Pleiotrophin Stimulates Fibroblasts and Endothelial and Epithelial Cells and Is Expressed in Human Cancer*

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Previously we reported the purification of the heparin-binding growth factor pleiotrophin (PTN) from supernatants of the human breast cancer cell line MDA-MB-231. To investigate further the biological activities of PTN and its potential role in cancer, we cloned a PTN cDNA and expressed the gene in a human kidney and in a human adrenal carcinoma cell line (SW-13). The supernatants harvested from cells transfected with PTN contained a heparin-binding specific protein of an apparent molecular mass of 18 kDa. These supernatants stimulated the proliferation of endothelial cells as well as the anchorage-independent growth of SW-13 cells and of normal rat kidney fibroblasts. Furthermore, SW-13 cells transfected with PTN acquired autonomous growth in soft agar and were tumorigenic in athymic nude mice. In contrast to these results with PTN from human cells, PTN obtained from insect cells (Sf9) using recombinant baculovirus as a vector was biologically inactive. We detected high levels of PTN mRNA in 16 of 27 primary human breast cancer samples (62%) as well as in 8 of 8 carcinogen-induced rat mammary tumors. Furthermore, 9 of 34 human tumor cell lines of different origin showed detectable PTN mRNA. We conclude that PTN may function as a tumor growth and angiogenesis factor in addition to its role during embryonic development.

Polypeptide growth factors have been shown to play important physiological roles in the timely development of tissues during embryonal and neonatal growth, and their expression is tightly regulated. On the other hand, polypeptide growth factor gene expression is deregulated in tumor cell lines as well as in solid tumors, and the activity of polypeptide growth factors appears to contribute significantly to autocrine and paracrine stimuli (for a review see Refs. 1 and 2).

Previously we have reported the purification of an 18-kDa heparin-binding growth factor from the conditioned medium of a human breast cancer cell line MDA-MB-231 (3). The N-terminus of this factor did not bear homology to any known heparin-binding FGFs but was homologous to a developmentally regulated protein which has recently been described by numerous laboratories under different names such as "heparin-affin regulatory protein (HARP)" (4, 5), heparin-binding neurotrophic factor (HBNEF) (6, 7) or p18 (8) from bovine brain, heparin-binding growth associated molecule (HB-GAM) from porcine rat brain (9-11), heparin-binding growth factor (H) (12), osteoblast-specific factor from mouse brain (13), and pleiotrophin (PTN) from human placenta or rat brain (11). Due to its numerous biological activities, we have decided to use the latter name for this growth factor. PTN belongs to a novel family of heparin-binding proteins which include the structurally related midkine proteins and appear to function during brain development (for a review see Ref. 14). The mitogenic activity of PTN, however, is still controversial (14). Several laboratories have described mitogenic activity of PTN purified from different sources for endothelial cells (4, 5, 7) and fibroblasts (6, 7, 11, 12). We reported that a purified preparation of PTN stimulates colony formation in soft agar of the epithelial cell line SW-13, and we identified PTN in this preparation by protein sequencing (3). However, other investigators have disputed an intrinsic growth factor activity of PTN and have attributed the activities to FGFs or other growth factors contaminating the respective preparations (15-17).

Our present study addresses this controversy by expressing the wild-type PTN cDNA under the control of a strong CMV promoter in two human cell lines. A PTN point mutant with a premature translation stop codon served as a negative control in these experiments. We show that supernatants from cells transfected with the wild-type construct but not the point mutant contain an 18-kDa heparin-binding protein that is immunoreactive and stimulates endothelial and SW-13 epithelial cells as well as fibroblasts. Furthermore, after expression of wild-type PTN, SW-13 cells acquired autonomous growth in soft agar and grew into tumors in athymic nude mice. This provides additional independent evidence that the PTN gene product can act as a growth factor in tumors. Furthermore, in agreement with a recent report from other authors (16), we were unable to obtain PTN in a biologically active form from insect cells which overproduced the protein after infection with PTN-recombinant baculovirus.

