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The potential role of the heparin-binding growth factor pleiotrophin in breast cancer

Anna Tate Riegel and Anton Wellstein
Vincent T. Lombardi Cancer Center and Department of Pharmacology, Georgetown University, Washington, DC 20007, USA

Summary

We propose that the secreted protein pleiotrophin (PTN) is a major factor in the malignant progression of breast cancer. This hypothesis is based on the growth-stimulatory effects of PTN on cells *in vitro* and *in vivo* and on its high levels of expression in 60% of tumor samples from breast cancer patients. The stimulation of proliferation and tube formation of endothelial cells by PTN suggests that it can serve as an angiogenesis factor during tumor growth. We hypothesize that PTN has the potential to support growth of breast cancer at its primary site and to enhance the ability of tumor cells to metastasize. Furthermore, we suggest that specific endocrine signals interact to regulate the expression of PTN *in vitro* and *in vivo*. Finally, we propose that understanding the functions of PTN and its hormonal regulation can lead to the development of novel therapeutic strategies for breast cancer.

The general role of polypeptide growth factors in the growth of breast cancer

Breast cancer tissues consist of a mixture of autonomously proliferating tumor cells and supportive normal tissue recruited by them. Generally speaking, the primary carcinogenic events lead to uncontrolled growth of transformed cells. The secondary development into breast cancer then induces and requires a network of growth signals between the tumor cells and the normal surrounding host tissue. Different polypeptide growth factors released from the tumor cells are believed to trigger these host responses and thus appear to be important for the growth of malignant tumors (reviewed e.g. in [1, 2]).

Physiologically, the expression of polypeptide growth factors is tightly regulated. They have been shown to play important roles in the timely development of tissues during embryonal and neonatal growth. However, 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 stimulation of the tumor cells themselves as well as to paracrine stimuli for the surrounding host tissues (reviewed for breast cancer e.g. in [3–5]; for a general review see [1,2]). Furthermore, hormones that promote breast cancer growth can induce expression of polypeptide growth factors. These growth factors can in turn positively or negatively modulate the hormone action locally (e.g. Refs. [6–9]; reviewed in [3–5]). Therefore expression of a growth factor could supplement for a lack of hormonal responsiveness of hormonal stimulation and thus could make a tumor insensitive to anti-hormone treatment (reviewed e.g. in [3, 5]).

Address for offprints: A. Wellstein, Department of Pharmacology, Georgetown University, 3900 Reservoir Road NW, Washington, DC 20007, USA

What do we know about the growth factor pleiotrophin (PTN)?

PTN is a secreted protein that belongs to a novel family of heparin-binding proteins which include the structurally related midkine protein [10]. PTN and midkine appear to play a major role in fetal and neonatal brain development. PTN expression is down-regulated to a low level after the perinatal period. However, based on its residual expression in a few distinct areas in the adult brain, it could also play a role in maintenance of the CNS in the adult (for a review see Ref. [11]). PTN does not bear homology to other proteins outside its own family and has been described by different names in the last few years by several laboratories using different tissue sources i.e. HARP [12, 13], HBNF [14, 15] or p18 [16] from bovine brain; HB-GAM from rat brain [17, 18], HBGF-8 [19], OSF-1 from mouse brain [20] and PTN from human placenta and rat brain [21].

The biological activity of the PTN protein has, until recently, been a matter of controversy between different laboratories (reviewed in [11]). Several laboratories described mitogenic activity of PTN purified from different sources for endothelial cells [12, 13, 15] and fibroblasts [14, 15, 19, 21]. 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 [22]. 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 [23–25]. We resolved this controversy by generating recombinant human PTN and testing its activities [26]. We expressed the PTN cDNA in two human cell lines and used a PTN point mutant with a premature translation stop codon as a negative control [26]. We demonstrated that recombinant human PTN stimulates endothelial and SW-13 epithelial cells as well as fibroblasts in the low nanogram/ml range [26]. In addition, we found (unpublished data) that recombinant PTN stimulates neurite outgrowth of primary newborn rat hippocampal cells and induces tube formation of endothelial cells (HUVEC) in matrigel. Due to the numerous biological activities of this protein, we decided to use the name 'pleiotrophin' (suggested by T. Deuel's laboratory [21]).

The structure of the PTN protein is characterized by 5 disulfide bridges that: (i) give this protein a unique three-dimensional structure, (ii) make it resistant to low pH, (iii) make it highly sensitive to reducing conditions and thus (iv) have made it very difficult to generate biologically active, properly folded, recombinant protein in prokaryotes and in non-mammalian eukaryotes [22, 26].

