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Pleiotrophin Can Be Rate-limiting for Pancreatic Cancer Cell Growth[1]

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ABSTRACT

Pancreatic cancer is one of the most aggressive malignant tumors, with an overall survival rate of 2%. The identification of growth factors that contribute to the malignant phenotype can help to identify new targets for therapy. In this study, we analyzed the growth factor pleiotrophin (PTN) that was originally described as a developmentally regulated cytokine during early embryogenesis. More recently, PTN was found to be overexpressed in a variety of neuroectodermal tumors and described as an essential angiogenic growth factor in choriocarcinoma and melanoma, promoting metastatic growth. Recently, we discovered high expression levels of PTN in patients with gastrointestinal malignancies, particularly in those patients with pancreatic cancer. However, it is not known whether PTN is a contributor to the growth of pancreatic cancer or is only a bystander. We used ribozymes to deplete PTN mRNA from Colo357 pancreatic cancer cells and studied the resulting phenotype. The reduction of PTN resulted in a decrease in the proliferation rate, soft agar colony formation, and tumor growth in animals. Supplementation of cells with PTN partially reversed the ribozyme effect. The autocrine function of PTN was confirmed by using PTN-binding antibodies that inhibited the proliferation rate by 50% in Colo357 cells but also in a different pancreatic cancer cell line, Panc89. Our study identifies PTN as a new and essential growth factor for pancreatic cancer. Due to the restricted expression pattern of PTN in adults, PTN is suggested as a target for pancreatic cancer therapy.

INTRODUCTION

Every year, 28,000 pancreatic cancer cases are diagnosed in the United States. A chance of cure exists for only a minority of the patients, those with a locally limited and surgically resectable tumor (1). However, even 70–80% of patients whose tumor could be completely removed will suffer from an incurable local relapse, distant metastases, or peritoneal carcinosis (2). Obviously, pancreatic cancer, which has an overall 5-year survival rate of only 2% (1), is one of the most aggressive tumors. It is hoped that an understanding of the molecular basis of pancreatic cancer may provide new therapeutic targets. Within the past years, several growth factors such as epidermal growth factor, transforming growth factor α, transforming growth factor β, and fibroblast growth factor, as well as their receptors, have been identified as important mediators of pancreatic cancer growth (3). Although these growth factors are frequently overexpressed in malignant tissues, they are found under physiological conditions in many tissue specimens, thereby limiting their application as therapeutic targets. The identification of growth factors that show a tumor-restricted expression profile and are rate-limiting remains a challenge.

We became interested in the potential role of PTN[4] after we found that PTN is frequently expressed in gastrointestinal cancer and particularly in pancreatic cancers (4) and was identified as playing a major role in neoangiogenesis and metastatic growth of melanoma cells (5).

PTN is a heparin-binding secreted growth factor with an apparent molecular weight of 18,000 that is highly conserved across species. Human, mouse, and rat PTN protein are identical, and they share a ~50% amino acid similarity to the other family member midkine. 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 is present only at minimal levels in very few tissues, with its highest levels in brain. An almost identical and limited expression profile was found in human adult tissues, making PTN highly attractive as a therapeutic target (6).

A role of PTN in human cancers was suggested after purification from conditioned media of the highly malignant breast cancer cell line MDA-MB-231 (7). 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 (8).

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 (6). Furthermore, PTN induces tube formation on endothelial cells and angiogenesis *in vitro* and in the rabbit corneal assay (9). PTN has been found to act as an essential paracrine and angiogenic factor for human breast cancer (9), choriocarcinoma (10), and melanoma (5, 11). Furthermore, PTN seems to be essential for the development of metastases in melanoma: the depletion of PTN by ribozymes dramatically reduced the ability of melanoma cells to form metastases by inhibiting angiogenesis. Therefore, PTN has been suggested as an essential angiogenic factor (5, 10, 11).

