in the PTHrP-MLL cells as compared with the pcDNA-MLL cells (Fig. 5A). The PTHrP signal in pcDNA-MLL cells was equivalent to that of the parental MLL cell line (data not shown). Additional parameters of the transfected cells were analyzed to determine whether PTHrP overexpression altered other cellular characteristics. Steady-state osteopontin (an adhesion protein thought to be associated with metastatic potential; Ref. 50) mRNA levels were unchanged in the three cell lines (Fig. 5A). The amount of PTHrP secreted by the transfected cells into the cell culture medium was quantitated by radioimmunometric assay to determine whether increased PTHrP mRNA resulted in increased protein production. PTHrP production by PTHrP-MLL cells was significantly higher than that of the pcDNA transfected cells (36 ± 1.6 versus 7.3 ± 2.0, respectively; P < 0.001; Fig. 5B).

In vitro proliferation of PTHrP-MLL cells and pcDNA-MLL cells was also monitored using the MTT metabolic assay. Over a 3-day period, there was no significant difference in the rate of proliferation between the cell lines (Fig. 5C), and they had growth rates similar to that of untransfected MLL cells. The lack of alteration in proliferation and osteopontin gene expression suggests that if there are any differences in the behavior of the cell lines in vivo, this would be attributable to PTHrP production and not to some artifact of the culture or transfection protocol.

PTHRP Influences Tumor Growth in the Dunning Rat Prostate Adenocarcinoma Model. To determine whether PTHrP expression affects tumor cell growth, three in vivo experiments were performed using the Dunning rat prostate adenocarcinoma metastasis model (see Fig. 1 for experimental design). For each of these studies, pcDNA-MLL and PTHrP-MLL cells were injected s.c. into the hind leg of male Copenhagen rats. This injection route does not lead to the formation of skeletal metastases. The LCT (10^5 cells injected) and HCT (2.5 × 10^6 cells injected) studies evaluated the ability of MLL transfectants (producing low and high levels of PTHrP) to influence primary tumor size at a fixed time (17 days and 14 days, respectively). The LCM study (10^5 cells injected) evaluated both the growth rate of the primary MLL tumors and the numbers of lung metastases based on a primary tumor of defined size (2 cm^3). The HCT study also evaluated the ability of MLL transfectants to metastasize to the lung.

The effects of PTHrP expression on primary tumor size are shown in Fig. 6A. In both the HCT and LCT studies, primary tumor weight was increased to a greater extent in tumors derived from PTHrP-MLL cells as compared with tumors derived from pcDNA-MLL cells. This trend, which was seen in the LCT study, reached statistical significance when larger numbers of cells were used to establish the primary tumor (HCT study).

The level of PTHrP expression also altered the rate of tumor growth, as measured in the LCM study. In this study, the number of days required for the primary tumors to reach 2 cm^2 was significantly lower in the high-PTHrP-producing tumors (PTHrP-MLL, 13.3 ± 0.94 days; n = 10) versus the tumors producing less PTHrP (pcDNA-MLL, 19.4 ± 2.0 days; n = 10; P < 0.05). This supports the data from the HCT experiment (Fig. 6A) that increased PTHrP expression promotes tumor growth in vivo.

To determine whether the level of PTHrP expression had an effect on the metastatic potential of prostate carcinoma cells, the extent of lung metastasis was analyzed 2 weeks after removal of the primary tumor when it reached a fixed size of 2 cm^2 (LCM study). There was no significant difference in the average number of lung lesions observed between the two cell lines injected (pcDNA-MLL, 161 ± 36; PTHrP-MLL, 113 ± 27). Similarly, there was no difference in the numbers of lung metastases in the HCT study, although the primary tumors were significantly larger in the PTHrP-MLL group.

