Parathyroid Hormone-related Protein as a Novel Tumor Marker in Pancreatic Adenocarcinoma


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Introduction: Parathyroid hormone-related protein (PTHrP) can act as an oncoprotein to regulate the growth and proliferation of many common malignancies, including pancreatic cancer. Previous studies have shown that PTHrP is produced by human pancreatic cancer cell lines, can be shown in the cytoplasm and nucleus of paraffin-embedded pancreatic adenocarcinoma tumor specimens, and is secreted into the media of cultured pancreatic adenocarcinoma cells. We hypothesized that PTHrP could serve as a tumor-marker for growth of pancreatic cancer in vivo.

Aim and Methodology: To test this hypothesis, we used an orthotopic model developed in our laboratory of the PTHrP-producing human pancreatic cancer line, BxPC-3. This tumor was stably transduced with green fluorescence protein (GFP) to facilitate visualization of tumor growth and metastases. At early (5 weeks) and late (13 weeks) timepoints after surgical orthotopic implantation, serum PTHrP was measured and primary and metastatic tumor burden was determined for each mouse by assessing GFP expression.

Results: By 5 weeks after surgical orthotopic implantation (early group), the mean serum PTHrP level was 33.3 pg/mL. In contrast, by 13 weeks after surgical orthotopic implantation (late group), the mean serum PTHrP level increased to 158.5 pg/mL. These differences were highly significant (p < 0.001, Student t test). Numerous metastatic lesions were readily visualized by GFP in the late group. Serum PTHrP levels measured by immunoassay correlated with primary pancreatic tumor weights and serum calcium levels (p < 0.01). PTHrP levels were not detectable (<21 pg/mL) in any of the 10 control mice with no tumor. Western blotting of BxPC-3-GFP tumor lysates confirmed the presence of PTHrP. BxPC-3-GFP tumor tissue stained with antibody to PTHrP.

Conclusion: These results indicate that PTHrP can serve as a tumor marker in animal models of pancreatic cancer and may be a useful tumor marker for clinical pancreatic adenocarcinoma.

Key Words: Pancreas—Pancreatic cancer—Parathyroid hormone-related protein.

Parathyroid hormone-related protein (PTHrP) is an oncoprotein that regulates the growth and proliferation of essentially every tissue in which it is expressed, including many common malignancies such as breast, colon, gastric, melanoma, and prostate cancer (1–4). In these tumors, PTHrP is processed into distinct peptides that mediate its unique biologic effects through intracrine (nuclear localization) and endocrine (autocrine and paracrine cell surface receptor) pathways (5–7). In addition to its endocrine effects, several studies have shown that increased expression of PTHrP in cancer is associated with accelerated tumor growth and a more malignant phenotype, suggesting that PTHrP may play a role in promoting tumor progression (2–4,7–13).

Recently, we showed that PTHrP is produced by human pancreatic adenocarcinoma cell lines and is present in the cytoplasm and nucleus of paraffin-embedded pancreatic adenocarcinoma tumor specimens (14,15). We also observed that PTHrP is secreted into the media of cultured pancreatic adenocarcinoma cell lines and could be measured by radioimmunoassay (14,15). Because of these findings, we hypothesized that PTHrP could serve...
as a tumor marker for growth of pancreatic cancer. To test this hypothesis in vivo, we used an orthotopic model, developed in our laboratory (16), of the PTHrP-producing pancreatic cancer cell line, BxPC-3, which expresses the green fluorescence protein (GFP) to facilitate visualization of tumor growth and metastases.

MATERIALS AND METHODS

Cell lines

The BxPC-3 human pancreatic cancer cell line was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and shown to express and secrete PTHrP (14). The cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Life Technologies, Inc., Grand Island, NY, U.S.A.). Cells were incubated at 37°C in a humidified 5% CO₂ 95% air incubator.

GFP DNA expression vector

The RetroXpress vector pLEIN was purchased from Clontech Laboratories, Inc. (Palo Alto, CA, U.S.A.). The pLEIN vector expresses enhanced GFP and the neomycin resistance gene on the same bicistronic message, which contains an internal ribosome entry site (17).