Recently, we showed that PTN is expressed in gastrointestinal and particularly in pancreatic cancer cells. We found high expression levels of PTN in 78% of tumor samples from pancreatic cancer patients. Furthermore, tissue expression of PTN resulted in elevated serum levels in more than 50% of the pancreatic cancer patients, suggesting PTN as a new tumor marker in pancreatic malignancies (4).

Melanoma, which is of neuroectodermal origin, differs in numerous molecular and pathophysiological aspects from epithelium-derived pancreatic cancer. The functional significance of PTN expression in pancreatic cancer tissue was not predictable, and it was not obvious whether PTN is only a bystander or a contributor to tumor growth.

Therefore, we analyzed in this study the biological function of PTN *in vitro* and *in vivo* in pancreatic cancer cells, and we provide evidence that PTN serves as a rate-limiting autocrine growth factor. Because PTN is found in only a few normal tissues at a low level and is overexpressed in the majority of tumor samples from pancreatic cancer patients, our study suggests PTN as an attractive new target for pancreatic cancer therapy.

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[4] The abbreviations used are: PTN, pleiotrophin; TBS, Tris buffer saline; wt, wild-type.

MATERIALS AND METHODS

Material. Polyclonal goat antihuman PTN antibody (Santa Cruz Biotechnology, Santa Cruz, CA) specifically binds to PTN and does not cross-react with the structurally related midkine. Antihuman von Willebrand factor antibody (which has cross-reactivity with mice) was purchased from DAKO (Glostrup, Denmark), and the MIB-1 antibody (Ki-67 antigen) was obtained from Dianova (Hamburg, Germany). To stain apoptotic cells, we used the ApopTaq kit from Intergen (Purchase, NY).

The cell lines BXPC-3, SW850, Capan2, and HPAF were obtained from American Type Culture Collection (Rockville, MD). Colo357, Panc89, A816-4, Panc-Tu1, ASPC-1, and PT45P1 were kindly provided by Dr. H. Kalthoff (University Hospital Kiel, Kiel, Germany).

Generation of Constructs. For PTN-ribozyme targeting, we used ribozyme Rz66, which has been extensively characterized and shows efficient and specific cleavage of PTN mRNA *in vitro* and *in vivo* without cross-reaction to any known human mRNA (5, 10, 11). In brief, a synthetic sense and antisense oligonucleotide hammerhead ribozyme sequence was synthesized that contained a *Hin*dIII and a *Not*I site at the flanking regions, respectively, to obtain unidirectional ligation into the pRc/CMV vector (Invitrogen, Carlsbad, CA). The target sequence is located at the 3′ site of nucleotide 66 in the open reading frame of the human PTN mRNA (GenBank accession number M53799).

Cell Lines and Cell Culture. Human pancreatic adenocarcinoma cell lines Colo357 (12) and Panc89 (13) were cultivated in IMEM culture medium (Life Technologies, Inc., Grand Island, NY) supplemented with 10% FCS (Life Technologies, Inc.).

Stable Transfection of Colo357 Cells. Colo357 cells were stably transfected with 10 μg of Rz66-pRc/CMV vector and 70 μl of LipofectAMINE (Life Technologies, Inc.). Transfection was performed in 50–70% confluent cells for 5 h in serum-free Optimem medium (Life Technologies, Inc.) at 37°C in 5% CO_2/95% air. Selection started 48 h later using 1mg/ml G418 (Life Technologies, Inc.).

Growth Assays. To measure the proliferation rate, we used a [^{3}H]thymidine incorporation assay as described previously (14). A total of 1×10^4 cells/100 μl were seeded in 96-well microtiter plates and continued to grow for 12–16 h. Methyl-[^{3}H]thymidine (20 μCi/ml; Amersham, Piscataway, NJ) was added, and was incubated for 3–5 h. The trypsinized cells were harvested, lysed, and transferred to a filter. The counts were measured using a Beckmann scintillation counter. At least six aliquots/sample were measured.

