16-kDa fragment of pleiotrophin acts on endothelial and breast tumor cells and inhibits tumor development

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Abstract

Pleiotrophin (PTN) is a 136-amino acid secreted heparin-binding protein that is considered as a rate-limiting growth and an angiogenic factor in the onset, invasion, and metastatic process of many tumors. Its mitogenic and tumorigenic activities are mediated by the COOH-terminal residues 111 to 136 of PTN, allowing it to bind to cell surface tyrosine kinase-linked receptors. We investigated a new strategy consisting in evaluating the antitumor effect of a truncated PTN, lacking the COOH-terminal 111 to 136 portion of the molecule (PTNΔ111-136), which may act as a dominant-negative effector for its mitogenic, angiogenic, and tumorigenic activities by heterodimerizing with the wild-type protein. In vitro studies showed that PTNΔ111-136 selectively inhibited a PTN-dependent MDA-MB-231 breast tumor and endothelial cell proliferation and that, in MDA-MB-231 cells expressing PTNΔ111-136, the vascular endothelial growth factor A and hypoxia-inducible factor-1α mRNA levels were significantly decreased by 59% and 71%, respectively, compared with levels in wild-type cells. In vivo, intramuscular electrophoresis of a plasmid encoding a secretable form of PTNΔ111-136 was shown to inhibit MDA-MB-231 tumor growth by 81%. This antitumor effect was associated with the detection of the PTNΔ111-136 molecule in the muscle and tumor extracts, the suppression of neovascularization within the tumors, and a decline in the Ki-67 proliferative index. Because PTN is rarely found in normal tissue, our data show that targeted PTN may represent an attractive and new therapeutic approach to the fight against cancer. [Mol Cancer Ther 2008; 7(9):2817–27]

Introduction

Tumor progression and metastasis depend on angiogenesis, a process inducing the outgrowth of new blood vessels from preexisting vessels (1). This process implies the presence of multiple controls and especially the up-regulation of growth factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor, which are capable of inducing the activation and expansion of endothelial cells. More recently, new growth factor members of the heparin-binding growth factor family such as pleiotrophin (PTN) and midkine (MDK) have been described, which also contribute to promoting tumorigenesis and angiogenesis. PTN was shown to induce the proliferation and migration of endothelial cells in vitro (2–6) and has been correlated with enhanced tumor growth and vascular density in vivo (5, 6). PTN was shown to be particularly involved in tumor growth of several cancer types (4, 7) and to also display transforming activity (8).

PTN is an 18-kDa secreted polypeptide of 136 amino acids, containing a heparin-binding site that plays a role in the modulation of PTN mitogenic activity (2) and in dimerization of the molecule (9).

Several PTN receptors have been described. In contrast to the members of the low-density lipoprotein receptor-related protein family (10) that are considered to be exclusively receptors of MDK (11), members of the proteoglycans including the receptor protein-tyrosine phosphatase (RPTP) have been assigned as the receptors of PTN called P122-131 (14). The interaction was mediated by the COOH-terminal part of PTN involving 10 amino acids of the COOH-terminal part of PTN called P122-131 (14).

Anaplastic lymphoma kinase is one of the major tyrosine kinase receptors of PTN, which induces the phosphorylation and recruitment of downstream effector molecules such as IRS-1, Shc, phospholipase Cγ, and phosphatidylinositol 3-kinase (15, 16). It has been shown that PTN binds to the extracellular domain of ALK via its COOH-terminal domain and that PTN lacking its COOH terminus is unable to interact with ALK, thus showing the importance of the COOH-terminal residues of PTN for its mitogenic and
tumorigenic activities (2, 17). Subsequently, a synthetic peptide corresponding to the deleted 26 amino acids residues of the COOH terminus of PTN, designated P111-136, has been shown to bind to the extracellular domain of ALK and to inhibit PTN-induced mitogenesis (2, 17). Furthermore, it has been shown that, when PTN is lacking its last 26 amino acids (PTNA111-136), it plays a dominant-negative effector for its mitogenic, angiogenic, and transforming activities by heterodimerizing with the wild-type protein (2).

In this study, we assessed the in vivo activities of the 111-136-deleted PTN and show for the first time that this truncated protein could act as a potent inhibitor of tumor growth and angiogenesis in an MDA-MB-231 breast carcinoma model. Furthermore, we focused our study on the mechanism of action of the PTN-deleted protein, which allowed us to explain the inhibition of PTN biological activities.