GFP retrovirus vector production

PT67, an NIH3T3-derived packaging cell line expressing the 10 A1 viral envelope, was purchased from Clontech Laboratories, Inc. PT67 cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Gemini Bioproducts, Calabasas, CA, U.S.A.). For GFP retrovirus production, packaging cells (PT67), at 70% confluency, were incubated with a precipitated mixture of DOTAP reagent (Boehringer Mannheim, Germany) and saturating amounts of pLEIN plasmid for 18 hours. Fresh medium was replenished at this time (17). The cells were examined by fluorescence microscopy after 48 hours. For selection of packaging cells producing high levels of GFP retrovirus, the cells were cultured in stepwise increasing amounts of 500 to 2,000 μg/mL G418 (Gibco-BRL, Life Technologies, Inc.) for 7 days.

GFP retroviral transduction and selection of high GFP expression BxPC-3 pancreatic cancer cells

For GFP gene transduction, 20% confluent BxPC-3 cells were incubated with a 1:1 precipitated mixture of retroviral supernatants of the PT67 packaging cells and RPMI 1640 (Life Technologies, Inc.) for 72 hours (17). Fresh medium was replenished at this time. BxPC-3 cells were harvested by trypsin and EDTA 72 hours after infection and subcultured at a ratio of 1:15 into selective medium that contained 200 μg/mL of G418. The level of G418 was increased to 800 μg/mL gradually. BxPC-3 clones expressing GFP (BxPC-3-GFP) were isolated with cloning cylinders (Bel-Art Products, Pequannock, NJ, U.S.A.) using trypsin and EDTA to detach the cells, which were then amplified and cultured by conventional methods. High GFP expression clones were then isolated in the absence of G418 in order for them to stably express GFP in vivo.

Surgical orthotopic implantation

BxPC-3-GFP pancreatic tumors at the exponential growth phase, grown subcutaneously in nude mice, were resected aseptically. Necrotic tissues were cut away, and the remaining healthy tumor tissues were cut with scissors and minced into approximately 3 x 3 x 3 mm pieces in Hanks’ balanced salt solution containing 100 U/mL penicillin and 100 μg/mL streptomycin. Each piece was trimmed with scissors until it weighed approximately 50 mg. Additional nude mice were anesthetized by isoflurane inhalation (Fort Dodge Animal Health, Fort Dodge, IA). The abdomen was sterilized with alcohol, and an incision was made through the left upper abdominal pararectal line and peritoneum. The pancreas was carefully exposed, and three tumor pieces were transplanted on the middle of the pancreas with an 8-0 Ethilon surgical suture (Ethicon Inc., Somerville, NJ). The pancreas was then returned into the peritoneal cavity, and the abdominal wall and the skin were closed with six Ethilon sutures. Animals were kept in a sterile environment. All procedures of the operation described above were performed using a stereo microscope (Model X7, Olympus, Japan) (16).

Chronologic analysis of tumor weight and metastases

Five weeks (early group, n = 15) and 13 weeks (late group, n = 32) after surgical orthotopic implantation (SOI), mice underwent exploratory laparotomy with resection of the primary tumor. Primary and metastatic tumors were weighed and preserved in formalin for further analysis. Each mouse was placed in an ultraviolet box and evaluated for GFP fluorescence to detect the presence and extent of metastatic disease. The location of metastases was recorded for each mouse.

Immuoassay of mouse serum

One hundred to two hundred microliters of blood was drawn at various times from mice that had previously undergone SOI of BxPC-3-GFP pancreatic tumors. PTHrP was measured by immunoassay, using modifications of previously described methods (10,14,18). In
brief, tyrosinized human PTHrP 38–64 was used to prepare tracer by chloramine-T radiodination and as an assay standard. Rabbit antiserum to the peptide standard were used in a nonequilibrium immunoassay format. Lack of cross reaction in the assay for at least a 100-fold excess of peptide was shown for the noncorresponding PTHrP peptides, calcitonin, calcitonin gene-related peptide, and human and rat atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP). All samples were assayed in multiple dilutions that paralleled the corresponding PTHrP standard. The intraassay and interassay variations were between 7% and 12%, respectively. All synthetic peptides were purchased from Peninsula Laboratories (Belmont, CA, U.S.A.). Cell protein was measured using a modified Bradford protein assay with bovine serum albumin as standard (Bio-Rad Labs, Hercules, CA, U.S.A.).