To determine whether PTN acts as an autocrine growth factor, we used a soft agar colony formation assay as described previously (15). Briefly, a single cell suspension of 1×10^4 cells was stirred in 0.35% Bactoagar (Life Technologies, Inc.) and layered on top of 1 ml of a solidified 0.6% agar layer in a 35-mm Petri dish. The agar was solved in IMEM culture media supplemented with 10% FCS. The dishes were incubated for 10–14 days at 37°C and 5% CO_2. Colonies larger than 60 μm in diameter were counted after 10–14 days using an image analyzer.

Immunohistochemistry. Immunostaining of tumor cell lines was performed as described previously (4). Tumor cells were grown on glass slides until confluence and then fixed with acetone, and endogenous peroxidase was blocked by incubation with 0.3% H_2O_2 in methanol. Sections were washed and incubated with 5 μg/ml anti-PTN antibody at 4°C overnight. A peroxidase-coupled rabbit antigoat IgG (Dianova) 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 5–15 min, the slides were washed, and the nuclei were counterstained using hematoxylin.

Immunostaining of paraffin-embedded tumor tissues was performed as described previously (5). To determine proliferation *in situ*, we incubated a 5-μm tumor section with 5 μg/ml MIB-1 antibody overnight at 4°C. Microvessels were stained after a 2-h incubation at 4°C with a 1:200 dilution of the monoclonal anti-von Willebrand factor antibody. As a secondary antibody, we used a rabbit antimouse peroxidase-conjugated antibody (Immunotech; 1:500 dilution) to detect the MIB-1 antibody, and a 1:200 dilution of a peroxidase-coupled rabbit antirat serum (Vector Laboratories) was used to stain the anti-von Willebrand factor antibody, respectively. Immunocomplexes were highlighted using the ABC kit (Biomedia, Foster City, CA) and diaminobenzidine (Sigma, St. Louis, MO). Finally, slides were counterstained using Harris hematoxylin (Lerner Laboratories, New Haven, CT).

To determine the apoptotic rate, we used the ApopTaq kit (Intergen), and the method was performed according to the manufacturer's manual.

PTN-ELISA. To determine the concentration of secreted PTN, we used a recently established ELISA (4). It is highly specific for PTN and does distinguish between PTN and the structurally related midkine. The ELISA was able to detect PTN at levels as low as 20–50 pg/ml. Briefly, 96-well microtiter plates (Corning, Inc., Corning, NY) were coated at 4°C overnight with 1 μg/ml in Tris-buffered saline of the 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 TBS were added (100 μl/well) and incubated for 1 h at room temperature. To achieve reliable levels of PTN for ELISA quantification, the supernatant of Colo357 cells was concentrated 10-fold (Centricon columns were from Amicon, Beverly, MA), and equal amounts of total protein (100 μg) were applied. 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 for 1 h at room temperature. After washing, 100 μl of a *p*-nitrophenyl phosphate substrate (Sigma) were added and incubated for 2 h at room temperature. The absorbance was measured with a microplate reader at $\lambda = 405$ nm.

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

RNA Preparation and Northern Blots. Total RNA was extracted from tumor cells and tumor tissue using RNA STAT-60 according to the manufacturer's manual (Tel-Test B Inc., Friendswood, TX). Total RNA (60 μg) was separated by electrophoresis on a 1% agarose gel containing 1× 4-morpholinepropanesulfonic acid and 10% formaldehyde (both from Sigma). Gels were blotted in 20× SSC buffer [1.5 M NaCl and 0.15 M sodium citrate (pH 7.0)] overnight. Blots were prehybridized for a minimum of 2 h in 5× SSC, 5× Denhardt's solution, 50% formamide, 0.5% SDS, and 100 μg/ml sonicated salmon sperm (Life Technologies, Inc.). Blots were hybridized at 42°C with an [α-^{32}P]dCTP-labeled cDNA probe directed to the open reading frame of human PTN overnight. After washing, the membranes were measured by using the phosporimager technique after 2–3 days of incubation. The PTN signal was quantified by densitometry in relation to β-actin, which was used as a loading control.

