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


Department of Periodontics/Prevention/Geriatrics [K. M. D., A. J. K., L. K. M.], University of Michigan Comprehensive Cancer Center [K. J. P.], The University of Michigan, Ann Arbor, Michigan 48109-1078, Department of Medicine, McGill University, Montreal, Quebec, Canada H3T 1E2 [J. E. H.]; and Department of Veterinary Biosciences, The Ohio State University, Columbus, Ohio 43210 [E. A. G. B., T. J. R.]

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 the 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 carcinomas, but they did have a detectable receptor message by reverse transcription-PCR analysis. In summary, PTHrP is expressed in many prostate carcinomas, but they did have a detectable receptor message by reverse transcription-PCR analysis. In summary, PTHrP is expressed in many prostate carcinomas, but they did have a detectable receptor message by reverse transcription-PCR analysis.

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 metastases 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 interconnections between prostate cancer and bone cells (4, 5) and likely reflects the presence of factors that either promote cell growth or inhibit cell death. However, the factors that influence the preferential spread of prostate tumor cells to the bone and the subsequent stimulation of bone formation are unknown.

PTHrP is an autocrine or paracrine factor that binds to receptors on osteoblasts and stimulates bone formation and resorption (6). Originally described as the factor responsible for humoral hypercalcemia of malignancy (7, 8), PTHrP is also produced by many normal cell types during development and adult life (9). PTHrP has limited homology to PTH at its NH2 terminus and can bind the same receptor as PTH with similar biological activity (6, 10). Suggested physiological roles for PTHrP include regulation of calcium transport, keratinocyte differentiation, smooth muscle relaxation, and cartilage development (11). In the latter instance, complete deficiency of PTHrP in mice leads to abnormal endochondral bone formation and lethal skeletal dysplasia (12, 13). PTHrP is secreted in a regulated or constitutive fashion, depending on the cell type (14, 15). Additionally, the protein contains a functional NLS (amino acids 87–107) that can target the protein to the nucleus or the nucleolus (16). Hence, in addition to functioning in a classical autocrine or paracrine manner, PTHrP has the capacity to act in an intracrine manner, bypassing the need for interaction with cell surface receptors.

Many features of PTHrP make it an attractive candidate for influencing prostate carcinoma growth. PTHrP is produced by normal prostate epithelial cells, from which prostate carcinoma arises, and PTHrP is found in the seminal fluid (17). PTHrP has been immunohistochemically identified in prostate cancer tissue in patients with clinically localized disease (18), is found in higher levels in prostate intraepithelial neoplasia than in normal prostate epithelium (19), and is found in higher levels in prostate carcinoma than in benign prostatic hyperplasia (20). There is also evidence that PTHrP can regulate malignant tumor growth in an autocrine manner in human renal cell carcinoma (21), enhance breast carcinoma metastasis to bone (22–24), and act as an autocrine growth factor for prostate cancer cells in vitro (25). Recent evidence indicates that expression of nuclear-targeted PTHrP can protect other cell types (i.e., chondrocytes) from apoptosis (16), bind RNA (26), or act as a mitogen in vascular smooth muscle cells in vitro (27); however, the ability of PTHrP to regulate apoptosis or cell proliferation in prostate carcinoma is unknown. The purpose of this study was to determine the role of PTHrP in the regulation of prostate carcinoma cell and tumor growth.

MATERIALS AND METHODS

Cell Culture. The cell lines used in this study are described in Table 1. PC-3 and LNCaP cells were obtained from American Type Culture Collection (Manassas, VA), PC-3 MB cells were obtained from Dr. Mark Stearns (Uni-
iversity of Pennsylvania, Philadelphia, PA), and AT-2.1, AT-3.1, MAT-Lu, and GP9F3 cells were a gift from Dr. John Isaacs (Johns Hopkins University, Baltimore, MD). MC3T3-E1 cells were obtained from Dr. M. Kempewaga (Meikai University, Sakado, Japan) via Dr. Renny Franceschi (University of Michigan, Ann Arbor, MI) and maintained as described previously (28). All prostate cell lines except DU-145 were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 μg/ml Gentamicin (Sigma, St. Louis, MO) in a humidified, 5% CO2, 37°C incubator. DU-145 cells were cultured in DMEM-F12 (Life Technologies, Inc.) supplemented as described above. ROS17/2.8 cells (29) were cultured in DMEM-F12 with 5% serum.

Plasmid DNA. The PTHRv3 vector contains a full-length rat PTHR cloning vector (pcDNA3.1+ vector control) was obtained from Invitrogen.

Cell Transfection. MLL cells were plated at 10,000 cells/cm² in 6-well plates and grown to 50–60% confluence. Cells were transfected with 2 μg of control plasmid pcDNA3.1+ (pcDNA-MLL) or PTHRv3 (pcDNA-MLL) using Lipofectin (Life Technologies, Inc.). The cells were placed under G418 selection (700 μg/ml; Sigma) during selection and subsequent expansion.