The intron/exon structure of the PTN gene is

Table 1. PTN mRNA in cell lines and tumor tissues. RNase protection assays and/or Northern blots

Origin	Characteristics	PTN+	PTN–
Human breast cancer *cell lines*	Estrogen receptor positive	T-47D/CO MCF-7/ADR	T-47/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
Human primary *breast cancer samples*	Most tumors were > 2 cm	n = 25	n = 19
Rat carcinogen-induced mammary cancer	DMBA treatment	n = 8	n = 0

known with respect to its open reading frame which is spread across more than 50 kb (published by us in Ref. [27] and subsequently by others in [28]). The PTN gene is arranged in at least five exons and four introns. Exon 1 is untranslated and exon 2 codes only for the PTN leader sequence. The transcription start site of the PTN gene is not clearly defined and several groups have proposed start sites that differ by over one hundred base pairs [29–31]. The promoter defined by Li *et al.* [30] has no TATA box but only a CAAT box and an initiator element [30]. A functional analysis of the human PTN promoter region from – 1984 to + 191 relative to this transcription start site defines a region from – 550 to + 191 as having the highest transcriptional activity [30]. These studies were conducted in NIH 3T3 cells and we have obtained similar results in transfection of SW13 cells (unpublished data). However, we have determined that neither of these cell lines express the endogenous PTN gene. Therefore, the promoter elements involved in tissue- and tumor-specific gene transcription have not been defined.

The potential role of PTN in tumor growth was first described by our laboratory [22, 26]. We showed that PTN can serve as a growth factor that supports tumor growth and angiogenesis [26]. Furthermore, we found PTN mRNA expressed in a high proportion (60%) of human tumor samples as well as in about one fourth of over forty human tumor cell lines of different origin (further details with respect to breast cancer are discussed below and see Table 1). Consistent with the pattern of expression in the normal adult animal we did not find the gene expressed in a number of non-tumor cell lines such as endothelial, melanocyte, some epithelial cells and fibroblasts [26]. Finally, expression of wild-type PTN in the non-tumorigenic SW-13 epithelial cells led to development of tumors in athymic nude mice [26]. Other investigators demonstrated recently that expression of PTN can support tumor growth in mice of 3T3-fibroblasts [32]. Recent data from our laboratory (unpublished) show that clonally selected high expressors of PTN from the SW-13/PTN transfectants form lung and liver metastases in the athymic nude mouse model. This provides additional supportive evidence that the PTN gene product can act as a growth factor in tumors.

Pleiotrophin in breast cancer

Expression of PTN

We detected PTN mRNA by Northern blot or RNase protection assay in 60% (25 of 44) of randomly selected human breast cancer samples and in 5 of 15 human breast cancer cell lines (see Table 1). The PTN mRNA levels in the positive breast cancer samples were equivalent or greater than those in the MDA-MB 231 breast cancer cell line from which we originally purified the PTN protein [22, 26] and were easily detected by a two-day exposure of a Northern blot with 20 or 30 μg of total RNA. On the other hand, even after longer exposures we observed no signal in the PTN-negative tumors (or cell lines). We have confirmed this with the more sensitive RNase protection assay [26]. For the tumor cell lines and for some of the cancer samples we also confirmed PTN-negativity by reverse transcriptase-PCR. These data taken together with data from expression studies of PTN strongly indicate a direct role of PTN in breast cancer growth.

Hormonal regulation of breast cancer growth and development

An essential requirement for the development of breast cancer is the presence of circulating steroid hormones (reviewed e.g. in [3]). Consequently one of the most widely used drug therapies of breast cancer is with the anti-estrogen tamoxifen. Thus, an important question regarding PTN is whether gene expression is sensitive to hormones *in vitro* and *in vivo*. PTN belongs to a family of genes that is developmentally regulated and includes the retinoic acid-responsive gene midkine (see e.g. [11]). Possibly the most intriguing studies of PTN will be on the potential interactions between the different nuclear hormone receptors and how these affect PTN gene expression. As we describe in the next paragraph, retinoic acid treatment can up-regulate PTN in breast cancer cells *in vitro* and it is conceivable that interactions between retinoic acid receptor pathways and the estrogen receptor can affect the level

of PTN gene expression. A recent report demonstrated inhibitory interaction by retinoic acid receptor (RXR-β) of estrogen up-regulation of gene expression in breast cancer cells [33]. As regards pleiotrophin gene expression positive and/or negative interactions between retinoids and steroid hormones (estrogen, progesterone and glucocorticoids) will be of interest for the understanding of how PTN is regulated during the development of breast cancer, and of great value for the development of novel therapeutic approaches.