Tumor Growth in Nude Mice. Tumor cells (5×10^6) were injected s.c. in both flanks of athymic nude mice (female Ncr nu/nu; Harlan Sprague Dawley, Indianapolis, IN). Tumors became visible after 3–4 days, and tumor size was measured every 2–4 days. After 3–4 weeks, the mice were sacrificed. Half of the tumors were fixed in 10% paraformaldehyde to obtain paraffin section (immunostaining), and the other half were frozen immediately in liquid nitrogen for RNA extraction.

Serum was prepared and stored at −20°C until used to determine the PTN level by ELISA.

The apoptotic rate and proliferation rate were determined after immunostaining (using the ApopTaq kit and MIB-1 staining, respectively) by counting 20 randomly chosen microscopic fields with a magnification of ×400. Additionally, we measured the number of mitotic cells by counting 20 microscopic fields (×400) after H&E staining.

Data Analysis. For statistical analysis, we used the unpaired *t* test.

RESULTS

PTN Expression by Pancreatic Cancer Cells. Using immunocytochemistry, we analyzed 10 different human pancreatic cancer cell lines and determined PTN expression. Staining for PTN was found in six cell lines: (*a*) A816-4; (*b*) BXPC-3; (*c*) Panc-Tu1; (*d*) SW850; (*e*) Panc89; and (*f*) Colo357. For additional studies, we choose the pancreatic cancer cell lines Colo357 and Panc89, which showed a strong and homogeneous staining pattern for PTN (Fig. 1) and were known to be tumorigenic in nude mice.

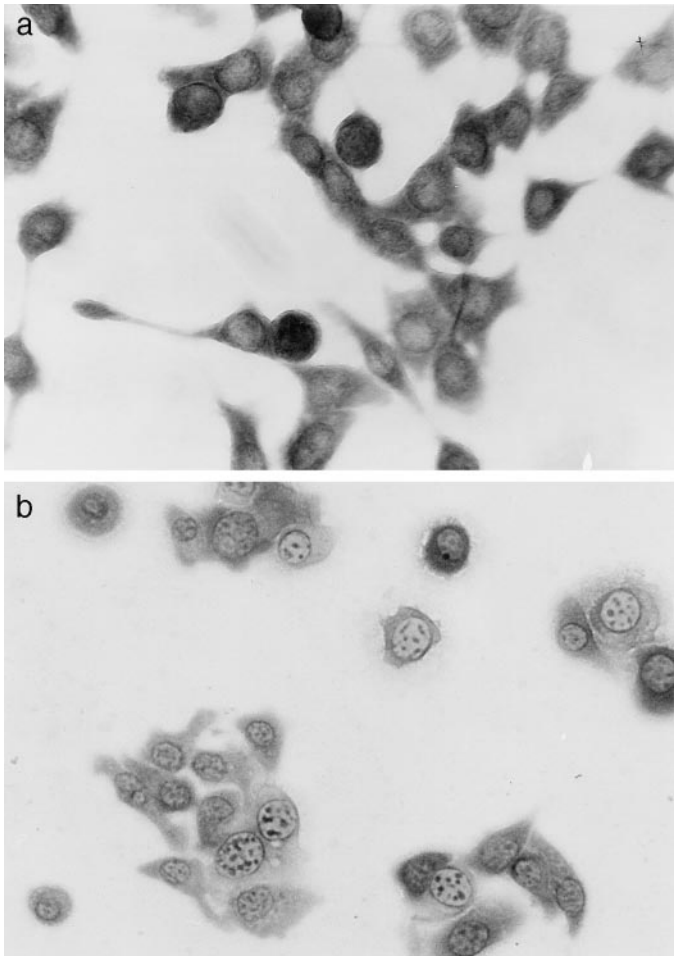

Fig. 1. PTN immunostaining of Colo357 (*a*) and Panc89 (*b*) human pancreatic cancer cells (×400).

