Parathyroid Hormone-related Protein Induces Interleukin 8 Production by Prostate Cancer Cells via a Novel Intracrine Mechanism Not Mediated by its Classical Nuclear Localization Sequence

Aneeta Gujral, Douglas W. Burton, Robert Terkeltaub, and Leonard J. Deftos

The Department of Medicine, University of California, and the Veterans Affairs Medical Center, San Diego, California 92161

ABSTRACT

PTHrP (parathyroid hormone-related protein) overexpression by prostate carcinoma cells has been implicated in tumor progression. Although the biological effects of PTHrP can be mediated by the G-protein-coupled PTH/PTHrP receptor, PTHrP also has intracellular actions mediated by a nuclear localization sequence at residues 87–107. We investigated the effect of PTHrP transfection and treatment on production by prostate carcinoma cells of IL (interleukin)-8, which can regulate prostate cancer growth by angiogenic activity and growth-promoting effects. Six prostate cancer cell lines exhibited constitutive expression of PTHrP and IL-8 that were significantly correlated ($r = 0.93; P < 0.01$). We transfected wild-type and mutant PTHrP into these cells. Wild-type PTHrP1-173 and PTHrP33-173 lacking the PTH/PTHrP receptor-binding domain induced a 3-fold stimulation of IL-8 production but not production of another angiogenic factor, vascular endothelial growth factor. Transfection of the COOH-terminal truncation mutant PTHrP1-87 induced a 5-fold stimulation of IL-8 and a 3-fold increase in IL-8 mRNA. Cells transfected with PTHrP1-87 and 1-173 also showed increased cell proliferation. In contrast, exogenous PTHrP1-34 and 1-86 peptides did not significantly affect IL-8 production; moreover, PTHrP-neutralizing antibodies did not inhibit the production of IL-8 by transfected PTHrP. Additional transfection studies with progressively COOH-terminally truncated PTHrP1-87 defined a 23-amino acid sequence, PTHrP65-87, required for PTHrP1-87 to induce IL-8 production by prostate cancer cells, Confocal microscopy and immunoassay demonstrated PTHrP1-87 nuclear localization. Our results demonstrate that PTHrP acts to induce IL-8 production in prostate cancer cells via an intracrine pathway independent of its classical nuclear localization sequence. This novel pathway could mediate the effects of PTHrP on the progression of prostate cancer.

INTRODUCTION

PTHrP is widely expressed in normal and malignant cells (1). The best-studied biological effects of PTHrP are mediated through the binding of its NH$_2$ terminus to a G-protein-coupled receptor that it shares with PTH. However, receptors have been postulated for other regions of the molecule that seem to have distinct effects (1). In addition, recent studies (2–7) have demonstrated that some of the biological actions of PTHrP are cell surface receptor independent and mediated through “intracrine” mechanisms based on nuclear localization of PTHrP; e.g., there is evidence for an intracellular mechanism for PTHrP in cell cycle progression and apoptosis mediated through its amino acids 87–107 that constitute a nuclear/nucleolar targeting sequence (3). This intracrine mechanism for PTHrP has also been reported to increase cell proliferation (4). Other related mechanisms may also mediate the effects of PTHrP; e.g., a region for importin-$eta$ binding was recently mapped to PTHrP66-94 (5). Furthermore, both endogenous and transfected PTHrP bind RNA, suggesting a role for PTHrP in the processing of RNA (2).

PTHrP is overexpressed by prostate cancer cells and may play a role in the growth of prostate cancer and its metastasis by effects that include interaction with cytokines (8, 9). In this study, we focused on the regulation by PTHrP of IL-8 in prostate cancer cells (10–13). Significant levels of IL-8 have been demonstrated in prostate cancer cells but not in benign prostate hyperplasia or normal prostate cells (13). Moreover, IL-8 has been demonstrated to promote the proliferation of prostate cancer cells and other cell types, including PC-3 cells, that exhibit a growth regulatory response to IL-8 in vivo and in vitro (10–12, 14–16). IL-8 also has angiogenic properties (13, 17), thereby giving this cytokine the potential to regulate tumorigenicity and metastasis in prostate cancer (16, 17). We focused our studies on PTHrP1-173 because it includes the structural motifs of all of the three PTHrP isoforms. We report in this study that PTHrP stimulates the production of IL-8 by prostate cancer cells through a novel intracrine pathway independent of its classical NLS (9).