The potential significance of PTN for tumor growth is addressed in further experiments studying expression of this gene in primary tumors and in tumor cell lines. About one-fourth of a series of established human tumor cell lines showed...
expression of PTN as assessed by RNase protection assays. Most significantly, more than half of a set of 27 primary human breast cancer samples contained high levels of PTN mRNA. Finally, carcinogen-induced rat mammary tumors also scored positive for PTN mRNA.

We conclude that the protein product of this developmentally active gene seems to play a significant role in the progression of human tumors. Based on its mitogenic activities it most likely functions as a tumor angiogenesis factor.

MATERIALS AND METHODS

Cell Lines and Tissues—BT-474, BT-579, Hs-578T, MCF-7, MDA-MB-154, MDA-MB-231, MDA-MB-361, MDA-MB-435, MDA-MB-468, SK-BR-3, T47D, ZR-75-1 (human breast cancer), DU-145, LNCaP, PC-3 (human prostate cancer), A-549 (human embryonal kidney), FBHE (fetal bovine heart endothelium), NRK (normal rat kidney fibroblasts clones 49F) were obtained from the American Type Culture Collection (Rockville, MD). The following cell lines or sub-lines were kindly made available by colleagues at the Lombardi Cancer Center: MCF/1,2Y (human breast cancer) by Dr. M. E. Lippman (University of Cincinnati, OH), A-431 (human epidermoid carcinoma), HL-60 (human promyelocytic leukemia), SW-13 (human adrenal cancer), HGT-1, KATOIII (human stomach cancer), 203 (human embryonal kidney), A-431 (human fibroblasts), Molaro (human breast cancer), MDDC (human bronchus), and IGROV-1 (human ovarian cancer). Cells were purchased from Clonetics (San Diego, CA). Rat mammary carcinoma tissues were kindly provided by Dr. M. Gottardis (Lombardi Cancer Center).

Tumor tissues were immediately snap-frozen in liquid nitrogen before being stored at −70 °C. Tumor tissues were obtained from the National Disease Research Interchange (Philadelphia, PA) and from Dr. S. Paik (Tumor Bank of the Lombardi Cancer Center). Rat mammary carcinoma tissues were kindly provided by Dr. M. Gottardis (Lombardi Cancer Center).

Cell preparations were added to 96-well plates by overnight drying at 37 °C. The remaining free binding sites in the wells were then blocked with 1% bovine serum albumin dissolved in phosphate-buffered saline with 0.5% Tween 20 (PBST) for 2 h at room temperature and washed three times with this buffer. After incubation with a peroxidase-labeled affinity-purified goat anti-rabbit IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), the plates were washed again three times with PBST. The second antibody was pre-equilibrated with phosphate-buffered saline.

Growth Assays—Proteins present in 100-μl aliquots were adsorbed onto 96-well plates by overnight drying at 37 °C. The remaining free binding sites in the wells were then blocked with 1% bovine serum albumin dissolved in phosphate-buffered saline with 0.5% Tween 20 (PBST) for 2 h at room temperature and washed three times with this buffer.

RNA isolation and Northern blot analysis of the RNA samples were carried out as described in Ref. 3. The preparation of total RNA was carried out as described previously by Klagsbrun et al. (20). In brief, 20 μg of total RNA were separated by electrophoresis in 1.6% formaldehyde-agarose gels and then blotted onto nylon membranes.
Schuell). The blots were prehybridized in 6 × SSC (0.09 M NaCl, 0.09 M sodium citrate (pH 7.0)), 0.5% (w/v) SDS, 5 × Denhardt's solution (0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) bovine serum albumin) (GIBCO/BRL) for 4 h at 68 °C and hybridized overnight at 68 °C in hybridization solution (same composition as the prehybridization solution with addition of 0.1 M EDTA (pH 8.0)) (GIBCO/BRL) containing a [α-32P]dCTP-labeled PTN cDNA probe. This probe was prepared by random primed DNA labeling (Boehringer Mannheim). After hybridization, blots were washed once with 1 × SSC and 0.1% SDS for 30 min, once with 0.2 × SSC and 0.1% SDS for 20 min at room temperature, and once for 20 min at 68 °C. Blots were then autoradiographed using intensifying screens at ~70 °C for 2 days.