**Materials and Methods**

**Plasmid DNA**

The pcDNA3-LacZ plasmid was obtained by cloning the LacZ fragment (isolated from the PavxLacZ plasmid) between the EcoRI and the NotI sites of the pcDNA3.1 plasmid (Invitrogen). The pcDNA3-PTNΔ111-136 (2), pcDNA3-LacZ, and pcDNA-HSA plasmids were purified using endofree Plasmid Maxi Kit (Qiagen) and solubilized in endotoxin-free 0.9% NaCl at working concentrations.

**Cell Culture and Conditioned Medium**

Human umbilical vein endothelial cells (HUVEC), human microvascular endothelial cells (HMEC-1), PC3 cells (ATCC CRL1435), MDA-MB-231 cells (ATCC HTB26), MCF-7 cells (ATCC HTB22), and stably transfected MDA-MB-231 cells expressing PTNΔ111-136 (MDA-MB-231-PTNΔ111-136) were cultured as described previously (2, 18). U138MG glioblastoma cell lines (ATCC HTB16), U38MG astrocytoma cell lines (ATCC HTB14), and L6 cell lines (ATCC CRL1458) were grown in DMEM-fetal bovine serum and Jurkat human T-cell lines (ATCC HTB14) were grown in RPMI supplemented with 10% fetal bovine serum. The MDA-MB-231-GFP cells were obtained using a fluorescence-activated cell sorting flow cytometer (Becton Dickinson).

**Western Blot Analysis and ELISA**

The PTN expression in supernatants, cell pellets, and extracellular compartments defined as cell surface and extracellular matrix MDA-MB-231 tumors was done in MDA-MB-231 and PC3 cells by Western blot analysis following heparin Sepharose precipitation as described previously (20). Quantification of the immunoreactive material was done by densitometric analysis using a ChemiGenius system and the Genetools software (Syngene). For ALK detection, proteins were transferred to nitrocellulose membrane that was blocked in TBS, 0.1% Tween 20, 5% powered milk and probed with rabbit anti-ALK antibody (17,000; Zymed). Goat anti-rabbit peroxidase-linked antibody (1:10,000; Promega) was used as secondary antibody. Actin was detected with monoclonal anti-β-actin peroxidase conjugate (1:50,000). The levels of PTN in cell supernatants were quantified by an ELISA test, using a goat anti-PTN antibody (R&D Systems), according to the procedure described previously (21).

**Real-time Reverse Transcription-PCR**

The theoretical and practical aspects of real-time quantitative reverse transcription-PCR (RT-PCR) using the ABI Prism 7900 Sequence Detection System (Perkin-Elmer Applied Biosystems) as well as the RNA extraction method, cDNA synthesis, and PCR conditions were described in detail elsewhere (22). Target gene expression was normalized relative to an endogenous RNA control (TBP gene), which encodes TATA box-binding protein as described previously (23). Results expressed as N-fold differences in target gene expression relative to the TBP gene and termed “NTarget” were determined as NTarget = 2ΔCt sample, where the ΔCt value of the sample was determined by subtracting the average Ct value of the target gene from the average Ct value of the TBP gene. The NTarget Values of the samples were subsequently normalized to a “basal mRNA level,” normalized to the smallest amount of target gene mRNA quantifiable (target gene Ct value = 35 and then scored as the smallest NTarget value; NTarget Value = 1). Samples with very low levels of Target gene mRNA, not quantifiable by means of the real-time quantitative RT-PCR assay (Ct < 35), were considered as “nonexpressed” (NTarget value = 0).
Proliferation and Soft Agar Assays

Cells were seeded in 24-well tissue culture dishes at a density of $5 \times 10^4$ per well for PC3, HUVEC, and HMEC-1 cells and $2 \times 10^4$ per well for MDA-MB-231 and MCF-7 cells in complete medium. After 24 h, the medium was replaced by 420 µL fetal bovine serum-free medium and 280 µL L6 conditioned medium containing or not PTNΔ111-136 or by 280 µL MDA-MB-231 or MDA-MB-231-PTNΔ111-136 conditioned medium. The culture mediums were refreshed every 24 h over 4 days, and cell survival was then quantified by a cell proliferation assay using the 3-(4',5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay as described previously (18). A proliferation assay and a soft agar assay were done with MDA-MB-231 cells in the presence of 0.1 and 0.01 µmol/L anti-PTN antibody (R&D Systems) as described previously (24).

