Human Breast Cancer Growth Inhibited in vivo by a Dominant Negative Pleiotrophin Mutant*

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Pleiotrophin (PTN) is a recently described 18-kDa heparin binding growth/differentiation factor. It also is a proto-oncogene; cells transformed by the Ptn gene form highly angiogenic tumors when implanted into the nude mouse. PTN may be an important regulator of transformation in other tumors, because constitutively high levels of expression of the pleiotrophin (Ptn) gene are found in human breast cancer and other malignant cell lines, and its levels of expression are high in many human tumor specimens. To determine whether PTN is an important regulator of the malignant phenotype of human breast cancer cells, we constructed a mutant cDNA to encode a truncated PTN designed to heterodimerize with the product of the endogenous Ptn gene during processing. The mutant gene product blocked transformation of NIH 3T3 cells by the wild type (wt) Ptn gene product. The mutant Ptn cDNA was then introduced into human breast cancer MDA-MB-231 cells, and clonal lines that stably express the mutant Ptn cDNA were selected. The truncated PTN was shown to form heterodimers with the endogenous Ptn gene product in these cells. Furthermore, the MDA-MB-231 cells that express the mutant Ptn gene were no longer transformed; they failed to form plaques or colonies in soft agar and were unable to form tumors in the athymic nude mouse. The results establish an important role of PTN in the dysregulated growth of human breast cancer cells and suggest that constitutive expression of PTN may be essential to the malignant phenotype of human breast cancers in vivo.

Pleiotrophin (PTN) is an 18-kDa heparin-binding protein that was purified from bovine uterus and from neonatal rat brain (1, 2). The Ptn cDNA (3, 4) encodes a lysine-rich, highly basic protein of 168 amino acids with a 32-amino acid signal sequence that is highly conserved in bovine, rat, human, and chicken (3, 5). PTN exhibits nearly 50% amino acid sequence identity and perfect conservation of 10 cysteine residues with the protein product of Mdk, a gene expressed in the early stages of retinoic acid-induced differentiation of mouse embryonal carcinoma cells (6), and with RI-HB, a protein isolated from chick basement membranes (7), linking Ptn with Mdk as a member of a new family of developmentally regulated genes (3). PTN promotes cell growth of NIH 3T3 and NRK fibroblasts (1, 3), endothelial cells (8–10), epithelial cells (9, 11), and osteoblasts (12–14). It induces neurite outgrowth from neuronal cells (1, 2) and process outgrowth from oligodendrocyte progenitors (15) and is angiogenic in the rabbit corneal pocket assay (8), suggesting that PTN may have a central role in regulating growth of cells of different lineages and organ systems. The importance of PTN in malignant cell growth was first established when introduction of the exogenous Ptn gene into NIH 3T3 and NRK cells led to morphological transformation, anchorage independent growth, and tumor formation with significant neovascularization in vivo in the nude mouse (16). It was shown subsequently that SW13 cells transformed by Ptn also develop highly vascular tumors in the flanks of athymic nude mice (9), that high levels of the Ptn gene are expressed in specimens of many human tumors, including neuroblastoma, glioblastoma, prostate cancer, lung cancer, and Wilms’ tumor, and that many breast cancer cells and about one-fourth of over 40 human tumor cell lines of different origins also express the Ptn gene in significant levels (9, 17, 18). These findings, strengthened by observations that the transformed phenotype of human melanoma cells (WM852) can be suppressed specifically by Ptn-targeted ribozymes (19, 20), strongly support the view that PTN has a central regulatory role in neoplastic cell growth. To directly test the role of PTN in human tumor cell lines, we constructed a mutant Ptn cDNA to encode a truncated PTN designed to heterodimerize with the wt Ptn gene during processing and thus to act as a dominant negative effector of PTN.

In this manuscript, we establish that expression of the truncated PTN prevents transformation of NIH 3T3 cells by wt PTN and that the mutant PTN forms heterodimers with PTN itself. We then demonstrate that expression of the truncated PTN reverts the transformed phenotype of clonally selected human breast cancer (MDA-MB-231) cells that constitutively express significant levels of the endogenous Ptn gene. The results establish that high levels of expression of PTN appear to be essential to maintain the transformed phenotype of MDA-MB-231 cells and that the Ptn gene may be a central regulator of the dysregulated growth of many human tumors.