**Detection of mRNA by RNase Protection Assays**—An [α-32P]UTP-labeled antisense riboprobe was prepared by *in vitro* transcription with SP6 RNA polymerase from linearized pPTNwt plasmid. Of this 832-bp probe, a 551-bp fragment will be protected by PTN mRNA present in the samples. As a loading control a 310-bp probe for the 832-bp probe, a 551-bp fragment will be protected by PTN mRNA, labeling (Boehringer Mannheim). After hybridization, blots were screens at -70 °C for 2 days.

**RESULTS**

We previously purified an 18-kDa heparin-binding growth factor from the conditioned medium of an estrogen receptor-negative human breast cancer cell line MDA-MB-231 (3). The NH2-terminal sequence of the purified protein was homologous to that of PTN purified from normal tissues (see Introduction), and an mRNA coding for PTN was expressed in the MDA-MB-231 cells. This purified protein was growth stimulatory in soft agar for the epithelial cell line SW-13 as well as for normal rat kidney fibroblasts (NRK). To study the function of PTN and to provide evidence that growth stimulatory activities observed with our purified preparation were due to PTN, we decided to clone the cDNA for PTN from MDA-MB-231 cells, express the gene, and study the function of the recombinant product. We opted for expression in human cells, since PTN is secreted after cleavage of a 32-amino acid signal peptide (3) and contains 1 cysteine residues linked by disulfide bridges (8). Our earlier studies indicated that these disulfide links are required for biological activity since treatment with the reducing agent dithiothreitol destroys mitogenic activity of PTN (3). We also produced PTN on a large scale in insect cells using baculovirus as a vector. However, PTN produced in insect cells was biologically inactive in the growth assays (see below).

**Constructs Used in the Expression Studies**

For the studies on PTN function, we transfected into human cell lines a PTN construct with a strong constitutive CMV promoter. Our earlier studies with SW-13 cells and the K-FGF gene (19) and preliminary studies with the human kidney cells 293 had shown that this promoter supports high levels of gene expression in these cell lines. We utilized the commercially available pRc/CMV vector (Fig. 1A) which contains a CMV promoter upstream of a multiple cloning site and a transcription unit for the G-418 resistance gene for selection of stably transfected eukaryotic cells.

A data base search with the PTN cDNA showed that a stretch of the initial 97 nucleotides at the 5' end of PTN is 90.7% homologous to the antisense strand of the 3' end of human heat shock protein 70 cDNA (hs70) (GenBank/EMBL accession number X04677). Furthermore, a stretch of 277 nucleotides at the very 3' end of the PTN cDNA is 98.6% homologous to the antisense 5' end of the human ribosomal protein L7 cDNA (GenBank/EMBL accession number X52967) (see Fig. 1B). To avoid interference of high levels of PTN gene expression with the expression of endogenous hs70 or L7, we decided to include only the open reading frame (ORF) of PTN. Shaded bars in the 5'- and 3' untranslated regions of PTN indicate areas that are homologous to regions in the 3'- untranslated strands of the human cDNAs of hs70 and L7, respectively (see "Results" for details). The start position of the primers used for PCR cDNA cloning is indicated by arrowheads, and the respective nucleotide sequence is given below. The position of the frame shift mutation in the ORF is symbolized by a vertical arrow and the respective nucleotide and deduced amino acid sequence in the wild-type and mutant construct are shown. The full-length PTN cDNA and amino acid sequence are accessible through GenBank/EMBL with the accession numbers M57399 and P21246, respectively.