PTN Depletion in Colo357 Cells by Ribozyme Targeting. The PTN targeting ribozyme Rz66 was stably transfected via G418 selection into Colo357 pancreatic cancer cells. Seven different clonal cell lines were isolated. They were screened for PTN expression by measuring PTN in the supernatant. Three clones (clones 5, 18, and 21) showed a significant reduction of PTN by 50–70%. The decrease of PTN protein was reflected by a 50–80% reduction of PTN mRNA compared with wt cells as determined by Northern blot (Fig. 2, *a* and *b*). Mass transfection of Colo357 cells using the empty pRc/CMV vector did not affect PTN on the mRNA or protein level.

PTN and Proliferation of Pancreatic Cancer Cells. The doubling time of ribozyme-transfected cells was reduced significantly in culture flask. This observation was confirmed by a [^{3}H]thymidine incorporation assay that showed a 30–70% decreased proliferation rate of ribozyme-transfected cells for clones 21, 18, and 5, respectively (Fig. 2*c*).

We also analyzed whether or not this effect was reversed by the exogeneous addition of PTN (Fig. 2*d*). Because functional recombinant PTN is not available, we used the culture supernatant of Colo357 wt cells as a source for biologically active PTN. Fresh culture medium was added to semiconfluently grown Colo357 wt cells and recovered after 36 h. We added this culture medium to ribozyme-transfected clones and, as a control, to Colo357 wt cells that were seeded on a microtiter plate. The proliferation rate was determined in comparison with cells that received fresh culture medium only. The proliferation rate of clones 21, 18, and 5 increased by 15%, 36%, and 42%, respectively, whereas the proliferation rate of Colo357 wt cells was not affected by this treatment.

PTN-dependent Colony Formation. The role of PTN was further studied in a colony formation assay with Colo357 ribozyme-transfected and wt cells. The colony formation was reduced by 40–80% in parallel with PTN depletion in clones 18, 21, and 5 (Fig. 3).

***In Vivo* Tumor Growth of PTN-depleted Pancreatic Cancer Cells.** For *in vivo* analysis, we injected Colo357 wt and Colo357 clone 5 cells s.c. into athymic nude mice. Clone 5 was chosen because it showed the strongest reduction of PTN expression at the mRNA and protein level (Fig. 2, *a* and *b*). Tumors became visible after 3–4 days. Whereas wt cells rapidly formed large s.c. nodules, the growth of PTN-depleted tumors were reduced dramatically (Fig. 4).

The histological evaluation showed numerous necrotic areas in PTN-depleted tumors, whereas no necrosis was detected in wt samples (Fig. 4, *inset*). Immunostaining for microvessels did not reveal any difference between wt and PTN-depleted tumors. However, the number of microvessels within wt tumors was extremely low (two to