The plasma PTHrP level in all rats was also determined after sacrifice. There was no detectable PTHrP in the plasma of uninjected control rats (n = 3), suggesting that the PTHrP measured in treatment rats was attributable to production by the tumor cells. The average plasma PTHrP level (pm) for the HCT studies (in which the rats were sacrificed, and plasma was collected 2 weeks after primary tumor removal) was significantly higher in the animals injected with PTHrP-MLL cells (9.03 ± 2.56; n = 10) than in animals injected with
pcDNA-MLL cells (0.67 ± 0.21; n = 8; P < 0.05; Fig. 6A). In the LCT study, the plasma levels were determined at the time of primary tumor removal. The average value was also significantly higher in the PTHrP-MLL group (3.61 ± 0.78; n = 7) than in the pcDNA-MLL group (0.56 ± 0.19; n = 8; P < 0.01; Fig. 6A).

In the LCT study, the plasma PTHrP levels were compared with tumor weight to determine whether the larger tumors observed in rats injected with the PTHrP-MLL cells could account for their increased plasma PTHrP levels. The average PTHrP level for animals injected with PTHrP-MLL cells was 0.75 ± 0.08 pmol/g tumor, whereas that for animals injected with pcDNA-MLL cells was 0.12 ± 0.02 pmol/g tumor (P < 0.0001). Therefore, the higher blood levels could not be attributed simply to larger tumors but appear to be dependent on the amount of PTHrP made by the tumor cells. This is supported by the Northern blot analysis of total RNA extracted from the primary tumors (Fig. 6B). The steady-state level of PTHrP mRNA, normalized to 18S rRNA, was increased 10-fold in tumors from PTHrP-MLL cells (n = 8) versus tumors from pcDNA-MLL cells (n = 6; P = 0.01). Thus, the tumor cells transfected with PTHrP maintained their ability to produce increased amounts of PTHrP in vivo.

**PTHrP Is Expressed by Metastatic Prostate Carcinoma in Vivo.** Primary human prostatic carcinoma is immunohistochemically positive for PTHrP, but little is known of PTHrP expression in bone metastasis. To determine whether prostate carcinoma metastases in bone produce PTHrP, immunohistochemistry was performed on sections from human patients. A polyclonal antibody against PTHrP positively stained the metastatic tumor tissue in all three patient samples evaluated. A representative section is seen in Fig. 7. Positive staining was specific to the tumor tissue, because the adjacent normal marrow in these samples were negative for PTHrP. Staining was observed throughout the cytoplasm, with intense staining localized at the nuclear envelope (Fig. 7B). No staining was observed in samples incubated without primary antibody or incubated with control egg yolk from nonimmunized chickens (data not shown).

**PTHrP Expression Protects LNCaP Cells from PMA-induced Apoptosis but Does Not Act as a Mitogen in Vitro.** Expression of full-length PTHrP localized to the nucleus has been shown to protect cells (chondrocytes) from apoptotic stimuli (16). Because metastatic prostate carcinoma showed nuclear PTHrP staining, the hypothesis was proposed that expression of full-length PTHrP in prostate cancer
cells could protect them from apoptosis and, consequently, contribute to primary tumor growth. To test this hypothesis, LNCaP cells, which do not produce detectable endogenous PTHrP, were transfected with a control plasmid (pcDNA-LNCaP), a plasmid containing full-length PTHrP (PTHrP-LNCaP), or PTHrP lacking the NLS, amino acids 87–107 (Δ-LNCaP). The cells were treated with PMA to induce apoptosis. It has been demonstrated previously that PMA promotes apoptotic death in LNCaP cells using light microscopy, transmission electron microscopy, and DNA laddering techniques (51). Using the APO-BRDU assay to end-label the fragmented DNA indicative of apoptosis, the cells expressing full-length PTHrP were found to have significantly less PMA-induced apoptosis than pcDNA-LNCaP cells (Fig. 8). Apoptosis in Δ-LNCaP cells was similar to that in pcDNA.

The parental LNCaP cell line did not have detectable PTH/PTHrP receptors by Northern blot analysis or by binding assay; however, a signal for the PTH/PTHrP receptor was detected by RT-PCR. The receptor status of the LNCaP transfected cell lines was examined as well, to determine whether the production of PTHrP could have an autocrine effect. Similar results for PTH/PTHrP receptor expression (negative by Northern blot analysis and binding, positive by RT-PCR) were seen with the pcDNA-LNCaP, PTHrP-LNCaP, and Δ-LNCaP cell lines as for the LNCaP parent line (data not shown).