MATERIALS AND METHODS

Mouse Ptn Mutant Gene Construct—A 345-base pair (bp) cDNA fragment containing 111-bp 5’-untranslated sequences and the sequences to encode mouse Ptn residues 32 to +40 was fused in frame with an 18-bp DNA fragment encoding 6 histidine residues and cloned into the Pet/I/BoaI site downstream from the p6K promoter of the eukaryotic expression vector pPGK-Neo-hpA (21). The resultant plasmid was designated pMMutPtn-1. The human wild type (wt) Ptn expression vector, pHpPtn, containing a full-length human Ptn cDNA fragment, was cloned into the Kpn/I/BoaRI site of pAGE103 vector driven by SV40 promoter as described previously (16). Amino acid residues 1–40 (after cleavage of the signal peptide) of mouse and human Ptn are identical, and thus the truncated PTN is equally effective in mouse and human lines.

Cell and DNA Transfection—Mouse NIH 3T3 and human breast
cancer (MDA-MB-231) cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum and 10% fetal bovine serum, respectively. 1 × 10^6 cells per dish were seeded on 100-mm culture dishes 20 h before transfection with 15 μg of pHuPtPn plus 15 μg of pBluescript II, 15 μg of pMuMutPtn-1 plus 15 μg of pHuPtPn, or 15 μg of the empty pAGE103 vector plus 15 μg of pBluescript II into NIH 3T3 cells as indicated, using a modified calcium phosphate precipitation technique (22). Cells were transfected on three separate occasions with DNA from three separate extractions. The results were effectively the same. After 3 days, cells were selected with neomycin (Life Technologies, Inc.) at 700 μg/ml and media were changed every 3 days until foci appeared. Cells from established foci were pooled and subsequently assayed for focus formation and anchorage-independent growth in soft agar.

MDA-MB-231 cells were transfected with the Lipofectin method (23) and selected with G418 at 500 μg/ml. Media were changed every 3 days until colonies formed. These clonal cell lines were selected by mechanical agitation, expanded for later use, and designated HBC (human breast cancer) Ptn-1, -2, and -3. The controls that lacked the Ptn mutant constructs ("empty" vector) were designated as HBC vector 1, 2, and 3.

**Northern Blot Analysis—** Total RNA was isolated by the guanidinium-thiocyanate method (24). Twenty μg of total RNA (20 μg) was separated on 1.2% formaldehyde-agarose gels, blotted onto nylon membranes (Hyalon, Micron Separations, Westboro, MA), prehybridized, and hybridized with an [α-32P]PdCTP-labeled SmaI fragment of mouse Ptn cDNA. After overnight hybridization, blots were washed twice with 0.5% SSC and 0.1% SDS for 20 min at room temperature and once for 20 min at 68 °C and autoradiographed with intensifying screens at -70 °C.

**Western Blot Analysis, Heterodimer Detection—** To detect heterodimers of mutant PTN and wt PTN, clonally selected MDA-MB-231 cells that harbored pMuMutPtn-1 or vector alone were lysed in 0.8 ml of cell lysis buffer (10 mM Hepes, pH 7.2, 142.5 mM KCl, 1 mM EGTA, 5 mM MgCl2, and 0.5% Nonidet P-40) per 100-mm dish for 30 min. After centrifugation, 25 μl of Ni-NTA Sepharose beads (Qiagen) were added to the supernatants, incubated at 4 °C for 24 h, washed 3 times with Tris-buffered saline containing 0.1% Tween 20 (TSBT, pH 7.35), and eluted with 15–30 μl of SDS-gel loading buffer. Eluates were separated by 12.5% SDS-polycrylamide gel electrophoresis, transferred to Immobilon membranes (Millipore), immunoblotted with an anti-histidine "tag" monoclonal antibody (1:250, Novagen), and visualized by the ECL system (Amersham) according to the manufacturer's instructions. To test whether the PTN mutant protein forms heterodimers with wild-type PTN of MDA-MB-231 cells, the same blot was stripped and probed with a chicken anti-human PTN antibody under the same conditions as above to establish that the human wt PTN molecule was isolated with the truncated mutant PTN using the affinity isolation technique specific for the His-tagged mutant PTN.