Migration Assay

The migration assay was done using 24-well microchemotaxis Boyden chambers (Costar) with 8-µm pore polycarbonate membranes. The bottom chamber was filled with L6 conditioned medium and the upper chamber was loaded with $5 \times 10^5$ HUVEC cells in 1 mL appropriate medium. After 24 h incubation, the membranes were fixed in acetone and cells were stained with May-Grunwald-Giemsa. Cells that had migrated through the membrane were quantified by counting eight fields of each membrane using a microscope with $\times 50$ magnification.

Plasmid Electrottransfer

Before the electrotransfer procedure, 6-week-old female athymic mice were anesthetized with an i.p. injection of 100 mg/kg ketamine (Ketalar, Panpharma) and 40 mg/kg xylazine (Rompun, Bayer). pcDNA3-PTNΔ111-136 plasmid or controls (pcDNA3-LacZ and pcDNA3-HAS; 20 or 30 µg) were resuspended in 30 µL sterile 0.9% normal saline and then injected into both tibialis cranialis muscles of mice using a Hamilton syringe. The three plasmids were then electrottransferred using a PS-15 electropulsator (Jouan) as described previously (25).

Tumor Cell Inoculation

MDA-MB-231, MDA-MB-231-PTNΔ111-136, and PC3 cells were harvested, washed, and resuspended in sterile PBS and 4 $\times 10^6$ cells in 100 µL were injected s.c. 7 days after the plasmid electrottransfer into the posterior flank of nude mice.

The tumor size was monitored measuring the largest (a) and the longest (b) tumor diameters and the tumor volume was calculated using the following formula: $[(a + b) / 2]^3 \times \pi / 6$. The tumor size was monitored over 1 month after the injection of tumor cells. At completion of each experiment, mice were sacrificed using a lethal dose of isoflurane. For each mouse, the two tibialis cranialis muscles and the tumor were collected and stored for immunohistochemical analysis.

Immunohistochemistry and Image Analysis

Muscles and tumors were fixed in FineFix (Milestone), and paraffin sections (4 µm thick) were prepared. Paraffin sections of MDA-MB-231 tumor cells were prepared to quantify the number of intratumor vessels by CD31 immunostaining and Ki-67 expression as described (18, 26, 27). Paraffin sections of muscles were prepared to evaluate β-galactosidase expression as described (28) and PTNΔ111-136 expression. For PTNΔ111-136 staining, slides were first incubated with the anti-PTN antibody (R&D Systems) and then incubated with a biotin-conjugated rabbit anti-goat antibody (DAKO) and with the streptavidin/peroxidase horseradish peroxidase complex (DAKO).

Statistical Analysis

Each experiment was done at least twice. Statistical significance was evaluated using the Student’s t test (unilateral and unpaired).

Results

Expression of PTN and Its Receptors

A Western blot was first done to analyze the expression of PTN in the supernatant and in MDA-MB-231 and PC3 cell extracts after 48 h culture as well as in the extracellular compartments defined as cell surface and extracellular matrix. PTN was detected in the cellular fraction as well in the surface of MDA-MB-231 cells (Fig. 1A, 1), whereas PTN was detected only in the supernatant of the PC3 culture medium (Fig. 1A, 2), suggesting that only the PTN secreted by MDA-MB-231 cells was correctly trapped on the cell membrane via an autocrine pathway. Endogenous PTN and MDK mRNA expression was then quantified by real-time RT-PCR in eight different breast carcinoma cell lines in comparison with PC3 prostate cell lines. As shown in Fig. 1B, MDK mRNA was expressed in all breast tumor cells, whereas PTN mRNA was only highly expressed in MDA-MB-231 cells compared with other cell lines and was 70-fold higher than the mRNA level detected in PC3 cells.

To evaluate the potential contribution of HBGH signaling, we then studied the expression of its receptors (ALK, RPTP, and lipoprotein receptor-related proteins) using real-time RT-PCR technology (see Materials and Methods). As shown in Fig. 1B, in contrast to U87MG positive for ALK, the amount of the ALK receptor transcripts was below detection in all extracts tested. An immunoblot analysis done on cell extracts from breast carcinoma cell lines (MDA-MB-231 expressing or not PTNΔ111-136 and MCF-7) confirmed these results (Fig. 1C). In contrast to Jurkat cells known to be ALK negative, two major isoforms of ALK were identified (220 and 140 kDa) in extracts from U38MG and U138MG cell lines that were undetectable when ALK was knocked down by siRNA in U138MG cells (Fig. 1D).