![Fig. 1. Construct used in the transfection studies (A) and comparison of wild-type and mutant PTN inserts. A](image-url)
cloned into pRc/CMV (Fig. 1A). Sequencing of the PCR product after bacterial amplification and selection of different bacterial clones showed that one construct, pPTNwt, contained the wild-type cDNA with the complete ORF. This ORF of pPTNwt contains 507 nucleotides and is identical to the reported human cDNA sequence of PTN (GenBank/EMBL accession number M57399). One of the bacterial clones picked contained a plasmid with a point mutation in the ORF of PTN. At position +130 (relative to the translation initiation site) an additional A was inserted (see Fig. 1B). This frame shift mutation results in a premature translation stop codon after amino acid 15 of the mature protein. The protein product expected from this frame shift construct is the signal peptide and a 15-mer peptide comprising 11 residues from the NH2 terminus of the mature PTN protein. We used this mutant construct as our negative control in further studies due to the fact that wild-type and mutant expression vectors only differ by a single nucleotide.

Demonstration of Secreted and Biologically Active PTN Protein in Transient Expression Experiments in Human Cells

In one series of experiments we transfected the human embryonal kidney cell line 293 with the wild-type (pPTNwt) and mutant (pPTNmu) constructs described above. CM from transiently transfected 293 cells were used for further analysis of secreted protein products. After in vivo metabolic [35S]cysteine labeling of transfected cells for 24 h, the cell supernatants were collected and passed through heparin-Sepharose columns. The eluted proteins from the columns were separated by SDS-PAGE (Fig. 2A). A labeled protein migrating at about 18 kDa was the only species in this protein affinity-purified material that is present in supernatants from pPTNwt but not from pPTNmu-transfected cells. The apparent molecular mass of this metabolically labeled protein was identical to that of the PTN protein originally purified from supernatants of the MDA-MB-231 breast cancer cells (3). This protein was also recognized in a Western blot using a rabbit polyclonal antiserum against PTN (data not shown). Furthermore, as shown in Fig. 2B, a high amount of PTN immunoreactivity was detected in the CM of pPTNwt-transfected 293 cells and no signal above background with CM from the pPTNmu controls.

Heparin affinity-purified CM collected from the transient expression experiment with 293 cells was also tested for growth-stimulating activity in SW-13, NRK, and endothelial cells. Fig. 2C shows that the CM from pPTNwt relative to pPTNmu-transfected cells stimulates the colony formation of SW-13 and of NRK cells in soft agar as well as the proliferation of endothelial cells from fetal bovine heart (FBHE) and human umbilical veins (HUVEC). Finally, we also tested whether SW-13 cells transiently transfected with pPTNwt would secrete autocrine-acting growth factor by plating them into soft agar. As shown in Fig. 2C, a 4-fold higher colony number in soft agar was observed from the transient pPTNwt versus pPTNmu-transfected SW-13 cells. Obviously, the activity of the transiently secreted growth factor is sufficient to induce an autocrine growth stimulation in this assay. Taken together, these data indicate that biologically active PTN is secreted into the media of human cells after transient pPTNwt transfection.

PTN Expression and Biological Activity after Stable Transfection of Human Cells

In a separate set of experiments we studied the effects of stable PTN transfection to rule out potential artifacts of a transient overexpression. SW-13 cells were chosen for these studies, since they do not form colonies in soft agar unless stimulated by added FGFs or PTN (3). Our earlier studies have also shown that expression of secreted K-FGF in these cells is sufficient to make them clonogenic in vitro and tumorigenic in vivo (19). From the transient transfection stud-
Pleiotrophin Function and Expression

Figure 3. Stable mass transfection of SW-13 cells with pPTNwt and pPTNmu. A, Northern analysis of PTN expression using \(^{32}\)P-labeled PTN probe. Total RNA (30 \(\mu\)g/lane) from parent SW-13 cells (lane 2), SW-13 cells transfected with pPTNwt (lane 3), pPTNmu (lane 4), and the vector without a PTN insert (lane 5) was used. Total RNA from MDA-MB-231 human breast cancer cells (lane 1) is shown for comparison. The bar indicates a size of 100 pm. C, heparin affinity chromatography of conditioned media from pPTNwt (circles)- and pPTNmu (triangles)-transfected SW-13 cells. Conditioned media were prepared and the samples were eluted by a stepwise NaCl gradient. Aliquots of the fractions were assayed for stimulatory activity on parent SW-13 cells in soft agar. FL, flow-through.

whereas a high pPTNmu expressor clonal cell line served as a negative control (see Table I). Tumor formation was checked on a weekly basis.