MATERIALS AND METHODS

Cell Culture. The human prostate cell lines DU 145, DuPro-1, LNCaP, PC-3, PPC-1, and 267B1-XR (18, 19) were maintained in RPMI 1640 or DMEM medium supplemented with 10% fetal bovine serum and 2 m M glutamine. Cells were passaged by trypsinization and cultured at 37°C in 5% CO$_2$ as described previously (20–22). The data shown represent the indicated number of replicate independent experiments. Ps were determined by Student’s t test.

IL-8 Assay. Cells were plated in 60-mm plates at 60% confluency and allowed to grow for 3 days to analyze the constitutive expression of IL-8. After experimental manipulation, the media were collected, and the cells were washed with PBS and extracted with lysis buffer [0.25 M Tris (pH 7.5), 0.25% NP-40, and 2 m M EDTA]. The cell extracts and media were assayed in triplicates for IL-8 by a sandwich ELISA following the manufacturer’s recommendations (BioSource International, Camarillo, CA). In brief, 96-well microtiter plates (Immulon 4 HB; Dynex Technology, Chantilly, VA) were coated with human IL-8 monoclonal antibody (0.6 µg/mL), and the measurement in 100 µl of cell media of IL-8 was accomplished by the addition of 50 µl/well of biotinylated IL-8 detection monoclonal antibody (0.4 µg/mL) for 18 h at 4°C followed by the addition of streptavidin-conjugated to β-galactosidase for 30 min and development with the fluorogenic substrate, 4-methyl umbelliferyl-β-D-galactopyranoside (Calbiochem, San Diego, CA). The assay sensitivity was 3 pg/mL of IL-8.

Cell Proliferation Assay. The cells were plated in replicates of 12 wells/group into 96-well plates at a density of 2 × 10$^3$ cells/well in normal growth media. Cells were synchronized by culturing in serum-free media for 18 h. Synchronized cells were then cultured in media containing 2% serum. At the conclusion of the experiment, the media were decanted, and the plates were then frozen at −70°C until further processing. A fluorogenic double-stranded DNA-binding dye, Hoechst H33258 bisbenzimide, was added to the thawed plates to quantitate cell number (23). The plates were scanned in a fluorometric plate reader (Wallac, Gaithersburg, MD) at an excitation wavelength of 355 nm and an emission wavelength of 460 nm. A reference standard curve was generated to convert sample fluorescence to cell number.
Plasmid Construction and Transfection. The following PTHrP expression plasmids (Fig. 1A) were used in this study: PTHrP1-173, PTHrP1-87, PTHrP1-64, PTHrP1-50, and PTHrP33-173. PTHrP1-87, 1-64, and 1-50 were created by site-directed mutagenesis following the protocols of Ditmer et al. (24). PTHrP33-173 was generated using a PCR-mediated cloning strategy wherein the prepro -36 to -1 region of the PTHrP, which signals the molecule to the ER and cell secretory pathway, was amplified using specific primers with the flanking restriction sites HindIII and PvuII for forward and reverse primers, respectively (25). The region of PTHrP containing amino acids 33 to 173 was amplified separately. The constructs were ligated and directionally subcloned into the CMVβ expression vector (a gift from Dr. Joel Habener, Harvard University, Boston, MA) or the pCI-neo vector (Promega, Madison, WI) and used for transfection by LipofectAMINE PLUS (Life Technologies, Inc., Gaithersburg, MD) as described previously (24).

PTHR1-173, which includes the structural motifs of the other two PTHrP isoforms, PTHrP1-139 and 1-141, was chosen as the parent plasmid. PTHrP1-173 and 33-173 were designed to study the NH2 terminus of PTHrP. PTHrP1-50 and 1-64 were designed to identify sequences within PTHrP1-87 that were responsible for its IL-8 effects. All of the plasmids except PTHrP33-173 contained the conserved PTHrP NH2 terminus, and PTHrP1-173 and 33-173 contained the classical NLS and importin-β binding sequence at residues 66–94 (5). The structure of all of the plasmids was confirmed by DNA sequencing, and all of the plasmids were demonstrated to express PTHrP by immunosassay of the media as well as in the extracts of transfected cells. A GFP expression plasmid, pEGFP (Clontech, Palo Alto, CA), and a β-galactosidase expression plasmid, CMVβGal (Stratagene, La Jolla, CA), were used to control for transfection efficiency.