Hormone receptor levels and PTN expression

As a first step in determining if there is hormonal regulation of PTN in breast cancer we have examined PTN and hormone receptor protein levels in breast tumors and in tumor cell lines. A preliminary indication of hormonal regulation of PTN would be a correlation between tumor estrogen receptor (ER)/progesterone receptor (PgR) levels and expression of the growth factor gene. A direct relationship would indicate a positive regulation of PTN, an inverse relationship of PTN and ER/PgR would indicate that the activity of the ER/PgR down regulates PTN. No apparent relationship could mean that the gene is regulated independently from the ER pathway or that other pathways interfere with the regulation by ER.

ER/PgR in vitro

The constitutive expression of PTN in breast cancer cell lines (Table 1) irrespective of ER status suggests that the presence of this hormone receptor does not seem to correlate with expression of PTN. However, it should be noted that the more aggressive and drug-resistant PTN-positive clonal cell lines (MCF-7/ADR = adriamycin resistant; T-47D/CO = Colorado clone) were derived from the wild-type estrogen or progesterone-responsive and PTN-negative MCF-7 and T-47D human breast cancer cells. Whereas the parent (wild-type) cell lines require hormone supplementation to grow into tumors in athymic nude mice, the PTN-positive clones are tumorigenic without hormone-supplementation. Thus an intriguing possibility is that PTN-expression in the more aggressive clones is the cause of their tumorigenic potential. These two isogenic pairs of tumor cell lines (MCF-7 versus MCF-7/ADR and T-47D versus T-47D/CO) provide an excellent model to study hormonal regulation of the PTN gene *in vitro* as well as *in vivo*.

ER/PgR in vivo

For a set of 17 tumor samples from different breast cancer patients we have compared PTN and steroid receptor hormone expression. The data suggest that in PTN-positive tumors the median ER-level is higher than in PTN-negative tumors. However, the median PgR levels were below detection in both PTN- positive and negative tumors. Interestingly, two of the cases that had the highest levels of PTN expression observed were ER and PgR positive. These data from patients' tumor samples suggest a role for the ER in the positive regulation of PTN levels. Further *in vivo* data from studies in rats also indicate that PTN-expression may be affected by estrogenic regulation. We found PTN expressed in all of the DMBA-induced rat breast tumors studied (n = 8; Table 1). The DMBA-induced rat breast tumors are estrogen responsive tumors and their development is dependent on estrogen and inhibited by the anti-estrogen tamoxifen [34].

In conclusion

The *in vivo* data lend support to the hypothesis that ER/PgR co-regulate PTN-expression.

Regulation of PTN expression by treatment of cells with hormones in vitro

To date we have performed only preliminary *in vitro* studies on retinoid effects on PTN mRNA. Interestingly, PTN steady-state mRNA levels were up-regulated 10-fold by treatment of PTN-positive, ER-negative human breast cancer cells (MDA-MB 231) with 10 nM of retinoic acid (all-trans) for 24 to 96 hours (unpublished data). This dose was chosen since it did not affect growth of these cells on the dish surface or in suspension culture (soft agar). On the other hand, parallel treatment of PTN-negative cells (SW-13) with retinoic acid did not activate PTN gene expression. These initial experiments suggest that retinoic acid affects the level of PTN gene expression in PTN-positive cells. The interplay of retinoids and steroid hormones in the regu-

lation of PTN gene expression will be an interesting subject for future studies.

Summary of 'PTN in breast cancer'

Our published data [22, 26, 27] and our preliminary studies as well as the data published by others show:

(1) PTN is a secreted growth factor expressed in a number of human breast cancer cell lines.
(2) PTN stimulates endothelial cells and can act as a tumor angiogenesis factor [12].
(3) PTN can support tumor growth of non-tumorigenic SW-13 cells [32].
(4) PTN mRNA is up-regulated by retinoic acid treatment of a PTN-positive breast cancer cell line.
(5) PTN mRNA is found at high levels in 60% of samples from patients with breast cancer.
(6) PTN is not expressed in normal breast epithelium (40% of the breast cancer samples were negative for PTN despite the fact that they obviously contain breast epithelial tissue).

PTN is obviously a potentially interesting marker of tumor progression as well as a therapeutic target in breast cancer.