After 8 weeks no tumors were observed in the animals injected with the high pPTNmu or with the low PTNwt expressing cells (Table I). Three of ten animals injected with the two medium PTNwt expressor cell lines, and all animals

ies, we selected pPTNwt and pPTNmu stable transfectant SW-13 cell lines using G-418, and we obtained several cell lines of clonal origin in addition to the mass-transfected SW-13 population.

Northern Blot—From our construct we expected a 0.8-kb PTN transcript in the pPTN-transfected cells which is clearly distinct from the constitutively expressed PTN mRNA of 1.4 kb in untransfected PTN-positive cells. A Northern blot with RNA from the PTN-positive MDA-MB-231 breast cancer cells indeed shows this latter transcript (Fig. 3A, lane 1) and no transcript in the parent SW-13 cells (lane 2). A high level of a 0.8-kb transcript hybridizing with the PTN probe was observed in the pPTNwt- and pPTNmu-transfected SW-13 cells (lanes 3 and 4), and no signal was seen from SW-13 cells transfected with the vector alone without a PTN insert (lane 5). Obviously the transfected SW-13 cells produce a high steady-state level of mRNA with both the mutant and wild-type PTN constructs, and the transfection itself does not induce transcription of the endogenous gene.

Growth in Soft Agar—Stably transfected SW-13 cells were placed in soft agar and Fig. 3B illustrates that cells harboring pPTNwt grow large colonies relative to the pPTNmu cells. Stable expression of PTN obviously leads to a phenotype of SW-13 cells that can grow anchorage-independently due to an autocrine action of the growth factor.

Heparin affinity chromatography of conditioned media from the stable pPTNwt and pPTNmu-transfected SW-13 cells was carried out to compare the affinity profile with that of native PTN originally purified from supernatants of MDA-MB-231 cells (3). We used the same step gradient of 0.4, 0.9, and 2 M NaCl employed previously to elute growth factor activity from the column. As can be seen from Fig. 3C, pPTNwt-transfected SW-13 cells release a heparin-binding growth factor into their media that is eluted by 0.9 M NaCl. This heparin affinity profile is identical to that of the original PTN purified from MDA-MB-231 cells. It is clearly distinct from acidic FGF and basic FGF which require higher salt concentrations for their elution (3).

Clonal Cell Lines—G-418 resistant clonal SW-13 cell lines transfected with pPTNwt or pPTNmu were generated and expanded to assay for PTN gene expression, biological activity of the secreted product, and for tumorigenicity in athymic nude mice. Fig. 4 shows a dose-response curve of conditioned media from three different clonal cell lines that express similar high levels of PTNwt or PTNmu mRNA. As is apparent, only the pPTNwt expressing cells secrete soft agar growth-stimulating activity for the parent SW-13 cells into their media. These experiments with stable transfections lend additional support to the growth factor activity of PTN shown above in the transient transfection studies.

Tumorigenicity of SW-13 Cells Transfected with PTN

Our earlier studies showed that expression of an autocrine-acting growth factor can make the non-tumorigenic SW-13 cells grow into tumors in athymic nude mice (19, 21). Since SW-13 cells respond to PTN by forming colonies in soft agar (see above), we used PTN-transfected SW-13 cell lines to assess the potential role of this growth factor for tumor development in vivo. Different clonal SW-13 cell lines transfected with pPTNwt and pPTNmu were injected subcutaneously into athymic nude mice. We chose clonal cell lines with different levels of PTN expression to test whether tumorigenicity is dependent on the level of PTN gene expression. PTN mRNA steady-state levels of the clonal cell lines were assessed by RNase protection assays. Four clones expressing PTNwt at different levels were chosen as positive controls,
PTN in Tumor Cell Lines and Tumor Tissues