Construction and Characterization of PTHrP-GFP Fusion Protein Chimeras. The PTHrP-GFP fusion protein chimeras were created following PCR-mediated cloning strategy (25). The PTHrP1-87, 1-173, and 33-173 were PCR amplified from their PTHrP plasmids cloned into the pCMVβ expression vector using specific primers. The sense primer contained a HindIII restriction site at the 5′ end. The antisense primer was designed to eliminate the stop codon at the 3′ end of PTHrP and contained a BamHI restriction site at the 5′ end. The PTHrP PCR products and pEGFPN1 (Clontech) were digested with HindIII and BamHI and then ligated so that the GFP fused in frame to the COOH terminus of PTHrP. The fidelity and function of the chimeras were verified by DNA sequencing, GFP expression, and RIA for PTHrP.

Quantitative PCR of IL-8 mRNA. Quantitative PCR was performed on PC-3 and PPC-1 cells using real time TaqMan technology and analyzed on an ABI PRISM 7700 sequence detecting system (Perkin-Elmer Corp., Foster City, CA). The assay was carried out using a TaqMan assay kit for human IL-8 mRNA (Perkin-Elmer Corp.). The reaction (25 μl) was conducted using 20 × target mix and 2 × PCR master mix provided in the kit, following the manufacturer’s recommendations in an optical 96-well plate. Serial dilutions of cDNA were used as standards for IL-8 and GAPDH. Cycle threshold values were converted to log ng DNA, and target cDNA (ng) was normalized with GAPDH cDNA. The cycling parameters were 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 1 min at 60°C.

Nuclear Extract Preparation. To quantitate nuclear PTHrP by immunoblot analysis, nuclear extract preparations were made from 30 × 106 cells, which were washed, scraped, and collected in PBS (26). The cell pellet was washed once in hypertonic buffer (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 mM DTT) before resuspending in 2.5 ml of hypotonic buffer. Cells were allowed to swell by incubating on ice for 20 min and then homogenized in a dounce homogenizer with 10 strokes. After centrifugation at 6000 rpm for 5 min, the supernatant (cytosolic fraction) was separated from the pellet, which was gently washed with hypotonic buffer and demonstrated by trypan blue staining to contain intact nuclei by microscopy. The intact nuclei were then lysed in a buffer containing 20 mM HEPES, 20% glycerol, 300 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT, 0.8 μM leupeptin, and 0.5 μM pepstatin followed by sonication for 20 s. Nuclear lysates were centrifuged at 15,000 × g for 10 min and assayed for PTHrP.

RT-PCR Analysis of the PTHrP/PTHrP Receptor. Total RNA was extracted from the prostate cells using TRIzol Reagent according to the manufacturer’s instructions (Life Technologies, Inc., Grand Island, NY). First strand cDNA synthesis was carried out using the Superscript PreAmplification system. In brief, total RNA (3 μg) and oligo[d(T)12] primer (1 μg) were denatured at 70°C for 10 min followed by chilling on ice for 2 min. Reverse transcription for the production of cDNA was carried out with 200 units of Superscript II reverse transcriptase in 2 μl of cDNA. The reaction (25 μl) was conducted using 200 units of Superscript II reverse transcriptase in 2 μl of cDNA and 4 μl of cDNA in 4 μl of cDNA. The cycling parameters were 2 min at 50°C and 10 min followed by 95°C for 1 min. RNase treatment for 15 min at 37°C was followed by heat inactivation at 70°C for 10 min. PCR amplification was performed using 2 μl of cDNA for 5 cycles at 94°C for 1 min, 42°C for 1 min, and 72°C for 1 min, followed by 30 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min. The oligonucleotides specific for human PTH/PTHrP receptor, 5’-CTCTTT-GGCGTCCACTACATTG-3’ and 5’-TTAGGAACCCACATGCCTTGTG-3’, amplified the expected 450-bp product (1, 3). Oligonucleotides used to amplify a housekeeping control gene for RT-PCR normalization, a 414-bp fragment of GAPDH cDNA.
GAPDH, were 5′ CATAAGAGAAGCTGCGGTC 3′ and 5′ CACGACAGCTGTGAGTGG 3′.

Confocal Microscopy. PPC-1 and PC-3 cells grown on glass coverslips were transiently transfected with PTHrP-GFP chimeric plasmids. GFP expression was studied between 24–48 h after transfection. The cells were fixed using 4% paraformaldehyde, and coverslips were mounted on glass slides using Prolong antifade kit (Molecular Probes, OR) and observed using a laser scanning confocal microscope (Zeiss LSM 510). A total of 300 transfected cells were evaluated. The gain and sensitivity of the confocal microscope were set so that no autofluorescence was observed in nontransfected cells. GFP was excited with a 488-nm argon/krypton laser. The images were manipulated with the Zeiss LSM software.

