Significance of the Expression of the Growth Factor Pleiotrophin in Pancreatic Cancer Patients

Hans-Jürgen Klomp, Oliver Zernial, Sabine Flachmann, Anton Wellstein, and Hartmut Juhl

Department of Surgery, University Hospital Kiel, 24105 Kiel, Germany [H.-J. K., O. Z.], and Lombardi Cancer Center, Georgetown University Medical Center, Washington, D. C. 20006 [S. F., A. W., H. J.]

ABSTRACT

Purpose: Recently, we found that pleiotrophin (PTN) acts as a rate-limiting autocrine growth factor in pancreatic cancer cells. The aim of this study was to determine the expression pattern of PTN in pancreatic cancer and to analyze the clinical significance of PTN in pancreatic cancer patients.

Experimental Design: We compared PTN expression in malignant (n = 24), inflammatory (n = 13), and normal (n = 14) pancreatic tissues using immunohistochemistry and in situ hybridization and determined PTN serum levels in pancreatic cancer patients (n = 77), in patients suffering from chronic pancreatitis (n = 21), and in healthy volunteers (n = 28). Two-year survival rates were determined for pancreatic cancer patients in relation to serum levels of PTN.

Results: The frequency of PTN expression increased from normal tissue (7%) to inflammatory (34%) and pancreatic cancer tissues (67%; P < 0.05). Compared with a healthy control group, we found elevated PTN serum levels in 30% of patients with chronic pancreatitis (mean, 143 ± 55 pg/ml) and in 53% of pancreatic cancer patients (mean, 200 ± 29 pg/ml; P < 0.05). Elevated serum levels of PTN dropped in patients after successful tumor resection but were unaffected when only palliative surgery was performed (P < 0.0001). High preoperative serum PTN levels correlated with a worse 2-year survival (P < 0.05).

Conclusions: This study supports the clinical significance of PTN for the malignant progression of pancreatic cancer.

INTRODUCTION

With an overall survival rate of 2–5%, pancreatic cancer has the highest lethality of all malignancies (1). The search for novel targets is an extremely important task, bearing in mind that e.g., in the United States 28,000 cases are diagnosed annually. Growth factor overexpression substantially contributes to pancreatic cancer development and progression, and these factors may serve as targets for therapeutic intervention (2). Epidermal growth factor, transforming growth factor α, and fibroblast growth factor as well as their receptors have been identified as important mediators of pancreatic cancer growth (2). However, these growth factors are found under physiological conditions in many tissue specimens, thereby limiting their application.

PTN3 is a M, 18,000 heparin-binding secreted growth factor that is highly conserved across species. Human, mouse, and rat PTN protein are identical, and they share an ~50% amino acid similarity with the other family member, midkine (3). PTN was originally described as a developmentally regulated cytokine based on its time- and tissue-specific expression pattern during rodent development. In rodents, it is highly expressed in many neuroectodermal and mesodermal cell lineages and is not expressed in endodermal, ectodermal, or trophoblast cells during late embryogenesis and perinatal growth. In the adult rodent, PTN is markedly down-regulated and present at minimal levels only in a very few tissues, with its highest levels in brain. An almost identical and limited expression profile was found in human adult tissues (3).

A role of PTN in human cancers was suggested after purification from conditioned media of the highly malignant breast cancer cell line MDA-MB231 (4). Screening of various human tumor cell lines and tumor specimens by RNase protection revealed that PTN is expressed in 18 of 36 tumor cell lines of different origin, such as melanoma, breast cancer, prostate cancer, and choriocarcinoma, and was found in 60% of primary breast cancer cases (5).

Besides the neurite and glial outgrowth activities of PTN in vitro, it was shown that PTN has growth-promoting and transforming activity on fibroblasts and epithelial cells and mitogenic activity on endothelial cells (3). Furthermore, PTN induces tube formation on endothelial cells and angiogenesis in vitro and in the rabbit corneal assay (6). PTN has been found to act as an essential autocrine, paracrine, and angiogenic factor for various malignancies including human breast cancer, choriocarcinoma, and melanoma (5–9).