PTN Expression in Cell Lines—Approximately one of four human tumor cell lines surveyed in our studies expressed the PTN gene as assessed by RNase protection assays (Fig. 5 and Table I). Some correlative evidence suggests that PTN gene expression could play a role in invasive and/or metastatic behavior of the cell lines or of the original tumors they were obtained from. One example is the highly tumorigenic and invasive breast cancer clonal cell line T47Dco which does express PTN, whereas the less malignant T47D parent cell line does not. Another example are the two cell lines derived from metastatic melanoma which did express PTN, whereas cell lines derived from melanocytes and from primary melanoma did not express PTN. On the other hand, expression of the PTN gene did not appear to be related to hormonal sensitivity of breast cancer cell lines, although the loss of hormone sensitivity is usually associated with a more malignant phenotype of breast cancer (23). Clinical studies will yield more conclusive evidence as to whether PTN expression in tumors is associated with a more malignant progression of the respective disease.

PTN Expression in Tumors—To address the issue of PTN expression and malignant progression, we have started to screen primary human breast cancer samples for PTN expression using RNase protection assays of freshly frozen tumor tissues. We had access to tumor samples from 27 randomly selected breast cancer patients. Seven of the samples are shown in Fig. 5 (lanes 4-10). As can be seen from the loading control (36B4 mRNA), the amount of RNA used in the different reactions was equivalent. In this experiment, PTN mRNA was detected in three of the samples at a level comparable with that of the MDA-MB-231 breast cancer cell line (cf. lane 11), whereas the other four breast cancer samples were negative for PTN. In our total survey so far we found PTN mRNA expressed in 16 of 27 (52%) of the breast cancer samples. Obviously, PTN is only expressed in a fraction of the tumors, and it remains to be seen in a larger survey whether PTN positive tumors indicate a different prognosis of the respective patient. It is worth pointing out that normal breast tissue, which is present in these tumor samples, does not express PTN at a significant enough level to be picked up by the RNase protection assay.

Finally, we assayed for PTN gene expression in carcinogen-induced mammary carcinoma in rats (Table II). This is a standard animal model of breast cancer and is frequently used to evaluate the efficacy of breast cancer treatments (24, 25). To our surprise all eight tumors studied showed high levels of PTN gene expression as assessed by Northern blot. Studies of other laboratories (26, 27) have shown that in carcinogen-
FIG. 5. Expression of PTN mRNA in human tumor cell lines and breast cancer samples analyzed by RNase protection assays. 32P-Labeled riboprobes for PTN (lane 1) and for the ribosomal protein 36B4 (lane 2) were used. After hybridization of yeast tRNA (lane 3), or total RNA (30 μg; lanes 4–17), the samples were digested with RNase, fractionated on a 6% 8 M urea polyacrylamide gel, and autoradiographed for 2 days. The arrowheads indicate specific protected fragments of PTN (top) and 36B4 (bottom). Lanes 4–10, primary human breast cancer tissues from different patients. Cell lines: lane 11, MDA-MB-231; lane 12, SW-13; lane 13, MCF-7; lane 14, ZR-75-1; lane 15, WM 239A; lane 16, WM 852; lane 17, Hs-578T (for further details see "Materials and Methods" and Table II).