Stable Transfection of PTHrP. PPC-1 cells, selected because they constitutively produced moderate levels of PTHrP, were used to prepare stable clones with PTHrP1-87, PTHrP1-173, and vector control using LipofectAMINE-PLUS (Life Technologies, Inc., Gaithersburg, MD) following the instructions of the manufacturer. In brief, 24 h after transfection, the cells were split 1:10 with trypsin into growth media containing the selection antibiotic, G418 (800 μg/ml). Individual G418-resistant clones were isolated and expanded, and specific clones were selected on the basis of PTHrP production as assessed by immunoblotting of conditioned media (24, 27). Six individual clones from each group were evaluated for IL-8 expression.

PTHrP Immunoblot. Cell extracts and media PTHrP were measured by RIA based on PTHrP1-34, PTHrP38-64, and PTHrP109-141, as described previously (28, 29). All of the samples were assayed in multiple dilutions and in triplicates. Because each of these three species was detected in all of the cell lines in proportionally related concentrations, except for Fig. 7B, only the data for the PTHrP1-34 assay are presented.

PTHrP Peptide Treatment. Cells plated the day before the experiment at 75% confluency in media containing 2% fetal bovine serum were then cultured in serum-free media for 18 h followed by treatment with PTHrP peptides 1-34 and 1-86 (BACHEM, Inc., Torrance, CA). These peptides were chosen because comparisons could be made between them and corresponding expression plasmids. Stock solutions of peptides diluted to a concentration range of 10^{-7} to 10^{-10} m were used to replace media aspirated from the cells, and the cells were incubated at 37°C with 5% CO2 for a period of 24 h, as indicated in the figure legends. As a positive control for IL-8 stimulation, cells were treated with 10 ng/ml IL-1β, which caused a >10-fold increase in media IL-8 (13). The negative controls received only the vehicle, distilled water.

Neutralizing Antibody Treatment. Cells transfected with PTHrP expression plasmids were treated with monoclonal antibodies to PTHrP1-34 and 109-141, previously shown to be neutralizing, for 48 h in normal growth media (21, 28, 29). PBS and irrelevant mouse antibody were used as controls. Cells were incubated in the media containing both antibodies at a concentration of 5 μg/ml each after the DNA- lipid complexes were aspirated from the cells. The media were collected and stored frozen until further processing.

RESULTS

Constitutive Expression of IL-8 and PTHrP in Prostate Carcinoma Cells. Constitutive expression of IL-8 and PTHrP was observed in both the conditioned media and cell extracts of the prostate cell lines DU 145, DuPro-1, LNCaP, PC-3, PPC-1, and 267B1-XR. Table 1 summarizes IL-8 and PTHrP secreted into culture media by the prostate cell lines, all of which produced PTHrP. There was a significant (P < 0.01) direct correlation (r = 0.93) between IL-8 and PTHrP expression.

Effect of Transfection of PTHrP on IL-8. To identify those cell lines that were suitable for studies of PTHrP and IL-8, we screened all of them with transient transfections (Table 2). We selected PC-3 and PPC-1 cells for additional studies because of their levels of IL-8 expression and transfection efficiency. Fig. 1B demonstrates that IL-8 levels were increased 5-fold in response to overexpression of PTHrP1-87 and 3-fold in response to PTHrP1-173 and 33-173 in PC-3 cells. Overexpression of PTHrP33-173, which lacks the PTH/PTHrP receptor-binding domain, stimulated IL-8 levels comparably with PTHrP1-173. The level of another angiogenic factor, vascular endothelial growth factor, was not significantly changed by PTHrP overexpression (data not shown). As seen in Fig. 2, PPC-1 prostate cancer cells stably transfected with PTHrP1-87 and 1-173 also showed an increase in IL-8 levels compared with vector alone.