More recently, we found PTN frequently expressed in pancreatic cancer tissue and detected the protein at an elevated level in serum samples of pancreatic cancer patients (10). In

Received 8/3/01; revised 11/19/01; accepted 1/3/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by a grant from the J. F. Buch Foundation (Hamburg, Germany), the Lombardi Cancer Research Center Core Facility, and USPHS Grant 2P30-CA-51008.

2 To whom requests for reprints should be addressed, at Departments of Oncology and Surgery, Lombardi Cancer Center, NRB Room E316A, Georgetown University Medical Center, 3970 Reservoir Road NW, Washington, D. C. 20007. Phone: (202) 687-1235; Fax: (202) 687-4821; E-mail: juhlh@georgetown.edu.

3 The abbreviations used are: PTN, pleiotrophin; TBST, Tris-buffered saline/Triton X-100.
subsequent studies, we analyzed the biological function of PTN in pancreatic cancer cells and demonstrated that PTN acts as a rate-limiting autocrine growth factor for pancreatic cancer cells and reported that riboyme targeting of PTN diminished tumor growth in vivo (7).

The experimental studies and the highly restricted expression pattern of PTN in human adults could make PTN an attractive circulating growth factor for prognostic monitoring as well as therapeutic targeting (3). In support of the significance of PTN for pancreatic cancer, we now expand our previous findings that PTN is overexpressed in pancreatic cancer relative to normal and inflammatory tissues and is elevated in serum samples from patients with chronic pancreatitis and pancreatic cancer. Most significantly, we demonstrate that elevated serum levels of PTN are associated with a worse clinical outcome in cancer patients.

MATERIALS AND METHODS

Patients and Samples. To study PTN tissue expression, we investigated 13 inflammatory tissues of the pancreas (chronic pancreatitis) and 24 samples that were classified as adenocarcinoma of the pancreas. In 14 samples from pancreatic cancer patients, a separate, normal, noninflammatory tissue was available and was used to determine PTN expression in normal pancreas.

Serum was obtained from 20 patients suffering from chronic pancreatitis and from 77 pancreatic cancer patients. In addition, serum samples from 28 healthy volunteers were analyzed. In 15 pancreatic cancer patients with elevated PTN serum levels, we measured PTN after surgical treatment before patients were discharged from the hospital.

Postoperative survival was determined in 49 surgically treated pancreatic cancer patients, 25 of whom underwent tumor resection (Whipple operation) and 24 of whom were palliatively treated (gastroenterostomy and biliodigestive anastomosis). Of these patients, 1 patient had stage I disease, 10 patients had stage II disease, 20 patients had stage III disease, and 18 patients had metastatic stage IV disease at time of operation (tumor classification according to Union International Coutre Candre tumor-node-metastasis (TNM) classification).

Immunohistochemistry. Staining of paraffin-embedded tumor tissues was performed as described previously (11). Briefly, deparaffinized sections of formalin-fixed tissues were microwaved (5 min), incubated for 15 min in 1% citrate buffer (pH 6) at 90°C treated with 0.3% hydrogen peroxide (30 min), and incubated overnight at 4°C with 5 μg/ml polyclonal goat antihuman PTN antibody (Santa Cruz Biotechnology, Santa Cruz, CA). A peroxidase-coupled rabbit anti-goat IgG (Dianova, Hamburg, Germany) diluted in 10% type ABO human blood serum was added and incubated for 1 h at room temperature. After washing, the substrate diaminobenzidine was added (Vector Laboratories, Burlingame, CA) for 15–30 min, the slides were washed, and the nuclei were counterstained using Harris hematoxylin (Lerner Laboratories, New Haven, CT).

Positive immunostaining was defined as staining of at least 20% of epithelial (normal and inflammatory tissue) or cancer cells, respectively. PTN staining intensity between experiments was controlled by parallel staining of testis tissue sections (positive control).

In Situ Hybridization. To confirm PTN immunostaining, we performed in situ hybridization using previously published PTN riboprobes and the according protocol (11). Briefly, deparaffinized sections of formalin-fixed tissues were treated for 15 min with proteinase K (10 μg/ml; Sigma Chemical Co.) at room temperature and washed with 2 × SSC before acetylation. Heat-denatured riboprobe (1 μl) was mixed with hybridization buffer, and the slides were incubated overnight at 50°C. After washing, immunological detection was performed using anti-digoxigenin-alkaline phosphatase conjugate.