Concerning the RPTPs and lipoprotein receptor-related protein profiles, most part of the breast tumor cells exhibited the mRNA of RPTPA and B (except MB468 and T47D) in contrast to the PC3 cell line, which expressed RPTPD and RPTPS, which seems to be highly ubiquitous in all cell lines tested (Fig. 1B). Furthermore, in contrast to PC3 cells, all breast cell lines exhibited the mRNA of LRP6 and some of them expressed also lipoprotein receptor-related proteins 1β and 2.

In vitro Expression and Functional Characterization of PTNΔ111-136

Experiments were first done to verify the expression and secretion of PTNΔ111-136. The amount of PTNΔ111-136...
was evaluated with a specific ELISA. As shown in Fig. 2A, an expression level up to 13 ng/mL culture medium was obtained in pcDNA3-PTN

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111-136 L6 conditioned medium, whereas no signal was detected in the supernatant of nontransfected L6 (<1 ng/mL). After 96 h culture, MDA-MB-231 cells expressing PTN

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111-136 exhibited a high quantity of the secreted immunoreactive protein compared with the control cells (15.3 versus <1 ng/mL), confirming satisfactory expression of PTN

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111-136 by both cell lines.

To evaluate a potential role of PTN

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111-136 in inhibiting angiogenesis, an endothelial cell proliferation assay was first done. We thus assayed PTN

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111-136, produced by transfected L6 cells, for their inhibitory activity on immortalized human endothelial cells (HMEC-1) or primary human endothelial cells (HUVEC) in a 96 h proliferation assay using the MTT test. Under these conditions, endothelial cell proliferation was significantly inhibited by 51% and 31%, respectively, for HMEC-1 (P < 0.007) and HUVEC (P < 0.05) compared with the control (Fig. 2B). As angiogenesis occurs through endothelial cell migration, it was therefore important to analyze whether PTN

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111-136 affects HUVEC cell migration or not using Boyden chambers. As shown in

Fig. 2C and D, the migration of HUVEC was inhibited by 31% when medium containing PTN

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111-136 was added compared with the migration of cells grown in control medium (P < 0.0009). These results clearly show that PTN

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111-136 exerts inhibitory activity on the proliferation and the migration of primary endothelial cells.

We then evaluated the PTN autocrine loop using polyclonal anti-human PTN antibody in a proliferation assay (Fig. 3A, 1) and in a soft agar colony formation assay (Fig. 3A, 2). Polyclonal anti-human PTN antibodies induced a significant dose-dependent decrease in colony formation and a significant inhibition of cell proliferation, whereas idiotypic immunoglobulins, used as the control, had no effect. These data clearly showed the existence of an autocrine PTN signaling loop for MDA-MB-231 cells. We thus did a proliferation assay using MDA-MB-231 in the presence of PTN

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111-136. As shown in Fig. 3B, 1, 66% of MDA-MB-231 cell proliferation was abolished compared with that of the control (P < 0.003), whereas PC3 cells expressing lower level of PTN were not affected (P = 0.2) by PTN

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111-136 (Fig. 3B, 2). To assess the paracrine effect of PTN

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111-136 produced by human cells, we used.
MDA-MB-231-PTNΔ111-136 and MDA-MB-231 cells that were mixed with MDA-MB-231 cells expressing GFP at a ratio of 1:1. MDA-MB-231-GFP-positive cells were quantified with flow cytometry after 24 h culture. As shown in Fig. 3C, 77% of MDA-MB-231-GFP-positive cell proliferation was abolished in the presence of MDA-MB-231 cells expressing PTNΔ111-136 (P < 0.02). These data show that the factor produced either by L6 muscle cells or by MDA-MB-231 tumor cells is biologically active and is capable of inhibiting the proliferation of breast cancer cells. MDA-MB-231 and MCF-7 cells were then tested in parallel with conditioned collected medium from MDA-MB-231 and MDA-MB-231-PTNΔ111-136 cells. As shown in Fig. 3D, the cell proliferation was reduced for both cell lines and was more pronounced in MDA-MB-231 cells (70.3% of inhibition; P < 0.0001) than in MCF-7 cells that do not express PTNΔ111-136 (34.7% of inhibition; P < 0.001).