Molecular Mechanism of PTHrP Induction of IL-8 Expression in PC-3 Cells. In contrast to the results for transfection with PTHrP (Fig. 1B), exogenously added PTHrP1-34 and 1-86 peptides did not influence the expression of IL-8 (Fig. 1C). As seen in Fig. 3, reverse transcription using primers specific for human PTH/PTHrP receptor showed the expected single band of 450 bp when resolved on 1.2% agarose gel in the lane containing amplified receptor cDNA from PC-3 and PPC-1 cells. A similar size band was obtained using RNA from ROS 17/2.8 cells, which served as a positive control for the receptor. To further confirm specificity, the PCR products eluted from the gel were sequenced, and BLAST analysis (National Center for Biotechnology Information, NIH, Bethesda, MD) of the sequence demonstrated full homology to the human PTH/PTHrP receptor sequence.

To determine whether PTHrP or another factor secreted into the medium of transfected cells stimulated IL-8 production, nontransfected PC-3 cells were incubated with conditioned medium collected after 48 h from PC-3 cells transfected with PTHrP1-87, 1-173, and 33-173 expression plasmids. There was no significant change in IL-8 levels in response to the conditioned medium (data not shown). As further assessment of the potential role of secreted PTHrP in IL-8 expression, the neutralizing antibodies to PTHrP had no effect on the induction of IL-8 in response to PTHrP (data not shown).

Fig. 4 demonstrates the requirement for IL-8 induction of the PTHrP1-87 moiety of the importin-β binding domain (at residues 66–94). Neither PTHrP1-64 nor 1-50 stimulated IL-8 to the extent observed with PTHrP1-87, so its robust effect on IL-8 expression resides within amino acids 65–87 of the PTHrP molecule.

Fig. 5 demonstrates a 3-fold induction of IL-8 mRNA in prostate cells transfected with PTHrP as assessed by quantitative PCR.

Effect of Transfection of PTHrP on Proliferation of Prostate Cells. Fig. 6 demonstrates the proliferation of stable clones of PPC-1 cells overexpressing PTHrP1-87 and 1-173. The cell clones stably expressing PTHrP1-87 proliferated at a rate 3-fold higher over vector control cells, whereas those expressing 1-173 showed only a 1-fold stimulation of proliferation over vector control.

Confocal Microscopy and Immunoblot Analysis of PTHrP Localization. Fig. 7 demonstrates the nuclear localization of PTHrP1-87 and 1-173. As seen in Fig. 7A, PPC-3 cells transfected with PTHrP1-173-GFP (A) showed nuclear staining in mitotic and postmitotic cells with a faint reticular staining in the rest of the cell. PTHrP1-87-GFP also concentrated more in the nucleus along with reticular staining in the cytoplasm (B). The fraction of cells showing nuclear staining was approximately 30–40%. The nuclear staining varied in intensity and homogeneity in the cells, perhaps because of

<table>
<thead>
<tr>
<th>Cell line</th>
<th>IL-8 (pg/μg protein)</th>
<th>PTHrP1-34 (pg/μg protein)</th>
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<tbody>
<tr>
<td>PC-3</td>
<td>45 ± 1.4</td>
<td>299 ± 1.9</td>
</tr>
<tr>
<td>PPC-1</td>
<td>8 ± 0.1</td>
<td>23 ± 1.9</td>
</tr>
<tr>
<td>Dupro-1</td>
<td>6 ± 1.9</td>
<td>23 ± 1.9</td>
</tr>
<tr>
<td>LNCaP</td>
<td>&lt;3 ± 0</td>
<td>35 ± 2.4</td>
</tr>
<tr>
<td>DU 145</td>
<td>11 ± 0.3</td>
<td>41 ± 3.3</td>
</tr>
<tr>
<td>267B1-XR</td>
<td>26 ± 1.3</td>
<td>44 ± 3.7</td>
</tr>
</tbody>
</table>

Table 1 Summary of the PTHrP and IL-8 levels expressed by human prostate cells as measured by immunoblotting.

Six prostate cell lines were grown to confluence for 72 h, and the media were analyzed by immunoblotting for PTHrP1-34 and IL-8 and cell extracts for total protein. The calculated means and SE are normalized to pg/μg cell protein. The SE of replicate measurements (n = 3) was <5%. There was a significant (P < 0.01) direct correlation (r = 0.93) between IL-8 and PTHrP expression.
cells in different phases of the cell cycle. Similar results were seen with PPC-1 cells. The nuclear association and quantitation of the amount of PTHrP in PPC-1 cells stably overexpressing PTHrP1-87, as well as 1-173 (and vector control), were demonstrated by PTHrP immunoassay, which provided quantitative confirmation of the qualitative cytology data (Fig. 7b).