Calcium measurements

We measured serum calcium levels in the available remaining samples for the mice in the study. Serum calcium was measured using a commercial calcium kit 587-M (Sigma Diagnostics, St. Louis, MO, U.S.A.). Briefly, 5 μL mouse serum was added to an alkaline solution containing o-cresolphthalein complex one in a 96-well plate. A purple complex formed and was quantitated at 570 nm in a plate spectrophotometer, and a reference calcium standard curve was generated at the same time to convert the sample optical density values into calcium concentrations.

Western blotting of cell extracts

Cells and tumors were lysed by sonication in a lysis buffer containing 10 mM Tris-HCl (pH 7.5) (Sigma Chemical Co., St. Louis, MO, U.S.A.), and 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1% NP-40, and protease inhibitor cocktail (Boehringer Mannheim). Protein lysates were reduced by incubation for 10 minutes at 100°C in Laemmli’s sample buffer (Sigma Chemical Co.) at a final concentration of 15% β-mercaptoethanol. Polypeptides were resolved at 150 V on 10% acrylamide–sodium dodecyl sulfate (SDS) gels and electrophoretically transferred to 0.45-μm nitrocellulose membranes (Millipore Co., Bedford, MA, U.S.A.) for 1 hour at 100 V. Membranes were blocked overnight in a Tris buffered saline (TBS) buffer (20 mM Tris, pH 7.5; 250 mM NaCl) containing 3% (w/v) casein. Blots were then probed for 90 minutes with monoclonal antibody to PTHrP 109–141 (18) and β-actin (control). Blots were developed using species-specific secondary antibodies. Immunoreactive material was then visualized by enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL, U.S.A.) according to the manufacturer’s instructions.

Immunohistochemistry of tumor specimens

Immunohistochemical localization of PTHrP was done by the streptavidin-biotineroxidase enzyme conjugate method using the PTHrP 109–141 antibody as previously described (10,18). All procedures were performed at room temperature unless otherwise specified. Formalin-fixed, paraffin-embedded cell blocks or tissue sections (3–5 μm thickness) were deparaffinized and hydrated through a series of isopropyl alcohol solutions. The slides were then incubated with a 1% zinc sulfate solution and microwaved for two 5-minute bursts to enhance antigenicity. After washing and blocking with a nonspecific protein solution (20% fetal bovine serum, 0.25% gelatin, and 0.01% azide in phosphate-buffered saline), protein A-purified anti-PTHrP antibodies at 10 μg IgG/mL were added to the slides overnight in humidified chambers at 4°C. Biotinylated goat antismouse IgG antibody was then applied for 1 hour, and the streptavidin-biotineroxidase enzyme complex was applied for 1 hour. Positive staining was developed by incubating the slides with a developing solution (1.3 mM 3,3’-diaminobenzidine-4 HCl with 0.02% v/v H2O2 in 10 mM Tris, pH 7.4) for 5 to 10 minutes. To determine the specificity of positive staining, the antibodies were preabsorbed with the corresponding antigen overnight. This antibody–antigen control solution was applied to serial tissue sections adjacent to the corresponding antibody-treated sections on the same slide.

Statistical analysis

The Pearson r correlation coefficient was used to determine the correlation among serum PTHrP, serum calcium, and tumor weight. The Student two-tailed t test was used to determine differences in serum PTHrP among the early, late, and control groups. A p value less than 0.05 was considered statistically significant.