Northern Blot Analysis. RNA was isolated from cells in culture by the RNA was isolated from tissue flash-frozen in liquid nitrogen. The tissue was homogenized briefly in cold guanidinium isothiocyanate using the Tissue Tearor homogenizer (Dremel, Racine, WI) at setting 4. After homogenization, tumor RNA isolation was carried out as described for tissue culture cells. Total RNA (20 μg) was electrophoresed on 1.2% agarose-formaldehyde gels and transferred to nylon membranes (Duralon U.; Stratagene Inc., La Jolla, CA), and UV cross-linked. [32P]dCTP-labeled probes for rat PTHR (32), PTHR receptor (R15B; Ref. 33), or human osteopontin (34) were made using the Redi-prime kit (Amersham, Arlington Heights, IL). After overnight hybridization followed by washes of increasing stringency, the nylon membranes were exposed to Kodak X-OMAT or Biomax film (Rochester, NY) at ~70°C. Quantitation of radioactivity (cpm) was determined using an Instant Imager (Packard Instrument Co., San Diego, CA). Loading was standardized by hybridizing with an 18S rRNA probe (35).

PTHrP Immunoradiometric Assay. The INCSTAR intact PTHR immunoradiometric assay (DiaSorin, Stillwater, MN) was used for the determination of biologically active PTHR (1–84) in cell culture supernatants and rat plasma. Initially, cell culture supernatants were collected either on ice in borosilicate glass tubes with no additives or on ice in Nicholls collection tubes containing protease inhibitors. Because there was no difference in the PTHR levels detected between the two methods, supernatants were subsequently collected on ice with no additives and stored at ~20°C. Rat blood was collected in EDTA (4 ml final concentration) on ice and centrifuged (9000 rpm, 15 min, 4°C). Plasma was transferred to borosilicate glass tubes and frozen. The immunoradiometric assay uses a first antibody specific for PTHR (1–40) and a second antibody specific for PTHR (57–80) that is labeled with 125I. The detection limit of the assay is 0.2 pmol/liter. The concentration of PTHR in experimental samples was determined using human recombinant PTHR (1–84) standards and calculated using Graph Pad Prism software (Graph Pad Software, Inc., San Diego, CA). All samples were assayed in duplicate.

Proliferation and Apoptosis Assays. PC-3 and MLL cells were plated at 20,000 cells/cm² and allowed to attach overnight. Cell culture supernatants were collected every 24 hr for 5 days (MLL) or 8 days (PC-3) for PTHR immunoradiometric assay. DNA levels were determined by collecting the cell layer in 10 ml Tris (pH 7.4), 0.5 M HCl, and 0.1% SDS and measuring Hoechst dye 33258 binding using a Hoefer TKO fluorometer as described previously (36). Calculations were done using a standard curve and Graph Pad Instat software (Graph Pad Software, Inc.).

The in vitro proliferation of transfected MLL and LCaP cell lines was monitored using the MTT colorimetric assay as described previously (37, 38) with slight modifications on cells seeded in 96-well plates (5000 (MLL) and 6000 (LCaP) cells/cm² in triplicate for each day).

Apoptosis was assessed in the three stably transfected LNCaP cell lines described above using the APO-BRDU Flow Cytometry Kit (Phoenix Flow Systems, San Diego, CA). The cells were grown to 60% confluence and then treated for 24 hr with 10 nM PMA (Sigma) or vehicle (0.1% ethanol). After treatment, floating and adherent cells were collected, fixed, and processed for flow cytometry following the manufacturer’s protocols. The assay was repeated four times with similar results.

PTHrP Immunohistochemistry. PTHR immunohistochemistry was performed as described previously (39–41). Metastatic prostate carcinoma specimens were collected from patients by biopsy or at autopsy, fixed in Bouin’s fixative, routinely processed, and paraffin-embedded. Tissue sections (5 μm) were deparaffinized in xylene and descending concentrations of ethanol and washed in water. Endogenous peroxidase was blocked with 3% H2O2 for 15 min. The slides were washed in PBS (pH 7.4) for 20 min, and nonspecific binding was inhibited by incubating the slides in dilute horse serum for 15 min. The slides were incubated in immunoperoxidized chicken antihuman PTHR antibody (42) for 12 hr at 4°C and then washed in PBS for 10 min, and the secondary antibody, biotinylated rabbit antichicken IgG (Zymed Laboratories, Inc., San Francisco, CA), was applied for 45 min at room temperature. The slides were washed in PBS (pH 7.4) for 20 min, and nonspecific binding was inhibited by incubating the slides in dilute horse serum for 15 min. The slides were incubated in immunoperoxidized chicken antihuman PTHR antibody (42) for 12 hr at 4°C and then washed in PBS for 10 min, and the secondary antibody, biotinylated rabbit antichicken IgG (Zymed Laboratories, Inc., San Francisco, CA), was applied for 45 min at room temperature. The slides were washed in PBS for 10 min and then incubated for 15 min with avidin-biotin-peroxidase complex (Immunopure ABC Staining Kit; Pierce Chemical, Rockford, IL), washed for 10 min in PBS, rinsed in 0.5% Triton X-100 and PBS for 30 s, and stained with 0.05% diaminobenzidine tetrachloride (Immunopure DAB; Pierce Chemical) for 7 min. The slides were washed in water for 5 min, counterstained with hematoxylin, dehydrated with ascending concentrations of ethanol and xylene, and coverslipped. Stains with no primary antibody or with control egg yolk IgG from nonimmunized chickens were used as negative controls.