DISCUSSION

PTHrP is overexpressed in prostate cancer and may regulate the proliferation and skeletal progression of the tumor (29–32). PTHrP has been demonstrated to have direct and indirect growth-regulating functions in a variety of cells, including prostate (9, 21, 33–35). However, reports of the effects of PTHrP in prostate cancer are not consistent (20–22). These inconsistencies may reflect experimental designs that do not accommodate the well-known complexities of PTHrP processing and the recently appreciated intracrine mechanism of action of PTHrP that is mediated through an NLS at residues 87–107 (3, 4, 9). To address some of these issues, we studied the intracellular and extracellular effect of PTHrP in prostate cells.

Table 2. Effect of transfected PTHrP expression plasmids on IL-8 levels (percentage of control) in prostate cell lines

The prostate cell lines of Table 1 were transiently transfected with the indicated PTHrP expression plasmids, and the media were assayed after 48 h for IL-8. The means and SE were normalized to pg/μg cell protein and expressed as percentage of vector control. PC-3 and PPC-1 cells demonstrated the most robust effects and were selected for additional studies.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>PTHrP expression plasmid</th>
<th>1-50</th>
<th>1-64</th>
<th>1-87</th>
<th>1-173</th>
<th>33-173</th>
</tr>
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<tbody>
<tr>
<td>DU 145</td>
<td>NT¹</td>
<td>NT</td>
<td>NT</td>
<td>112 ± 31</td>
<td>172 ± 45</td>
<td>79 ± 33</td>
</tr>
<tr>
<td>DuPro-1</td>
<td>NT</td>
<td>144 ± 25</td>
<td>157 ± 15</td>
<td>123 ± 24</td>
<td>126 ± 5</td>
<td></td>
</tr>
<tr>
<td>LNCaP</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PC-3</td>
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<td>754 ± 61</td>
<td>230 ± 94</td>
<td>326 ± 35</td>
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<tr>
<td>PPC-1</td>
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<td>194 ± 42</td>
<td>300 ± 54</td>
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<tr>
<td>267B1-XR</td>
<td>NT</td>
<td>NT</td>
<td>121 ± 18</td>
<td>162 ± 33</td>
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</table>

¹ NT, not tested. ND, not detectable.

Fig. 2. Effect of PTHrP overexpression on IL-8 in PPC-1 cells. Stably transfected PPC-1 cells overexpressing PTHrP1-87 and PTHrP 1-173, as well as vector control, were plated at 70% confluency and incubated for 48 h at 37°C. The IL-8 levels were measured by immunoassay and corrected for cellular protein. The data represent an average of three independent clones from each group. Significant differences in IL-8 expression relative to the vector group are indicated by *, where *P < 0.001. n = 6; Bars, SE.

Fig. 3. RT-PCR analysis of PTH/PTHrP receptor in prostate cells. Total RNA isolated from PC-3, PPC-1, and ROS 17/2.8 cells was reverse transcribed followed by amplification using PTH/PTHrP-specific primers. A single band corresponding to 450 bp was seen in Lane 1 (PC-3) and Lane 2 (PPC-1), similar to the band seen in Lane 3 for the positive control ROS 17/2.8 cells. Lane 4, the 100-bp DNA ladder.

Fig. 4. Effects of overexpression in PC-3 cells of PTHrP 1-50, 1-64, and 1-87 on IL-8 levels. PC-3 cells were transfected with PTHrP1-87, 1-50, and 1-64 and vector control. Media collected 48 h after transient transfection were assayed for PTHrP and IL-8, and cell extracts were assayed for total protein. The data (IL-8; pg/ml/mg) are expressed as a percentage of vector control. Ps compared with vector control are indicated by *, where *P < 0.01 and **P < 0.005. n = 4; Bars, SE.