TABLE II

| PTN mRNA expression in cell lines and tumor tissues | Human cell lines | Breast cancer | Prostate cancer | Melanocytes and melanoma | Ovarian carcinoma | Others | Tumor tissues |
| xsi| | | | | | | |
| Origin | Special characteristics | PTN mRNA-positive | PTN mRNA-negative |
| xsi| | | | | | | |
| Human cell lines | Breast cancer | Estrogen receptor-positive | T47Dco | T47D/wt, MCF-7, MCF-7/LY-2, ZR-75-1 |
| | | Estrogen receptor-negative | MDA-MB-231, MDA-MB-361, Hs-578T | BT-474, BT-549, MDA-MB-134, MDA-MB-435, MDA-MB-453, MDA-MB-468, SK-BR-3 |
| | Prostate cancer | Melanocytes, primary melanoma, metastatic melanoma | PC-3 | DU-145, LNCaP, LNCaP/H26 Melanocytes, 39, D |
| | Ovarian carcinoma | Epidermoid carcinoma, leukemia, rhabdomyosarcoma, stomach cancer | A1827, PA-1 | A2780, OVCAR-2, -3, -4 |
| | Others | | A-431 | A-204 |
| | Tumor tissues | Human primary breast cancer | n = 16 | n = 11 |
| | Rat carcinogen-induced mammary cancer | n = 8 | n = 0 | HGT-1, KATO III |

induced tumors, neoangiogenesis is one of the early signs of malignant growth, and we are currently investigating whether activation of PTN gene transcription and of angiogenesis in the tumorous lesions coincide.

DISCUSSION

Our current studies focus on the biological activities of the heparin-binding growth factor PTN and its potential as a tumor growth factor. Originally PTN was purified from developing rat brain and bovine uterine tissues and preliminary NH2-terminal amino acid sequence data of PTN were reported in 1989 (9, 12). It was postulated by different laboratories (11, 14, 28) that PTN is a protein that functions during neuronal growth and development, but other data indicated that PTN also acts as a mitogen for fibroblasts (11) and for endothelial cells (5). We were the first to report the purification of PTN as a growth factor from the tissue culture supernatants of a human cancer cell line (MDA-MB-231), and we also detected active PTN in supernatants from human melanoma cells (3). We therefore postulated that PTN may play a role in the promotion of tumor growth in addition to its presumed function during neuronal growth (3).

Very recently the mitogenic activities of PTN have been questioned by different laboratories (14–17), and we will discuss this point first. In our purification protocol that resulted in the detection of PTN in conditioned media from tumor cells, we used a soft agar cloning assay to select chromographic fractions containing biologically active growth factor (3). Although the major protein in that fraction was identified as PTN by NH2-terminal sequencing, it is conceivable that other contaminating minor proteins could contribute in part or completely to the biological activity observed. It should, however, be noted that none of the known growth factors for SW-13 cells (19) were detected by us in the purified PTN preparation by immunoblots (3), and these considerations led us to believe that PTN was the active growth factor present in the purified material.

The final proof that PTN acts as a growth factor can only stem from production of recombinant material and our current study provides this evidence. The autocrine activity of PTN is apparent from experiments with SW-13 cells transfected with PTN (Figs. 2C and 3B). The paracrine activity of PTN is demonstrated in studies in which PTN is harvested from the supernatants of transfected cells and added back onto different cell lines to study its mitogenic or soft agar colony stimulating effects (Fig. 2C, 3B, and 4). We show that
transient expression of PTN in human kidney cells as well as SW-13 cells results in the secretion of immunoreactive and biologically active PTN from these cells (Fig. 2). The growth factor activity was not only assessed with SW-13 cells but also using NRK fibroblasts and two different endothelial cell lines. Furthermore, stable transfection and selection of clonal SW-13 cells gave the same result, namely that PTN functions as a secreted growth factor (Figs. 3 and 4). A final experimental proof that PTN can act as a growth factor in tumors are results from animal studies which show that clonal SW-13 cells expressing PTN form tumors in athymic nude mice (Table I). It should be emphasized that in all of these studies, our negative controls were cells expressing PTN with a frame shift point mutation leading to a truncated product (Fig. 1B).

We conclude that PTN acts as a tumor growth factor and that its mitogenic activities on endothelial cells make it a candidate tumor angiogenesis factor.