Receptor Binding Assay. The PTHR receptor binding assays were performed as described previously (38) on cells grown to confluence in 24-well plates. Each well was incubated with 25,000 cpm of mono-siodinated [125I] labeled [Tyr36] PTHR (1–36) in addition to varying concentrations of non-radioactive PTHR (1–36). The cells were incubated for 2 hr at 4°C with gentle...
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-CAGGAAACAGGTGCATG-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 electrophoresis on a 2% agarose gel, and positive bands were visualized 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 105 of the transfected MLL cells described above (10 rats/group). Two-dimensional measurements of the primary tumors were taken daily using callipers. Tumors were removed when they reached a size of 2 cm3 by amputation of the tumor-bearing leg under ketamine (100 mg/kg; Fort Dodge Laboratories, Fort Dodge, IA) and methoxyflurane anesthesia. The tumors were weighed and frozen in liquid nitrogen for RNA isolation. Two weeks after the primary tumor was removed, the animals were sacrificed, plasma was collected and frozen for the PTHrP immunoradiometric assay, and the lungs were fixed in Bouin’s solution (Polysciences Inc., Warrington, PA). The numbers of metastatic lesions in the lung were determined by counting the surface lesions (43).

To determine the effects of MLL cell transfectants on primary tumor size, rats were injected as described for the metastasis study with either 105 (LCT study; 10 rats/group) or 2.5 × 105 cells (HCT study, 10 rats/group), and the animals were sacrificed on day 14 (HCT study) or day 17 (LCT study). After sacrifice, plasma was collected and frozen, and tumors were weighed and then frozen for RNA isolation.

All animal experiments were performed using protocols approved by the University of Michigan Animal Investigation Committee.

**Statistical Analysis.** The results of the animal experiments were analyzed by unpaired t test with Welch correction using the InStat 3.0 biostatistics program (GraphPad Software, Inc.). The receptor binding assay and PTHrP immunoradiometric assay were analyzed using the GraphPad Prism program. All in vitro experiments were repeated at least three times.

**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. 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. 4B).

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 growth 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 (1 × 10⁶ cells injected) and HCT (2.5 × 10⁶ 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 (10⁵ 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 cm³). 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 cm² 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 cm² (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; 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 was 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

---

**Fig. 5. Analysis of MLL stable transfectants in vitro.** A, Northern blot analysis of total RNA from pcDNA-MLL cells and PTHrP-MLL cells. Blots were probed with PTHrP (top panel), osteopontin (OPN; middle panel), and 18S rRNA (bottom panel). B, PTHrP levels in tissue culture supernatants from pcDNA-MLL (7.3 ± 2.0) or PTHrP-MLL (36 ± 1.6) cells were quantitated by RIA (mean ± SE for three separate experiments). C, proliferation of pcDNA-MLL and PTHrP-MLL cells in vitro as determined by the MTT assay.

**Fig. 6. Analysis of MLL stable transfectants in vivo.** A, analysis of pcDNA-MLL and PTHrP-MLL cells injected s.c. into the hind leg of male Copenhagen rats as described in "Materials and Methods." Primary tumor weight at the time of amputation and plasma PTHrP levels at sacrifice are shown for two separate experiments. Data are expressed as the mean ± SE (+, P < 0.05; **, P < 0.01; ***, P < 0.001). B, Northern blot analysis of total RNA from tumor tissue using radiolabeled rat PTHrP cDNA as a probe.
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-suppressor 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 proliferation, 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 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 line 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 trabeculae from pcDNA-MLL lesions. Similar skeletal metastatic findings were observed by Rabbani et al. (53).

Studies of LNCaP cell lines 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.

ACKNOWLEDGMENTS

We thank Dr. Mary Jo Pilat, Jeff Lehr, and Susan Brumfield for assistance with the animal studies; Dr. Mark and Kathy Day for advice and direction on the animal studies; Mark Kukuruga for fluorescence-activated cell-sorting analysis; Dr. Andrew Karaplis for the PTHrP plasmids; Dr. Harald Jüppner for the PTH/PTHrP receptor cDNA; Dr. Kirk Wojno for assistance with tissue procurement; and Thomas Hullinger for technical assistance.

REFERENCES

7. Gleave, M. E., Wyss, M., and Becher, C. Parathyroid hormone-related protein: structure, function, and measure-


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


Updated version

Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/59/23/6015

Cited articles

This article cites 51 articles, 18 of which you can access for free at:
http://cancerres.aacrjournals.org/content/59/23/6015.full#ref-list-1

Citing articles

This article has been cited by 22 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/59/23/6015.full#related-urls

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, use this link:
http://cancerres.aacrjournals.org/content/59/23/6015.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.