**Antibodies—** Chicken anti-human PTN antibodies were prepared using recombinant human PTN expressed in Escherichia coli as antigen and purified as described (1). It is used at a dilution of 1:500.

**Focus Formation, Soft Agar Assay, and Tumor Formation in Nude Mice—** For focus formation assays, 2 × 10^5 NIH 3T3 cells transfectedants were pooled and plated in triplicate onto 60-mm dishes. Media were changed every 3 days. After 16 days, cultures were stained with crystal violet, and the foci on each dish were photographed and counted. To test anchorage-independent growth on soft agar, 5 × 10^4 cells from the pooled NIH 3T3 or clonally selected MDA-MB-231 cell lines were suspended in 5 ml of agar containing Dulbecco's modified Eagle's medium, 10% calf serum or 10% fetal calf serum and were overlaid over 0.7% agar in 60-mm dishes. After 16 days, colonies with more than 20 cells were scored as positive using an inverted microscope equipped with a measuring grid.

For tumor formation, female athymic nude mice (Ncr nu/nu; Harlan Sprague Dawley, Indianapolis, IN) were injected subcutaneously in each flank with 2 × 10^6 cells from the above 6 clonally selected cell lines, and tumor size was measured daily, starting at 10 days following the injections.

**RESULTS**

The Mutant Ptn Gene Product Prevents Transformation of NIH 3T3 Cells by the Full-length Ptn cDNA—PTN contains 10 cysteine residues that establish intramolecular disulfide bonds (25). We therefore constructed a mutant cDNA (pMuMutPtn-1) to encode mouse PTN amino acid residues -32 to +40 that likely would establish non-functional heterodimers with the product of the wt Ptn cDNA (wtHuPtPn) after cleavage of the signal sequence and tested the ability of the mutant cDNA product to inhibit transformation of NIH 3T3 cells by PTN. Northern blots established that the NIH 3T3 cells that were transfected with wt HuPtPn alone or with wt HuPtPn and pMuMutPtn-1 expressed both genes at essentially equal levels (data not shown).

Cells that were co-transfected with wt HuPtPn and pMuMutPtn-1 together were normal in appearance and growth rate. The growth characteristics and appearance of these cells did not differ from cells transfected with the empty vector or from non-transfected NIH 3T3 cells. In contrast, cells transfected with the wt HuPtPn construct alone were typically transformed; they had a higher growth rate and were clustered, highly refractile, and spindle-shaped in appearance. At confluence, these cells grew to a density ∼2-fold higher than the cells co-transfected with the wt HuPtPn and pMuMutPtn-1 plasmids together or cells co-transfected with the empty vector alone.

Cells that were transfected with the wt HuPtPn construct had readily detected foci after 16 days. However, the number of foci was dramatically reduced in NIH 3T3 cells co-transfected with both the wt HuPtPn and pMuMutPtn-1 constructs as scored in Table I. However, when lysates were prepared from the foci that developed from cells that were co-transfected with wt and mutant Ptn constructs and analyzed in Northern blots, transcripts of the exogenous Ptn mutant gene were not detected (data not shown), indicating that the mutant gene was not expressed at detectable levels. Although these foci were scored as observed, a (0) was placed after the number of foci observed in Table I to indicate that in the foci examined by Northern blots, the mutant Ptn transcripts were not detected; it is presumed that these foci arose from clonally selected cells that express the neomycin resistance gene and the wt Ptn cDNA but failed to express the mutant Ptn cDNA.

In soft agar assays, expression of the mutant Ptn gene also effectively prevented transformation by the wt Ptn gene (Table I). Colonies that arose from the clonally selected cells transfected with both wt Ptn and mutant Ptn cDNAs were analyzed by Northern blots as above, in no instance were transcripts of the mutant Ptn cDNA detected. These colonies were scored as observed; the (0) following the number of colonies indicates that in the colonies tested, none expressed the mutant Ptn cDNA in detectable levels.

The data suggest that the truncated PTN product of pMuMutPtn-1 functions as a true dominant negative effector of PTN and establish that PTN-dependent transformation in both focus and clonary formation assays is effectively prevented by expression of the Ptn mutant cDNA to encode PTN residues 1–40.