PTNΔ111-136 Down-regulates the Expression of Genes Linked to Angiogenesis, Proliferation, and Apoptosis In vitro

The mRNA level from MDA-MB-231 cells and stably transfected MDA-MB-231-PTNΔ111-136 cells was evaluated using real-time RT-PCR (Table 1). To obtain further evidence confirming the inhibition of angiogenesis, we examined the expression profile of genes involved in hypoxia and angiogenesis pathways, namely the VEGF and platelet-derived growth factor families and hypoxia-inducible factor (HIF-1α). Whereas VEGF-B, VEGF-C, platelet-derived growth factor-α, and platelet-derived growth factor-β did not seem to be affected by PTNΔ111-136 expression, VEGF-A and HIF-1α were significantly down-regulated by 59% and 71%, respectively, compared with levels in wild-type cells (P < 0.05).

We also examined the expression profiles of key proteins such as TOP2A and BCL2 and TNFAIP3/A20 (an anti-apoptosis nuclear factor-κB pathway-associated gene), respectively, involved in proliferation and in cell protection against apoptosis. Significant down-regulation of mRNA levels of these three genes was observed: TOP2A expression was reduced by 64%, whereas BCL2 and TNFAIP3/A20 expression was lowered by 72% and 73% (P < 0.05), respectively, in MDA-MB-231 cells expressing PTNΔ111-136 compared with wild-type cells. In this study, p21/CDKN1A and GADD45B mRNA levels involved in the apoptosis pathway were not affected.

Inhibition of Tumor Growth of PTNΔ111-136 In vivo

PTN has been described previously to induce tumor formation in nude mice (2). Hence, 4 × 10⁶ MDA-MB-231 human adenocarcinoma mammary cells expressing PTNΔ111-136 were injected s.c. into nude mice and their tumorigenicity was compared with that of injected parental cells (n = 11 per group). Five days after injection, 100% of nude mice receiving nontransfected MDA-MB-231 cells started to develop small tumors that reached a mean volume of 608 ± 3.8 mm³ at day 33. In contrast, 5 days after the injection of MDA-MB-231 cells expressing PTNΔ111-136, only 3 of 11 mice developed tiny tumors, which have completely disappeared after 10 days (Fig. 4A).
We then conducted two other experiments to confirm the antitumor potency of PTNΔ111-136 in a prophylactic protocol after muscle electrotransfer of the pcDNA3-PTNΔ111-136 plasmid. We first electrotransferred 60 μg pcDNA3-PTNΔ111-136 or pcDNA3-HSA (pcDNA3 control plasmid expressing human serum albumin) into the tibialis cranialis muscles of female nude mice (n = 10 per group). Seven days later, 4 × 10⁶ MDA-MB-231 cells were inoculated s.c.

To evaluate electrotransfer efficiency and to detect in vivo expression of PTNΔ111-136, tibialis cranialis muscles of mice were collected at day 37 and analyzed by a specific ELISA. As shown in Fig. 4B, all muscle extracts, except one (mouse 7), exhibited a high amount of immunoreactive PTNΔ111-136 that was not observed in the control group. The mean value of PTNΔ111-136 was 1724 ± 214 pg/mg tissue (range, 341-8,441 pg/mg). As shown in Fig. 4C, tumor growth in treated animals was significantly inhibited compared with tumor growth in animals in the control group: 37 days after electrotransfer, the tumor volumes were reduced by 60% (P < 0.0001). The tumors had reached a mean volume of 321 ± 23 and 790 ± 17 mm³ in the
As shown in Fig. 5A, pcDNA3-LacZ plasmid expressing a nonsecreted molecule. The efficiency of the electrotransfer was first evaluated at completion of the first experiment (day 47) on muscle sections receiving the plasmid. The efficiency of lacZ gene were electrotransferred. The efficiency of the transgene product on the tumor vasculature. Interestingly, for mouse 7, which was negative for the PTN111-136 ELISA done on the muscle extract, we observed a high vessel index (2.69), showing good correlation between PTN111-136 expression in the muscle and the effect on the tumor vessel network.