PTN ELISA. To determine the concentration of secreted PTN, we used a recently established ELISA (10). It is highly specific for PTN and distinguishes between PTN and structurally related midkine. The ELISA was able to reliably detect PTN at levels as low as 20 pg/ml, with a minimum detection level of 5–10 pg/ml. Briefly, 96-well microtiter plates (Corning, Inc., Corning, NY) were coated at 4°C overnight in Tris-buffered saline with 1 μg/ml monoclonal mouse antihuman PTN antibody 4B7, kindly provided by Dr. D. Raulais (INSERM, Paris, France). After washing, the plate was blocked with 200 μl of TBST containing 1% BSA for 2 h at 4°C. After washing, samples diluted 1:1 in Tris-buffered saline were added (100 μl/well) and incubated for 1 h at room temperature. The wells were washed three times with TBST, and a biotinylated goat antihuman PTN antibody (R&D Systems, Inc.) was added at a concentration of 500 ng/ml and incubated for 1 h at room temperature. After washing three times with TBST, 100 μl of streptavidin-conjugated alkaline phosphatase (50 ng/ml) were added to each well (1 h at room temperature). After washing, 100 μl of a p-nitrophenyl phosphate substrate (Pierce Chemical Co.) were added and incubated for 2 h at room temperature. The absorbance was measured with a microplate reader at λ = 405 nm.

As a standard we used different dilutions of recombinant PTN protein purchased from R&D Systems, Inc.

Data Analysis. For statistical analysis we used the χ² test. Survival curves were analyzed by the log-rank test.

RESULTS

Tissue Expression of PTN. PTN expression was detected in 7% of normal tissue samples. In contrast, 34% of inflammatory pancreatic tissues and 67% of pancreatic cancer specimens showed PTN expression in epithelial and cancer cells, respectively (P < 0.05). In situ hybridization confirmed the finding of positive PTN immunostaining. Fig. 1 shows a typical immunostaining (Fig. 1A) and corresponding in situ hybridization (Fig. 1B) of malignant pancreatic tissue. Fig. 2 summarizes the results of PTN expression in pancreatic tissue samples. Tissue expression of PTN did not correlate with the tumor stage (P = 0.2011).

Serum Level of PTN. We determined serum PTN level in 28 healthy volunteers, 21 patients with chronic pancreatitis, and 77 histologically proven pancreatic cancer patients. The majority of healthy volunteers had PTN levels around the 10 pg/ml detection limit of the ELISA (mean value, 27 ± 14 pg/ml). The highest PTN value of 107 pg/ml, found in one
A healthy volunteer, defined our cutoff level. The baseline for elevated PTN serum levels was set at 110 pg/ml. We chose this high cutoff level, which is far above the usually applied 2×SD of 55 pg/ml, to achieve the highest possible specificity of the assay.

Mean serum levels of patients with chronic pancreatitis (143 ± 55 pg/ml) and pancreatic cancer (200 ± 29 pg/ml) were significantly higher than that of the healthy control group (Fig. 3). The difference in mean values between chronic pancreatitis and pancreatic cancer did not achieve statistical significance (P = 0.3919), but elevated serum levels were seen significantly more often in cancer patients, i.e., in 30% (6 of 21) of patients with chronic pancreatitis versus 53% (41 of 77) of patients with pancreatic cancer (P < 0.05). In both groups, minimal values included patients with PTN levels below the detection limit.

**Serum PTN Level after Surgical Treatment.** To demonstrate that elevated serum PTN levels depend on tumor growth in pancreatic cancer patients, we correlated preoperatively elevated PTN serum levels with the outcome of surgical treatment in 15 patients (Fig. 4). In nine patients, the tumor mass was resected by Whipple operation, and all of these patients had declining PTN serum levels of <110 pg/ml at the time of hospital discharge. In contrast, six patients who were surgically treated but did not receive tumor resection demonstrated unchanged elevated PTN levels (P < 0.0001).