Because Massfelder et al. (27) have found that full-length PTHrP acts as a mitogen and NLS-deleted PTHrP acts as a growth-supressor in vascular smooth muscle cells, the effect of PTHrP expression on proliferation was tested in the transfected LNCaP cells in vitro. The pcDNA-LNCaP and PTHrP-LNCaP cells exhibited identical rates of growth using the MTT proliferation assay, and no antimitogenic effects were observed in the Δ-LNCaP cells (data not shown).

DISCUSSION

Determining the factors involved in promoting prostate cancer growth, its preferential metastasis to bone, and induction of blastic lesions is important for understanding the pathogenesis of the disease and for attempts to develop more effective therapies. This study has examined the potential role of PTHrP in the regulation of prostate carcinoma growth.

PTHrP was expressed in many, but not all, of the prostate cancer lines examined. PTHrP produced by the cells increased during the proliferative phase, suggesting bioavailability of PTHrP during tumor formation in vivo. There was no apparent correlation between expression of PTHrP and the aggressiveness or androgen dependency of the tumor from which the cell lines were derived. These same cells did not have detectable PTH/PTHrP receptors using Northern blot analysis or binding assays. The detection of transcript by RT-PCR suggests that receptors may be present at a low level, but its physiological relevance is unknown. In a previous study, PTHrP was found to stimulate prostate cancer cell growth in vitro via an autocrine loop (25); however, we did not find any alteration in proliferation in vitro when cells overexpressed PTHrP.

Does PTHrP play a role in the establishment or growth of primary tumors? Several lines of evidence from this study support this idea. The first is the observed trend that rat prostate cancer cells which overexpress PTHrP form larger primary tumors than cells producing lower levels of PTHrP. Second, the rate of tumor growth was increased with PTHrP expression in vivo, whereas these same cells did not show an increase in growth rate in vitro. This suggests that PTHrP may be exerting its effects through a paracrine path in vivo or, alternatively, that expression of PTHrP could protect cancer cells from apoptotic stimuli present in the in vivo microenvironment via an autocrine or intracrine mechanism.

The s.c. MLL model was also used to assess the effect of PTHrP
expression on lung metastasis. Our results showed that the number of lung metastases was not influenced by the level of PTHrP produced by the tumor cells. This is in agreement with results obtained using the same cell lines in an intracardiac injection study to evaluate metastasis to bone (52). The incidence of metastasis was not affected by the amount of PTHrP made by the cell line, although osteoclast numbers were increased in the PTHrP-MLL lesions compared to trabecular bone from pcDNA-MLL lesions. Similar skeletal metastatic findings were observed by Rabbani et al. (53).

Studies of LNCaP cells in vitro provide other insights into potential mechanisms of PTHrP action. This cell line provides a good model for assessing the effects of PTHrP expression because the parental cell line produces no detectable PTHrP. The present study revealed that expression of full-length PTHrP in this cell line was protective against PMA-induced apoptosis, whereas the expression of NLS-deleted PTHrP in the same cells had no effect on apoptosis. Therefore, PTHrP must reach the nucleus to exert its antiapoptotic effect. These data are in agreement with Henderson et al. (16), who originally suggested that PTHrP could act as an inhibitor of apoptosis. Because PTHrP expression protects prostate cancer cells from an apoptotic stimulus, PTHrP could give cells a growth advantage during primary tumor formation and/or subsequent metastasis. In these transfected cells, expression of PTHrP did not have a mitogenic effect in vitro, nor did the NLS-deleted PTHrP transfected cells have altered proliferation rates. This is in contrast to what has been observed in similar experiments using vascular smooth muscle cells (23). However, as Massfelder et al. (27) point out in their vascular smooth muscle study, the cellular response to PTHrP appears to be cell type specific, inhibiting proliferation in some cells and stimulating proliferation in others. Although the present study indicates that PTHrP expression stimulates primary tumor growth, whether this occurs via a specific mitogenic effect in vivo remains to be tested.

In summary, the findings that PTHrP is expressed in many prostate cell lines, in primary and metastatic prostate cancer, and that PTHrP can influence tumor growth in vivo and apoptotic rates in vitro support the hypothesis that this protein plays an important role in the progression of prostate cancer.

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