We then conducted a second experiment, under the same conditions, except that only 40 μg pcDNA3 plasmid expressing PTN111-136 or the pcDNA3-LacZ control plasmid expressing the β-galactosidase encoded by the Escherichia coli lacZ gene were electrotransferred. The efficiency of the electrotransfer was first evaluated at completion of the experiment (day 47) on muscle sections receiving the pcDNA3-LacZ plasmid expressing a nonsecreted molecule. As shown in Fig. 5A, 1 and 2, a high level of β-galactosidase staining was observed only in the muscles of mice receiving the control plasmid confirming that plasmid electrotransfer in mouse muscles was significantly effective.

As shown in Fig. 5B, 3 and 4, MDA-MB-231 tumors from the PTN111-136-injected group were significantly smaller than those from the control group (P = 0.003) at day 47: tumors had reached a mean volume of 231.5 ± 12 mm² for the pcDNA3-PTN111-136-treated mice versus 1127 ± 93.5 mm² for control mice. This 80% inhibition correlated with the detection of a high amount (776 ± 36 pg/mg tissue) of immunoreactive PTN111-136 in muscle extracts (Fig. 5B, 2) and sections (Figs. 5A, 5 and 6) assessed by ELISA and immunohistochemistry, respectively, and in tumor extracts (Fig. 5C, 1), assessed by Western blot analysis following heparin Sepharose precipitation. PTN-immunoreactive material with a molecular weight of 18 kDa (lane 1) corresponding to native PTN molecule was detected in tumors from treated and nontreated animals. Furthermore, immunoreactive PTN with lower molecular weight corresponding to proteolytic fragments of PTN was detected in treated and nontreated mice with molecular weight of 15 kDa corresponding to PTN111-136 and fragments with molecular weight less than 15 and 10 kDa. These immunoreactive fragments corresponding to proteolytic degradation of PTN and/or PTN111-136 were found significantly increased by 42% and 57% in tumors from the treated mice. As shown in Fig. 5A, 3 and 4, MDA-MB-231 tumors treated with PTN111-136 were very smaller and much less vascularized compared with untreated tumors.

During this experiment, tumor proliferative activity was assessed by means of Ki-67 immunostaining, which is now widely used in clinical pathology diagnostics. Collected tumor tissues were stained with anti-Ki-67 antibody and nonnecrotic areas on histologic slides were scored. The proliferation appeared to be significantly decreased in the group electrotransferred with pcDNA3-PTN111-136 compared with the control group (Figs. 5C, 2, and 5A, 7 and 8). According to the immunohistochemical analysis, the percentage of Ki-67-positive MDA-MB-231 cells was significantly reduced by 41% (P = 0.003).

We carried out an in vitro experiment, under the same conditions, using PC3 human prostate carcinoma cells; as shown, no significant difference was observed (P = 0.2) in tumor growth in either group (Fig. 5D).

<table>
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Mol Cancer Ther 2008;7(9). September 2008
Discussion
PTN is expressed in many human tumors and tumor cell lines including neuroblastoma, melanoma, pancreatic, lung, and breast cancers (29–32) and can be a rate-limiting growth and an angiogenic factor in tumor development and metastasis in vivo (30). This finding sparked off efforts to design technologies aimed at interfering with the PTN signaling pathway or with its receptors, thereby blocking tumor development and angiogenesis. Various strategies have been developed such as the use of ribozymes, RNA interference, or a monoclonal antibody to down-regulate PTN expression or that of its tyrosine kinase-linked receptors (33,34).

In the present study, we investigated a new strategy evaluating the antitumor effect of a truncated PTN lacking the COOH-terminal 111 to 136 portion of the molecule (PTN Δ111-136), which is known to be involved in the mitogenic and in the transforming activities of this growth factor and in its binding to high-affinity receptors (2).

The antiproliferative activity of PTN Δ111-136 was first evaluated on tumor cell lines that express high and low level of PTN using MDA-MB-231 breast cells and PC3 prostate cell lines, respectively. Furthermore, we have evaluated PTN Δ111-136 on breast MCF-7 cells that do not express PTN. In contrast to U87MG or U138MG cell lines, known to express ALK, all the three cell lines do not express ALK in our experimental settings cells and do not express all the RPTP receptors members. Whereas RPTPS seems to be ubiquitarily expressed in all the three cell lines, RPTPA and RPTPB are especially expressed by both breast tumors cells in contrast to PC3 prostatic cell line expressing only RPTPD.