**Correlation of Serum PTN Levels with Survival.** Follow-up data for up to 2 years were studied in 49 surgically treated pancreatic cancer patients. Twenty-two patients had elevated PTN serum levels, and 27 patients had normal PTN serum levels. Both groups did not differ with respect to surgical
treatment (resection versus palliative treatment; \( P = 0.1299 \)), nor did the number of cases with advanced (stage III and IV) and localized tumors (stage I and II) vary between both groups (\( P = 0.7625 \) and \( P = 0.945 \), respectively). As shown in Fig. 5, patients with elevated PTN serum levels had a significantly worse 2-year survival compared with patients with serum levels below 110 pg/ml (\( P < 0.05 \)).

**DISCUSSION**

Our aim was to determine the clinical significance of PTN expression in pancreatic cancer patients and thereby to better define its potential role in novel therapeutic modalities. We have already shown in a previous study that PTN is expressed by pancreatic cancer specimens and becomes detectable in serum using a novel PTN ELISA (10). However, that study was rather small with regard to the number of samples studied and, more importantly, did not include patients suffering from chronic pancreatitis, a disease that frequently precedes pancreatic cancer (12). In addition, from a clinical point of view, pancreatic cancer is often difficult to distinguish from chronic pancreatitis (13). A marker that improves current diagnostic tools and would allow distinction between inflammatory and malignant pancreatic disorders would be of high clinical value.

PTN expression in normal tissue was rare. Only 7% of the samples (1 of 14) showed PTN-expressing epithelial cells. PTN expression in normal tissue may be even less frequent because in this study “normal” tissue was derived from pancreatic tissue that was adjacent to pancreatic cancer tissue. This includes a risk of wrong classification because epithelial cells may appear normal although pathological up-regulation has already occurred. However, the rare finding of PTN expression is in accordance with other studies that also found PTN expression to be restricted to pathological disorders, specifically malignant diseases (3). The frequency of PTN expression increased in both tissue samples and serum from patients with chronic pancreatitis, but PTN tissue expression and elevated serum levels were most frequently found in pancreatic cancer patients (\( P < 0.05 \)). Furthermore, patients with elevated serum PTN levels showed a significantly worse survival rate compared with patients with normal PTN levels. It is noteworthy that, in both groups, we did not find significant differences with respect to surgical procedure (resection versus palliation) and tumor stage. This supports our assumption that elevated PTN serum levels indicate an aggressive tumor biology and do not only reflect a higher tumor burden.

Our clinical observations, the finding of a stepwise increase of PTN expression from normal to inflammatory and malignant disorders, together with experimental studies (5–9, 14) strongly suggest that PTN expression has a significant impact on the biology of pancreatic cancer and represents an early event in pancreatic cancer development.

Although PTN failed to become a diagnostic marker that helps distinguish between inflammatory and malignant disorders, determination of the serum PTN level may still be of high value for monitoring therapeutic effectiveness. In our study, we found that PTN levels dropped under baseline levels in all patients whose tumor was completely resected, whereas all patients who received palliative surgical treatment had stable serum levels in the follow-up. Determination of PTN serum levels offers an attractive approach to monitor disease by using a biologically relevant marker that may also serve as a target for novel anticancer agents.

In summary, this study strongly suggests PTN up-regulation as an early event in pancreatic cancer development. Experimental evidence of the important role of PTN in tumor growth, together with our clinical observation that PTN overexpression occurs in 67% of pancreatic cancer patients and correlates with a worse outcome, demonstrates its clinical significance in pancreatic cancer progression.

**REFERENCES**

Significance of the Expression of the Growth Factor Pleiotrophin in Pancreatic Cancer Patients

Hans-Jürgen Klomp, Oliver Zernial, Sabine Flachmann, et al.


Updated version Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/8/3/823

Cited articles This article cites 11 articles, 8 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/8/3/823.full.html#ref-list-1

Citing articles This article has been cited by 7 HighWire-hosted articles. Access the articles at:
/content/8/3/823.full.html#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, contact the AACR Publications Department at permissions@aacr.org.