These results obtained with PTN expressed in human cells are in contrast with those obtained with recombinant PTN protein from insect cells (see "Results" and Ref. 16) as well as from bacteria (17). The failure to obtain biologically active protein from bacterial expression systems may be due to improper processing of this protein in prokaryotes or due to the loss of activity during extraction under the harsh conditions required to harvest the recombinant protein (see Ref. 22). This reasoning does, however, not suffice to explain the negative data obtained with expression of PTN in insect cells. In agreement with data published very recently from the laboratory of H. Rauvala (16), we found PTN properly secreted into the media of insect cells infected with recombinant baculovirus carrying the PTN gene (see "Results"). This recombinant material was, however, not active as a growth factor, and we conclude that this expression system is not adequate to obtain large quantities of biologically active material for further studies. We wish to emphasize that the lack of biological activity of proteins obtained from the baculovirus expression system is not uncommon and different laboratories have attributed this to poor processing of the protein (29), improper folding (30, 31), or to inadequate posttranslational modifications (phosphorylation (32, 33) or glycosylation (34). We are currently testing if the recombinant PTN from the baculovirus system can be activated by refolding the protein (22, 30) or by removing potential posttranslational modifications with phosphatases (35) or with glycosidases. It is worth pointing out that at extremely high PTN concentrations of about 1 μg/ml from insect cells (16) and from bacterial expression (17) some neurite-outgrowth and minor mitogenic activity was actually observed. This activity could be due to traces of active PTN present in these preparations and makes it worthwhile trying to recover a fully active preparation. Alternatively, transfection with pPTNwt provides high levels of PTN in supernatants from human SW-13 cells (2–10 ng/ml; see Fig. 4) and could be used as a source for recombinant protein. These levels are about 10-fold above those of conditioned media from the breast cancer cell line MDA-MB-231 originally used in our purification of PTN (3).

An aspect closely related to the above discussion of the biological activity of PTN is the potential significance of PTN gene expression in human cancer growth. Our survey of different human cell lines showed that PTN is expressed in about one fourth of the cell lines and it appears as if gene expression might be related to a more malignant phenotype (Table II and Fig. 5). This is true for the comparison between cell lines derived from melanocytes and from primary melanoma versus metastatic melanoma (36, 37) as well as for the comparison between T-47Dwt and the more invasive and tumorigenic T-47Dco subclone (see Table II). Whether or not PTN expression supports a more malignant phenotype is currently being addressed by expressing the gene in PTN-negative tumor cell lines.

We have expanded our studies with cell lines to tissues from primary human tumors and found 16 of 27 breast cancer samples positive for PTN (Table II, Fig. 5). Since breast cancer samples contain mixtures of normal and of tumor tissue, the fact that 11 of the samples were negative for PTN mRNA indicate that PTN is not present in normal breast tissue at levels high enough to be detected with RNase protection assays. PTN detected in the other 16 breast cancer samples is thus most likely derived from the tumor cells and not from activated normal host parenchyme. In a pilot study with four lung cancer samples, we found all of them highly positive for PTN mRNA in contrast to five samples from normal lung that showed no or very faint bands in the RNase protection assay. In line with the observations in human tumor tissues, carcinogen-induced rat mammary carcinoma showed PTN mRNA in eight of eight samples (Table II). The data with the carcinogen-induced rat mammary cancer samples indicate that the PTN gene is turned on during the transforming events, and it is tempting to speculate that PTN gene expression is the switch from the nonangiogenic to the angiogenic phenotype (27, 38, 39). Careful studies monitoring tumor angiogenesis and PTN gene expression after carcinogen exposure in this model will be required to obtain conclusive evidence. Finally, whether or not PTN gene expression in human cancer indicates a less favorable outcome of the disease will require a larger number of samples from patients with longterm clinical follow-up.

CONCLUSION

We demonstrate that PTN functions as a tumor growth factor in vivo, stimulates endothelial, fibroblast, and epithelial cells in vitro, and may contribute to a more aggressive phenotype of tumors. Further clinical studies will have to show if the PTN gene expression observed by us in a small set of human tumors could signify a more malignant phenotype.

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