We showed in our study that PTN Δ111-136 was able to significantly reduce in vitro the proliferation of tumors cells that only expressed high levels of PTN as MDA-MB-231 breast-tumor cells. In this case, heterodimerization of the COOH-terminal mutant with endogenous PTN probably mainly explains its dominant-negative effect and that PTN Δ111-136 was able to dissociate PTN homodimers, thereby inducing the formation of nonfunctional heterodimers. Thus, the inhibition of PTN in MDA-MB-231 cells has a wide effect on different important cellular systems. In this context, we believe that the inhibition of PTN by PTN Δ111-136 abolishes the PTN/RPTP signaling pathways independently of ALK, as it was suggested previously using human prostatic adenocarcinoma cells DU145 (14). The RPTP receptor was found to be involved in...
Figure 5. Tumor growth and inhibition of tumor cell proliferation. A, high level of β-galactosidase staining was observed only in muscles of mice receiving the pcDNA3LacZ-expressing plasmid (1) compared with muscle injected with pcDNA3PTNΔ111-136 (2). Tumors from the pcDNA3LacZ-treated (3) and PTNΔ111-136-treated (4) groups are shown 47 d after the electrotransfer. Muscle sections of PTNΔ111-136-transfected mice showed a high expression of PTN Δ111-136 (6) that was not observed in the control group (5). A representative image of MDA-MB-231 tumors showing Ki-67-positive immunostaining; control tumors showed a high level of Ki-67 expression (7) and PTNΔ111-136-treated tumors showed a low level of Ki-67 expression (8). B, effect of PTNΔ111-136 transgene expression after in vivo muscle pDNA electrotransfer on MDA-MB-231 tumor growth in nude mice. Female mice were electrotransferred with 40 μg pcDNA3PTNΔ111-136-expressing plasmid or the control (pcDNA3LacZ). Tumor growth was inhibited in mice electrotransferred with pcDNA3PTNΔ111-136 compared with the control (2). Tumor growth inhibition was correlated with the detection of a high amount of PTNΔ111-136 detected by ELISA in the muscle at day 47 (2). C, immunodetection of PTN and proteolytic fragments of PTN within MDA-MB-231 tumor extracts (1). Western blot analysis was done following heparin Sepharose precipitation 47 days after muscle electrotransfer of pcDNA3-PTNΔ111-136 or pcDNA3LacZ and quantification of the immunoreactive material was done by densitometric analysis. The signals correspond to native PTN with a molecular weight of 18 kDa (lane 1), to PTNΔ111-136 with a molecular weight of 15 kDa (lane 2), and to proteolytic peptides with a molecular weight less than 15 kDa (lane 3) and less than 10 kDa (lane 4). Quantitation of Ki-67-positive cells in cancer cell compartment (2). D, effect of PTNΔ111-136 transgene expression after in vivo muscle pDNA electrotransfer on PC3 tumor growth in nude mice. Male mice were electrotransferred with 40 μg pcDNA3PTNΔ111-136-expressing plasmid or control (pcDNA3LacZ) and tumor growth was monitored until day 34.
PTN-induced cell migration and neurite outgrowth (35). Binding of PTN to RPTPB was shown to inactivate the catalytic phosphatase activity, leading to further activation of the Src/Fyn kinase family and β-catenin phosphorylation pathway (36). In keeping with this hypothesis, we found that PTNΔ111-136 did not affect the proliferation of PC3 cells and thus believe that the autocrine PTN signaling loop is very marginal because these cells do not exhibit high PTN level or the major cell surface tyrosine kinase-linked receptor such as ALK or RPTPB.

On the other hand, we have shown that PTNΔ111-136 inhibited the in vitro growth of MCF-7 cells, expressing as MDA-MB-231 the same surface tyrosine kinase-linked receptors. MCF-7 cell line is known to express MDK and not PTN (37); therefore, we can conclude to the existence of an autocrine MDK signaling loop through the RPTPB receptor because high levels of MDK transcript were found for this cell line. In this context, we believe that PTNΔ111-136 could induce the formation of nonfunctional heterodimers with MDK and thus inhibit the MDK/RPTPB pathway. MDK and PTN are members of the same neurotrophic family factors and are both basic, cysteine-rich polypeptide containing identical heparin-binding motifs, which are essential for the dimerization of PTN (9). They have about 50% of homology and no sequence homology with other heparin-binding proteins, such fibroblast growth factors. In this context, the formation of MDK/PTNΔ111-136 heterodimers are not excluded and further experiments have to be conducted to explore this hypothesis.