Fig. 5. Real-time PCR of IL-8 mRNA in prostate cells. Real-time PCR was conducted on RNA isolated from PPC-1 cells stably transfected with PTHrP1-87, PTHrP1-173, and vector control. The RNA was reverse transcribed to make cDNA. IL-8 mRNA levels were normalized to GAPDH. The samples were assayed in triplicate. The data are presented as an average (n = 6) of fold increase in IL-8 compared with vector control. Significant differences in IL-8 mRNA relative to vector control are indicated by *, where *P < 0.001.
We demonstrated the constitutive expression of IL-8 in several prostate cancer cell lines that directly correlated with their PTHrP expression. To study PTHrP effects on IL-8 expression, we transfected and treated the cells with different forms of PTHrP. Treatment with PTHrP peptides 1-34 and 1-86, both containing the NH\textsubscript{2}-terminal motif that PTHrP shares with the cell-surface PTH/PTHrP receptor, had no effect on IL-8, although the cells expressed this receptor. However, transfection with PTHrP1-87 and PTHrP1-173 significantly increased expression of IL-8 by several cell lines, especially PC-3 and PPC-1, whereas treatment with PTHrP1-34 and 1-86 peptides did not. Furthermore, the addition of PTHrP-neutralizing antibodies to the transfected cell media did not inhibit the stimulatory effect on IL-8. This stimulatory effect by PTHrP transfection but not peptide treatment was consistent with the recently described intracrine effect of PTHrP and suggested that the stimulation of IL-8 expression is mediated through this mechanism (2–7). The comparable effect of PTHrP1-173 and 33-173 in stimulating IL-8 levels further demonstrated that this effect of PTHrP on IL-8 can be mediated by a domain other than PTHrP1-34, which mediates the cell surface receptor effects of PTHrP(1). Stable transformation of PPC-1 cells had comparable effects on IL-8 and corresponding effects on cell proliferation. The stimulation of IL-8 by PTHrP1-173 and 33-173 to almost the same extent could result from the fact that these two forms of PTHrP both contain the NLS found in PTHrP at residues 87–107. However, this hypothesis does not accommodate our observation that PTHrP1-87, which does not contain this classical NLS, also stimulated IL-8 levels more than PTHrP1-173 and 33-173, suggesting the operation of other molecular mechanisms.

Additional transfection studies with PTHrP constructs 1-64 and 1-50 helped to identify the sequences within PTHrP1-87 responsible for its stimulation of IL-8. Transfection with these two plasmids had little effect on IL-8 compared with PTHrP1-87. One possible explanation for this observation is that truncation of 1-87 changes confor-
mation of the protein, rendering it no longer able to interact with other regulatory proteins (5).

To further investigate the intracrine stimulation of IL-8 by PTHrP, we studied the intracellular distribution of PTHrP1-87 and 1-173 using PTHrP-GFP chimeras. Immunostaining of nuclear PTHrP confirmed and quantitated the nuclear localization of PTHrP1-87 demonstrated by confocal microscopy. This localization corresponded to the greater effect of PTHrP1-87 on IL-8 and cell proliferation when compared with PTHrP1-173 and 33-173. Although PTHrP1-139 has been demonstrated to exert proliferative effects in an intracrine manner in vascular smooth cells (4), proliferative effects via nuclear localization of human specific PTHrP1-173 and PTHrP1-87 are identified by this study and for PTHrP1-87 are 2–3-fold higher than PTHrP1-173.

PTHRP1-87 does not possess a conventional NLS but appears to act like a number of proteins that still have nuclear mechanisms of action by association with other nuclear targeting proteins (36–44); e.g., upon activation by certain cytokines and growth factors, the transcription factor STAT, although not possessing an NLS, is translocated to the nucleus (43). The reverse process, retention of NLS-bearing proteins in the cytoplasm, has also been demonstrated (44), and cell type-specific differences in terms of NLS activity have also been observed (45–47). Although the basic NLS that contains the nuclear import pathway is the best characterized, other nuclear pathways exist, such as the karyopherin β pathway (48, 49). Furthermore, certain RNA-binding proteins not possessing NLS have been found to shuttle between nucleus and cytoplasm (48, 49). In fact, it has been demonstrated that PTHrP binds to RNA in a manner similar to HIV Rev protein (2), which gets transferred into the nucleus after an interaction between an arginine-rich domain and importin β (49). Because PTHrP has already been demonstrated to follow an importin β-mediated nuclear transport independent of the classical NLS (5), its nuclear localization may not be dependent on the classical NLS, which relies on importin α.