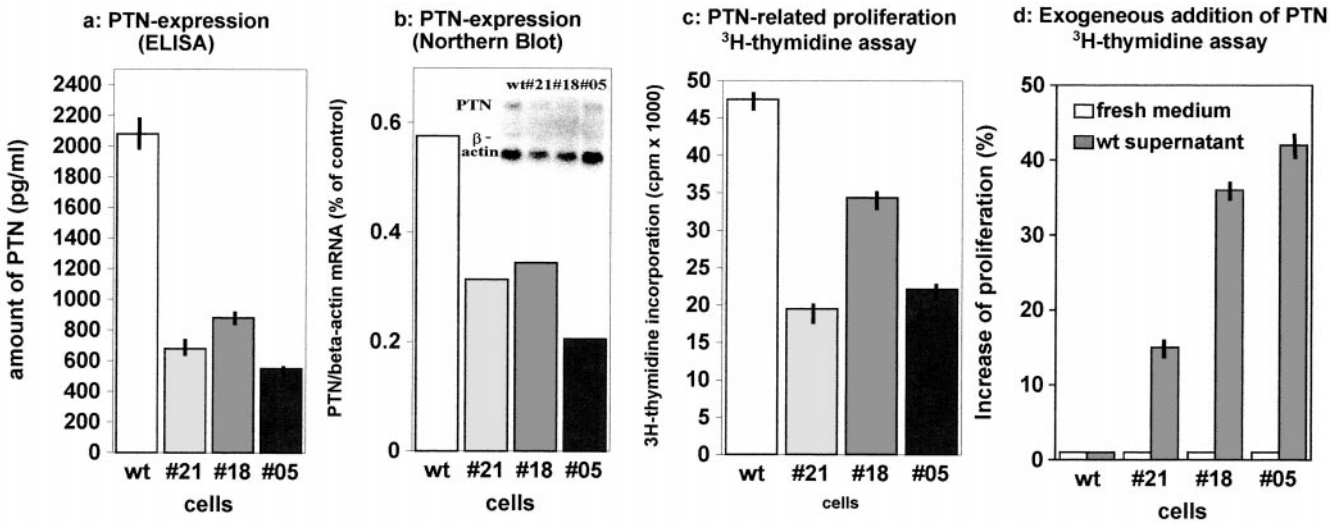

Fig. 2. *a*—*d*, effect of PTN-targeted ribozymes on Colo357 cells. Data from Colo357 wt cells and ribozyme-transfected clones 5, 18, and 21 are shown. *a*, ELISA for PTN protein levels in the cell supernatant. *b*, quantification of the Northern blot analysis (*inset*). PTN mRNA was quantified relative to β-actin mRNA by densitometry. *c*, proliferation rate as assessed by [^{3}H]thymidine incorporation. *d*, proliferation assay to determine the effect of exogeneously added PTN. Supernatant of Colo357 wt cells was used as a source for biologically active PTN that was recovered 36 h after its addition to semiconfluently grown Colo357 wt cells. The increase in proliferation compared with cells that received fresh medium is shown.

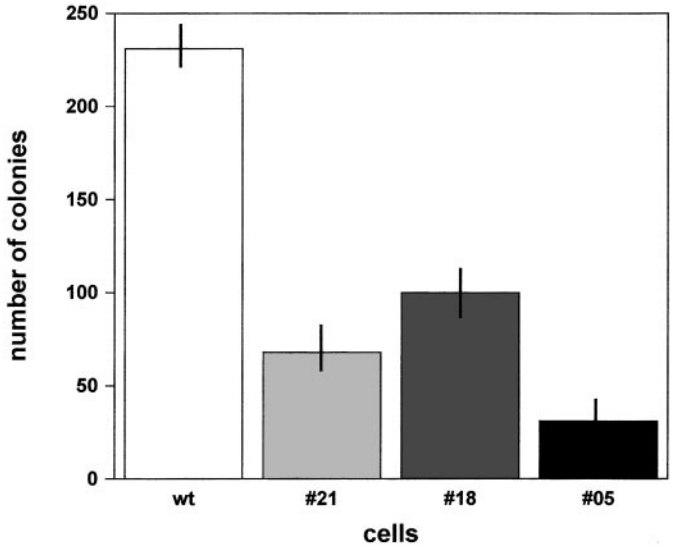

Fig. 3. Soft agar colony formation of Colo357 wt and PTN-depleted clones (clones 5, 18, and 21). Mean ± SD of three Petri dishes.