Because angiogenesis is also known to be a key mechanism via which PTN promotes tumorigenesis (5), we investigated the effects of PTNΔ111-136 on endothelial cell proliferation and migration. We were able to show that the inhibition of PTN by PTNΔ111-136 has major consequences on different stages of angiogenesis, because PTNΔ111-136 strongly inhibited the proliferation and the migration of endothelial cells in vitro. Our in vitro results were confirmed in vivo, because tumor formation in nude mice usually obtained with MDA-MB-231 cells was completely abolished when PTNΔ111-136-expressing MDA-MB-231 cells were injected, suggesting that both the growth advantage and the angiogenic activity were inhibited. Furthermore, we showed that PTNΔ111-136-expressing MDA-MB-231 cells were not efficient in promoting VEGF- and HIF-1α-induced angiogenesis compared with MDA-MB-231 cells. Indeed, we determined by quantitative RT-PCR that PTNΔ111-136 significantly down-regulated the expression of genes involved in hypoxia and the angiogenesis pathway such as VEGF-A as suggested previously (38). Moreover, further experiments are warranted to explain these results and more particularly to explore the role of PTNΔ111-136 in phosphoinositide 3-kinase/Akt/mammalian target of rapamycin signaling, because the expression of HIF-1α and VEGF-A was recently shown to be dependent on the mammalian target of rapamycin pathway (39, 40).

To complete and confirm our in vitro experiments, we investigated the antitumor efficacy and antiangiogenic properties of PTNΔ111-136 using an in vivo therapeutic approach using intramuscular electrophoresis of a plasmid delivering a secretable form of PTNΔ111-136. The long-term and durable expression of the molecule we observed reflects the quiescent status of the electrotransferred myofibers, thus showing that this method does not induce significant muscle damage, as confirmed at histologic examination (data not shown).

A single intramuscular injection of pcDNA3-PTNΔ111-136 but not of the control plasmid was shown to dramatically inhibit primary tumor growth in two independent experiments, without apparent toxicity. This inhibitory effect was observed only with the MDA-MB-231 tumor xenografted in nude mice. These observations were correlated with the results of in vitro experiments and suggest that the inhibitory effects of PTNΔ111-136 observed on MDA-MB-231 tumor growth seem to be exclusively a consequence of the inhibition of endogenous PTN. This antitumoral inhibitory effect was tightly correlated with markedly decreased vascularization within tumors and with the detection of PTNΔ111-136 immunoreactive material in muscle sections and extracts together with the detection of high levels of immunoreactive materials in the tumor extracts corresponding to little peptides that could be produced from cleavage of PTNΔ111-136 by extracellular proteolytic enzymes such as plasmin or matrix metalloproteinase-2, as it has been recently suggested (41, 42). We also showed that PTNΔ111-136 induced significant G0 quiescence in a large fraction of MDA-MB-231 cells as revealed by Ki-67-positive immunostaining. Furthermore, we showed in vitro that genes promoting cellular proliferation and genes protecting against apoptosis were deregulated in PTNΔ111-136-expressing MDA-MB-231 cells. We were able to show that the inhibition of PTN by PTNΔ111-136 significantly down-regulated the expression of TOP2A and the antiapoptosis genes BCL2 and TNFAIP3/A20, this latter gene being a well-known antiapoptosis nuclear factor-κB pathway-associated gene.

In conclusion, we have shown that specific targeting of PTN by PTNΔ111-136 involves strong inhibition of a breast tumor model and inhibition of the angiogenesis process. Nevertheless, the fact that cDNA muscle electrophoresis failed to completely abolish tumorigenesis underlines the importance of improving the gene transfer method, so that sustained expression can be achieved for maximum clinical benefits. We also intend to combine PTN inhibitors with cytotoxic approaches to enhance PTN inhibitor potency and thus improve the clinical outcome of patients with malignant diseases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank I. Laurendeau and E. Connault for technical assistance, L. Saint Ange for editing, M. Mackenthun for critical reading, and F. Louache for providing a retrovirus vector encoding the GFP gene.
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Mol Cancer Ther 2008;7:2817-2827.

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