Alternate mechanisms of nuclear translocation of proteins involving posttranslational modification of arginine residues has been reported (50). Because there are five arginine residues for potential methylation in PTHrP1-87, this mechanism may be operative for PTHrP. In addition, PTHrP1-87 contains arginine at position 66, needed for importin-β binding (5). This site may allow PTHrP1-87 to mediate a nuclear effect through importin-β binding. However, in the study by Lam et al. (5), PTHrP66-87 peptide did not demonstrate significant binding with importin β in comparison with 66-94, nor did the peptide PTHrP83-103 that encompasses the entire NLS along with the nucleolar transport sequence. Differences in experimental conditions may contribute to the different results observed between the two studies. Our transfection studies, in contrast to the studies with peptides, allow for a properly folded protein within the cell, the conformation of which may permit binding or interaction with importins or other proteins. Furthermore, because PTHrP1-87 does not have an intact CDK domain required for the phosphorylation of PTHrP to exit the nucleus at G2-M phase of the cell cycle (4), PTHrP1-87 could remain in the nucleus for a longer time than 1-173 and 33-173, which contain the intact (CcN) motif. In fact, it has been demonstrated that PTHrP in a dephosphorylated state remains in the nucleus, whereas in the phosphorylated state, it is excluded from the nucleus into the cytoplasm (7).

The stimulatory effects of PTHrP on IL-8 might contribute to the metastatic potential of PTHrP-producing cancers (11, 16). IL-8 is a potent angiogenic factor produced by prostate and contributing to tumorigenic activity in several cancers (16, 51). Our finding that PTHrP overexpression selectively stimulates IL-8 over vascular endothelial growth factor is consistent with the observation that different cell types use unique chemokines to mediate their tumor growth (10, 52). PTHrP1-87 was found to up-regulate transcription of IL-8 by 3-fold, with a lesser effect of PTHrP1-173. The greater induction of IL-8 could result from the posttranscriptional stability of IL-8 mRNA, but other mechanisms are possible (53).

A role for PTHrP and IL-8 in prostate cancer progression is also provided by other studies of the PTHrP-producing PC-3 cells. Moore et al. (10, 11) demonstrated that IL-8 is responsible for tumorigenesis of PC-3 cells in a SCID mouse model in which neutralizing antibodies to IL-8 inhibited PC-3 tumor growth. Furthermore, Reiland et al. (12) showed that PC-3 cells treated with IL-8 had a 2-fold-increased invasion through a reconstituted basement membrane.

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REFERENCES


Announcements

MEETING OF THE RADIATION RESEARCH SOCIETY

The annual meeting of the Radiation Research Society will be held at the State University of Iowa, Iowa City, on June 22–24, 1953. The Society will be the guest of the University, and all meetings will be held on the campus. The program will consist of: (1) Two symposia, one on “The Effects of Radiation on Aqueous Solutions,” which includes the following speakers: E. S. G. Barron, Edwin J. Hart, Warren Garrison, J. L. Magee, and A. O. Allen. The second is “Physical Measurements for Radiobiology” and companion talks by Ugo Fano, Burton J. Moyer, G. Failla, L. D. Marinelli, and Payne S. Harris. (2) On Monday night, June 22, a lecture by Dr. L. W. Alvarez on meson physics has been tentatively scheduled. On Tuesday night, June 23, Dr. L. H. Gray of the Hammersmith Hospital, London, will speak on a topic to be announced. Dr. Gray’s lecture is sponsored by the Iowa Branch of the American Cancer Society. Those desiring to report original research in radiation effects, or interested in attending or desiring additional information, please contact the Secretary of the Society, Dr. A. Edelmann, Biology Department, Brookhaven National Laboratory, Upton, L.I., New York.

ERRATUM

The following correction should be made in the article by Beck and Valentine, “The Aerobic Carbohydrate Metabolism of Leukocytes in Health and Leukemia. I. Glycolysis and Respiration,” November, 1952, page 891; substitute for the last paragraph:

The data in Table 3 permit several interesting calculations. If one compares the amount of glucose actually disappearing with the sum of the amount equivalent to lactic acid produced plus that equivalent to O2 consumption, it is seen that the amount of glucose “cleavage products” exceeds the amount of glucose utilized by 12 per cent in N and 27 per cent in CML and is exceeded by the glucose utilized by 16 per cent in CLL. If the assumption is made that, in this respect, the myeloid and lymphoid cells of leukemia are similar to those of normal blood, it may be that the computed normal figure represents a summation of the myeloid (M) and lymphoid (L) cells that make up the normal leukocyte population. Thus, if M = +0.27 and L = −0.16 and the normal differential is 65 per cent M and 35 per cent L, then

\[ 0.65 (+0.27) + 0.35 (-0.16) = +0.12 \]

a figure identical to the observed +0.12 for normal leukocytes.
Parathyroid Hormone-related Protein Induces Interleukin 8 Production by Prostate Cancer Cells via a Novel Intracrine Mechanism Not Mediated by its Classical Nuclear Localization Sequence


Cancer Res 2001;61:2282-2288.

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