The Dominant Negative Ptn Effector Reverses the Malignant Phenotype of MDA-MB-231 Cells in Culture and in Vivo—MDA-MB-231 cells are derived from a highly malignant human
breast cancer and constitutively express the endogenous Ptn gene in high levels (9), raising the important question whether the endogenous Ptn gene and the PTN signaling pathway have an important regulatory role in the aggressive growth of these cells. To test the mutant Ptn gene construct in this highly transformed cell line, we introduced pMMutPtn-1 into MDA-MB-231 cells and isolated, expanded, and analyzed G418-resistant clones by Northern blots (Fig. 1A). The exogenous Ptn mRNA (~1.0 kilobase) is easily differentiated from the endogenous Ptn mRNA (~1.5 kilobases). Clones HBC Ptn-1 and HBC Ptn-2 express high levels of the mutant Ptn gene whereas it was not detected in HBC Ptn-3 or in cell lines that harbor the pGK-Neo-pba vector alone (HBC vectors 1, 2, and 3). Since the mouse PTN mutant protein was fused with a six-histidine “tag” at its C terminus, the mutant protein is readily detected with a His-tag monoclonal antibody. As shown in Fig. 1B, only HBC Ptn-1 and HBC Ptn-2 expressed the mutant PTN protein. Clones transfected with the neomycin resistance gene alone and clone HBC Ptn-3, which failed to express the mutant Ptn gene, also failed to express the protein in Western blots probed with the anti-6× histidine tag monoclonal antibody.

All six of the clonally selected, stably transfected MDA-MB-231 cells were expanded and placed in soft agar. Cells harboring pMMutPtn-1 and pMMutPtn-2 formed substantially fewer colonies in soft agar (Table II). These colonies also were substantially smaller than colonies of HBC Ptn-3 in soft agar. The results indicate that the dominant negative PTN effector greatly attenuates focus formation and anchorage-independent growth of MDA-MB-231 cells and suggest that the PTN signaling pathway has an important and perhaps essential regulatory role in the ability of these human tumor cells to sustain the transformed phenotype.

The clones that stably express mutant PTN(1–40) were also tested for their ability to form tumors in the nude mouse. The two clones that expressed the mutant PTN(1–40) protein at high levels (HBC Ptn-1 and HBC Ptn-2) were compared with HBC Ptn-3 cells and with cells expressing vector alone (HBC vector 1, 2, and 3); after 6 weeks, tumors at the sites of injection of MDA-MB-231 cells that express mutant PTN(1–40) were strikingly smaller than tumors in animals injected with control MDA-MB-231 cells that stably express the neomycin gene alone (Table II). These results indicate that the ability of MDA-MB-231 cells to form tumors in vivo in the nude mouse is markedly attenuated by the dominant negative PTN effector.

The results confirm the results of the tumorigenic assays in soft agar and argue strongly that in MDA-MB-231 cells, PTN has a central regulatory role in maintaining the transformed phenotype in vivo.

Heterodimers of wt PTN and Ptn Mutant 1–40 Were Detected in pMMutPtn-1 Stably Transfected MDA-MB-231 Cells—Fully processed PTN is a monomer with internal cysteine residues −15 and −44, −23 and −53, −30 and −57, −67 and −99, and −77 and −109 linked as disulfide bonds (25). The dominant negative cDNA construct was designed to encode residues −32 to +40. There are 3 cysteine residues (+15, +23, +30) in the truncated PTN product; it was anticipated that these residues would dimerize with the wt PTN gene product during processing.

To determine if the mutant PTN protein establishes heterodimers with wt PTN in MDA-MB-231 cells that stably express the mutant Ptn gene, cell lysates were incubated with Ni-NTA to “capture” the His-tagged PTN mutant, and eluates were prepared and analyzed by Western blotting with chicken polyclonal anti-human PTN antibodies. 2 The chicken polyclonal anti-human PTN antibody recognized the endogenous (wt) PTN in lanes in which lysates of HBC clones Ptn-1 and Ptn-2 were analyzed (Fig. 2). When the membranes used in Western blotting were stripped and reprobed with the anti-His tag antisera, the truncated PTN (data not shown) was recognized as well, indicating that the Ni-NTA captured both the mutant His-tagged truncated PTN and the wt PTN together.