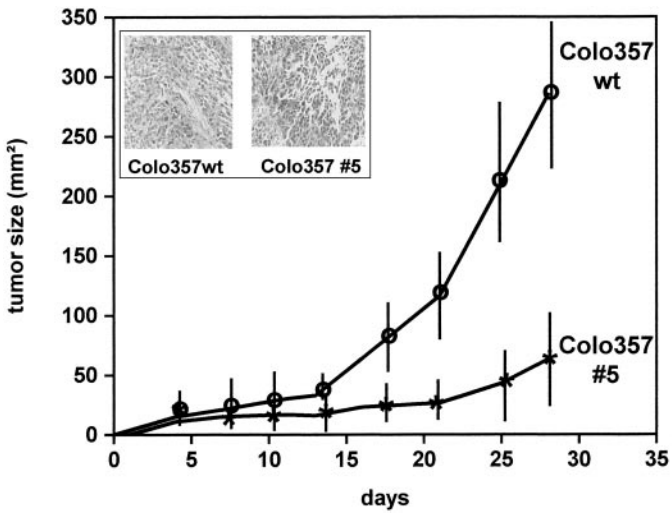

Fig. 4. Tumor growth of Colo357 wt cells and ribozyme-transfected clone 5, which showed the highest down-regulation of PTN protein. Tumor size is the product of perpendicular diameters. The *inset* shows a representative histological picture (H&E staining).

four stained vessels/section), which limits the comparative analysis. We compared tumors regarding the number of apoptotic cells and also could not see any difference. All tumors showed a comparable rate of 3–5% apoptotic cells.

However, as shown in Fig. 5 and in accordance with our *in vitro* data, we found a significantly higher number of proliferating cells in wt cells (50% *versus* 20%) as determined by MIB-1 staining ($P = 0.0163$). Additionally, the mitotic rate was reduced by 67% in PTN-depleted cells, which had an average of 4 mitotic cells/microscopic field, in contrast to wt cells, which had 12 mitotic cells/microscope field ($P = 0.0062$). These results are shown in Fig. 5.

The experiment was repeated with a lower cell number (5×10^5 cells/mouse), resulting in visible tumor growth after 1 week. Tumor growth of wt and PTN-depleted cells showed the same differences as the immunohistochemical evaluation (data not shown).

Antibody-mediated Inhibition of Pancreatic Cancer Cell Proliferation. Our data strongly suggest that PTN is produced by tumor cells and acts as an autocrine growth factor. To further prove this, we used PTN-binding antibodies to inhibit PTN function. PTN-targeting antibodies were added to the supernatant of Colo357 wt cells, and the proliferation rate was determined using a [^{3}H]thymidine assay. Additionally, the PTN-secreting pancreatic cancer cell line Panc89 (Fig. 1) was studied in the same way. We found a comparable and dose-dependent inhibition of the proliferation rate in both cell lines ranging from 10% (5 μg/ml anti-PTN antibody) to 50% (40 μg/ml anti-PTN antibody). An irrelevant isotype control IgG antibody (40 μg/ml) did not affect the proliferation rate of either Colo357 or Panc89 cells.

DISCUSSION

PTN was originally described as a developmentally regulated cytokine that regulates the growth of neuroectodermal and mesodermal cell lineages during early embryogenesis but becomes down-regulated during the late phase of embryogenesis and shows a very restricted expression pattern in adults (6). The finding of PTN expression in the supernatant of a highly malignant breast cancer cell line (7) was surprising, and additional studies revealed that PTN functions as an angiogenic growth factor that acts mitogenic on endothelial cells (6). Due to its mesodermal and neuroectodermal origin, it was not surprising that the highest expression levels of PTN were found in glioblastoma, melanoma (6), and small cell lung cancer cell lines (16). Functional analysis using PTN-targeted ribozymes showed that PTN is a rate-limiting angiogenic factor in melanoma and choriocarcinoma cells. The depletion of PTN resulted in a dramatic reduction of microvessels and consequently inhibited the development of metastases in nude mice (5). Overexpression of PTN in 3T3 fibroblasts, MCF-7 cells, or SW-13 cells resulted in increased *in vivo* tumor growth due to its angiogenic properties (8, 9, 17).