RESULTS

PTHrP serum levels increased chronologically with metastatic progression

Parathyroid hormone-related protein levels were not detectable (<21 pg/mL) in all 10 control mice with no tumor. PTHrP levels increased in a chronologic manner in the mouse pancreatic cancer model (Fig. 1). By 5 weeks after SOI (early group), the mean serum PTHrP level was a mean of 33.3 pg/mL. In contrast, by 13 weeks after SOI (late group), the mean serum PTHrP level increased to 158.5 pg/mL. These differences were highly significant (p < 0.001, t test) for the 5-week and 13-week groups compared with the control group. Numerous metastatic lesions were easily visualized by GFP in the
late group (Fig. 4). The most common sites were to the retroperitoneum and the spleen, followed by the mesentery, liver, and colon (Table 1).

**PTHrP serum levels correlate with tumor weight**

Serum PTHrP levels measured in 47 tumor-bearing mice correlated with primary pancreatic tumor weights (Fig. 2). The correlation was significant ($p < 0.01, n = 47$). The correlation coefficient was 0.38832 for $n = 47$ at >99% confidence limits.

**Serum calcium levels correlate with tumor weight and serum PTHrP**

Serum calcium levels correlated with tumor weight ($p < 0.01, r = 0.643$ for $n = 39$ at >99% confidence limit).

**TABLE 1. Metastases of pancreatic adenocarcinoma: sites of metastases 13 weeks after surgical orthotopic implantation of BxpC-3-GFP human pancreatic cancer (n = 32 mice)**

<table>
<thead>
<tr>
<th>Site of metastasis</th>
<th>No.</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td>26</td>
<td>82</td>
</tr>
<tr>
<td>Retroperitoneum</td>
<td>25</td>
<td>63</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>20</td>
<td>63</td>
</tr>
<tr>
<td>Mesentery</td>
<td>14</td>
<td>44</td>
</tr>
<tr>
<td>Liver</td>
<td>11</td>
<td>34</td>
</tr>
<tr>
<td>Colon</td>
<td>8</td>
<td>25</td>
</tr>
<tr>
<td>Stomach</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>Kidney</td>
<td>5</td>
<td>16</td>
</tr>
<tr>
<td>Mediastinum</td>
<td>5</td>
<td>16</td>
</tr>
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and serum calcium correlated with serum PTHrP ($p < 0.01, r = 0.481$ for $n = 37$ at >99% confidence limit).

**Western blot analysis of pancreatic tumor lysates**

Western blotting of BxpC-3-GFP tumor lysates confirmed the presence of PTHrP (Fig. 3). The approximate molecular weight of PTHrP was 37 kilodaltons. The BEN cell line, which is known to produce high levels PTHrP, was used as a positive control (10). Equal loading on the gels was controlled with β-actin and showed comparable PTHrP production in both tumor types.

**PTHrP immunohistochemistry in human pancreatic adenocarcinoma tumors**

The BxpC-3-GFP tumor was stained with PTHrP antibody (18). PTHrP staining of the BxpC-3 tumor was strong (Fig. 4C). In all cases of positive staining, incubation of sections with the antibody–antigen control mixture showed no positive staining (data not shown).

**DISCUSSION**

In this study, we have shown that PTHrP is secreted into the blood of mice orthotopically implanted with human pancreatic adenocarcinoma and that serum PTHrP levels correlate with tumor burden. The use of GFP helped visualize the extensive metastases that develop in the later stages of this pancreatic cancer. This study shows that PTHrP can serve as a tumor marker for this
model of pancreatic cancer and raises the possibility that PTHrP may be a useful clinical marker for pancreatic cancer (14–16).