As anticipated, endogenous (wt) PTN was not “captured” with the His-tagged PTN mutant in lysates of HBC Ptn-3 or lysates of the control HBC vector 1, 2, and 3 cells, suggesting that the Ni-NTA column effectively captured the His-tagged mutant PTN as a heterodimer with wt PTN. These results thus indicate that the His-tagged mutant PTN(1–40) associates with wt PTN to form heterodimers in MDA-MB-231 cells and suggest that the mechanism of the dominant negative effect of the truncated PTN(1–40) is the formation of non-functional heterodimers with wt PTN.

| Stably transfected MDA-MB-231 cell line | Colonies in soft agar assay | Tumor size |
|-----------------------------------------|-----------------------------|------------|
| HBC Ptn-1                               | −0.8                        | 10 ± 4     |
| HBC Ptn-2                               | −0.75                       | 15 ± 8     |
| HBC Ptn-3                               | −8.5                        | 65 ± 12    |
| HBC vector 1                            | −8.2                        | 70 ± 8     |
| HBC vector 2                            | −7.9                        | 68 ± 10    |
| HBC vector 3                            | −8.1                        | 61 ± 14    |

2 N. Zhang, R. Zhong, Z.-Y. Wang, and T. F. Deuel, unpublished data.
Fig. 2. Detection of heterodimers of mutant and wild type PTN. Lysates from stably transfected MDA-MB-231 cells were mixed with Ni-NTA beads to bind His-tagged PTN mutant. The bound proteins were eluted in sample buffer and immunoblotted with chicken anti-human PTN antibody after SDS-polyacrylamide gel electrophoresis. The product of the endogenous PTN gene is indicated by the arrow. Lane 1, HBC vector 3 cells; 2, HBC vector 2 cells; 3, HBC vector 1 cells; 4, HBC Ptn-3 cells; 5, HBC Ptn-2 cells; 6, HBC Ptn-1 cells.

DISCUSSION

The data demonstrate that the mutant Ptn cDNA to encode PTN residues −32 to +40 functions as a dominant negative effector of wt PTN. The mutant PTN-(1–40) effectively prevents transformation of NIH 3T3 cells by wt PTN and effectively reverses the malignant phenotype of the human breast cancer MDA-MB-231 cell line that constitutively expresses high levels of the endogenous PTN gene. Finally, the data establish that mutant PTN-(1–40) forms heterodimers with wt PTN, suggesting that the mechanism of the dominant negative effect of PTN-(1–40) is to establish non-functional complexes presumably through disulfide interactions with wt PTN during processing. Because Mdk retains perfect conservation of cysteine residues with PTN (3), it may also be possible for Mdk to heterodimerize with the truncated PTN and be inactivated through heterodimerization. To further establish the specificity of the mutant PTN for PTN itself in MDA-MB-231 cells, Northern blots were prepared and Mdk transcripts were not found (data not shown). This result is consistent with published experiments that established a negative correlation of the levels of expression of the Mdk gene as rat mammary tumors progress in malignancy (26).

The most important conclusion of this work is the dependence of a highly malignant human breast cancer cell line on the constitutive high expression of PTN and PTN-mediated signal transduction to maintain the transformed phenotype in culture and in vivo. The ability to block transformation with the dominant negative mutant suggests a central role of the PTN signaling system in tumorigenesis and supports the contention that this signaling system may be a major regulator of neoplastic transformation in the highly malignant MDA-MD-231 human breast cancer cell line. The results suggest that expression of the Ptn gene and its signaling pathway in appropriately high levels may play a crucial regulatory role in many neoplasms of diverse origins. This potentially highly important regulatory role of PTN in transformation is consistent with previous results that established that the Ptn gene itself directly transforms NIH 3T3 cells (16) and that a Ptn ribozyme construct reverses transformation of human melanoma cells in culture (19, 20). In addition to breast cancer, Ptn mRNA is also highly expressed in a large number of samples of other human tumors including neuroblastoma, glioblastoma, prostate cancer, lung cancer, and Wilms’ tumor, and in over 10 tumor cell lines derived from human tumors (9, 17, 18). The identification of PTN and its signaling pathway as an important regulator molecule that is specific and crucial for solid tumor progression in vivo suggests a number of strategies for clinical applications in which PTN is a promising target for the development of specific anti-tumor drugs.

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