For us, it was a surprise to find strong PTN immunostaining in 41% of stomach, 56% of colon, and 78% of pancreatic cancer patients and to detect elevated PTN serum levels in 24% of the stomach cancer patients and 15% of colon cancer patients but in more than 50% of pancreatic cancer patients (4). We have also confirmed the observation of a frequent and high PTN expression level in pancreatic cancer

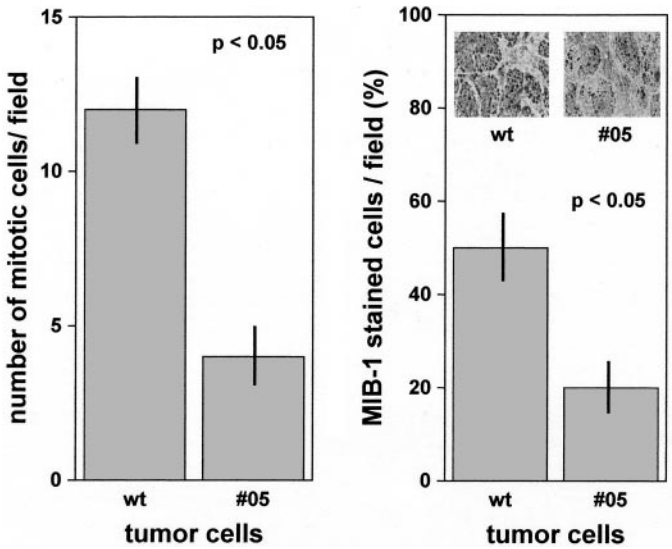

Fig. 5. Proliferation of Colo357 wt and PTN-depleted pancreatic cancer cells (clone 5) *in vivo*. The number of mitotic cells/microscopic field (*left panel*) and the percentage of MIB-1-stained tumor cells/microscopic field (*right panel*) are shown. The difference between wt and clone 5 cells was significant in both assays (*t* test, $P = 0.0062$ and $P = 0.0163$, respectively). *Inset*, immunostaining with anti-MIB-1 antibody of wt and clone 5 tumors.

patients using *in situ* hybridization.[5] The high serum levels of PTN in pancreatic cancer patients and PTN overexpression in more than 75% of the primary tumors were reasons to elucidate the function of PTN in pancreatic cancer cells.

We used ribozymes directed against the open reading frame of PTN mRNA that were previously shown to efficiently cleave the PTN mRNA (11). We transfected PTN-positive Colo357 pancreatic cancer cells with ribozyme expression vectors. Depletion of PTN resulted in a dramatic inhibition of the proliferation and colony formation rate. A supernatant of cultivated Colo357 wt cells reversed the ribozyme effect by almost 50%, and PTN-binding antibodies inhibited Colo357 wt and Panc89 cell proliferation by approximately 50%. In summary, these data demonstrate that PTN acts as an autocrine growth factor in pancreatic cancer. The *in vivo* tumor growth of PTN-depleted cells was dramatically reduced, and we detected a decreased proliferation fraction in the tumor. PTN has been described as a rate-limiting angiogenic factor in a variety of tumors (6). Because the microvessel density in wt tumors was extremely low (two to four vessels/slide), it was not possible to detect significant differences in PTN-depleted tumors, and PTN might have angiogenic properties in pancreatic tumors as well.

In summary, in this study, we identify PTN and prove that it is an essential autocrine growth factor for pancreatic cancer. This finding is of high clinical significance because PTN is overexpressed in more than 75% of tumors from pancreatic cancer patients. Furthermore, elevated PTN serum levels can be detected frequently in a majority of pancreatic cancer patients and help to monitor the disease (4). Because PTN is rarely found in normal tissue, PTN appears to be an attractive target for cancer therapy.

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[5] Unpublished observations.

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