Secretion of PTHrP into the serum has been described in many case reports for various pancreatic tumors. However, most of these are endocrine neoplasms (19–27). Ratcliffe et al. (19) described a patient with a neuroendocrine tumor of the pancreas associated with hypercalcemia, which was attributed to production of PTHrP by the tumor. Plasma PTHrP levels were significantly increased and decreased after surgical resection of the tumor. Mitlak et al. (20) described a patient with an islet cell carcinoma and hypercalcemia in whose serum PTHrP was measured during the course of therapy by an immunoradiometric assay directed toward the midportion of the molecule. The concentration of PTHrP increased with time and decreased after the patient received chemotherapy directed toward the islet cell tumor. Wu et al. (21) measured PTHrP levels in 17 patients with islet cell carcinoma and 110 healthy subjects. PTHrP levels were significantly higher in 10 patients with hypercalcemia and islet cell carcinoma than in 7 patients with eucalcemia and islet cell carcinoma or in the 110 healthy subjects. Despite these reports in endocrine pancreatic tumors, serum PTHrP levels have not been extensively evaluated in patients with exocrine pancreatic adenocarcinoma.

Our recent studies have shown that PTHrP is commonly expressed in exocrine pancreatic cancer (14). To study PTHrP in pancreatic exocrine cancer, we studied its expression in eight pancreatic cancer cell lines and 14 surgical specimens. Cellular PTHrP was detected in all cell line extracts by Western blotting and immunoassay. PTHrP secretion measured by a PTHrP 38–64 assay was highest for BxPC-3, which is consistent with our use of the PTHrP 38–64 immunoassay to measure the serum PTHrP in BxPC-3 orthotopic mice. This assay is also our most sensitive and technically reliable PTHrP immunoassay. CFPAC-1, derived from a pancreatic liver metastasis, had the highest concentration of PTHrP, and MIA PaCa-2, derived from primary pancreatic adenocarcinoma, had the lowest. PTHrP was localized by immunocytochemical staining in the cytoplasm in all but one cell line, and nuclear and cytoplasmic immunostaining was observed in the MIA PaCa-2 and PANC-1 cells. Secre-
tion of PTHrP into cell media was also observed for each cell line and paralleled intracellular PTHrP levels. Evidence for differential processing of PTHrP expression was provided by studies showing different patterns of PTHrP among the cell lines when assessed by PTHrP immunoassays directed against different PTHrP peptides. Growth of AsPC-1 cells was stimulated in a dose-dependent manner by PTHrP 1–34. Immunostaining from archival tissue of patients with pancreatic adenocarcinoma showed robust PTHrP expression in 14 of 14 specimens. These results showed that PTHrP is commonly expressed in pancreatic cancer.

Although the function of PTHrP in pancreatic cancer is unknown, it appears to regulate growth in other tumor types (28). We have previously shown that PTHrP 1–34 stimulated thymidine uptake in prostate cancer cells more than threefold over control under serum-free and steroid-free conditions; in addition, the PTHrP-induced DNA synthesis was completely neutralized by our mouse monoclonal antibody against PTHrP 1–34 (29). We also have shown that PTHrP 1–34 regulates the growth of cultured type II epithelial cells (30). Recent preliminary studies from our laboratory indicate that PTHrP may similarly regulate the growth of pancreatic cancer (15). Future studies in orthotopic models of pancreatic cancer will also address the growth properties of PTHrP.

In our study, PTHrP levels were highest in late stages of the disease, when multiple metastatic lesions were present in the mice. Some of these metastases, such as those of the liver, were hematogenous, whereas other metastases, such as those to the retroperitoneum, were by direct extension. This may indicate that PTHrP may be a useful tumor marker for clinical pancreatic adenocarcinoma. However, serum levels of PTHrP of patients with pancreatic cancer must be examined and correlated with clinical stage, prognosis, and pathologic tumor features before PTHrP can be validated as a useful clinical tumor marker for pancreatic cancer.
In summary, we have shown that PTHrP is secreted into the blood of mice that have undergone surgical orthotopic implantation of PTHrP-expressing human pancreatic tumors. Furthermore, serum PTHrP correlated with the primary pancreatic tumor weight and the extent of metastatic burden. Future studies will determine whether there is differential expression of PTHrP in the primary tumor and specific metastatic sites. Additional studies are needed to elucidate the role of PTHrP in the development and progression of pancreatic cancer and to determine whether PTHrP could be useful in the early detection or clinical treatment of patients with this disease.

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REFERENCES