References

1. Cross M, Dexter TM: Growth factors in development, transformation, and tumorigenesis. Cell 64: 271–280, 1991
2. Liotta LA, Steeg PS, Stetler-Stevenson WG: Cancer metastasis and angiogenesis: An imbalance of positive and negative regulation. Cell 64: 327–336, 1991
3. Dickson R, Lippman ME: Estrogenic regulation of growth and polypeptide growth factor secretion in human breast carcinoma. Endocr Rev 8: 29–43, 1987
4. Wellstein A, Lippman ME: Fibroblast growth factors and breast cancer. In: Broder S (ed) Molecular Foundations of Oncology. Williams and Wilkins, Baltimore, 1991, pp 403–418
5. Clarke R, Dickson RB, Lippman ME: Hormonal aspects of breast cancer. Growth factors, drugs and stromal interactions. Crit Rev Oncol Hematol 12: 1–23, 1992
6. Dickson RB, McManaway ME, Lippman ME: Estrogen-induced factors of breast cancer cells partially replace estrogen to promote tumor growth. Science 232: 1540–1543, 1986
7. Knabbe C, Lippman ME, Wakefield LM, *et al.*: Evidence that transforming growth factor beta is a hormonally regulated negative growth factor in human breast cancer cells. Cell 48: 417–428, 1987
8. Huff KK, Knabbe C, Lindsey R, *et al.*: Multihormonal regulation of insulin-like growth factor-I-related protein in MCF-7 human breast cancer cells. Mol Endocrinol 2: 200–208, 1988
9. Dickson RB, Huff KK, Spencer EM, Lippman ME: Induction of epidermal growth factor-related polypeptides by 17 beta-estradiol in MCF-7 human breast cancer cells. Endocrinology 118: 138–142, 1986
10. Kadomatsu K, Huang RP, Suganuma T, Murata F, Muramatsu T: A retinoic acid responsive gene MK found in the teratocarcinoma system is expressed in spatially and temporally controlled manner during mouse embryogenesis. J Cell Biol 110: 607–616, 1990
11. Böhlen P, Kovesdi I: HBNF and MK, members of a novel gene family of heparin-binding proteins with potential roles in embryogenesis and brain function. Prog Growth Factor Res 3: 143–157, 1991
12. Courty J, Dauchel MC, Caruelle D, Nguyen TT, Barritault D: Purification and characterization of a new endothelial cell growth factor named HARP (Heparin Affin Regulatory Peptide). J Cell Biochem 15F: Abstr. 221, 1991 (abstract)
13. Courty J, Dauchel MC, Caruelle D, Perderiset M, Barritault D: Mitogenic properties of a new endothelial cell growth factor related to pleiotrophin. Biochem Biophys Res Commun 180: 145–151, 1991
14. Kovesdi I, Fairhurst JL, Kretschmer PJ, Böhlen P: Heparin-binding neurotrophic factor (HBNF) and MK, members of a new family of homologous, developmentally regulated proteins. Biochem Biophys Res Commun 172: 850–854, 1990
15. Huber D, Gautschi-Sova P, Böhlen P: Amino-terminal sequences of a novel heparin-binding protein from human, bovine, rat, and chick brain: high interspecies homology. Neurochem Res 15: 435–439, 1990
16. Kuo MD, Oda Y, Huang JS, Huang SS: Amino acid sequence and characterization of a heparin-binding neurite-promoting factor (p18) from bovine brain. J Biol Chem 265: 18749–18752, 1990
17. Rauvala H: An 18-kd heparin-binding protein of developing brain that is distinct from fibroblast growth factors. EMBO J 8: 2933–2941, 1989
18. Merenmies J, Rauvala H: Molecular cloning of the 18-kDa growth-associated protein of developing brain. J Biol Chem 265: 16721–16724, 1990
19. Milner PG, Li YS, Hoffman RM, Kodner CM, Siegel NR, Deuel TF: A novel 17 kD heparin-binding growth factor (HBGF-8) in bovine uterus: purification and N-terminal amino acid sequence. Biochem Biophys Res Commun 165: 1096–1103, 1989
20. Tezuka K, Takeshita S, Hakeda Y, Kumegawa M, Kikuno R, Hashimoto-Gotoh T: Isolation of mouse and human cDNA clones encoding a protein expressed specifically in osteoblasts and brain tissues. Biochem Biophys Res Commun 173: 246–251, 1990
21. Li YS, Milner PG, Chauhan AK, *et al.*: Cloning and expression of a developmentally regulated protein that induces mi-

togenic and neurite outgrowth activity. Science 250: 1690–1694, 1990

22. Wellstein A, Fang WJ, Khatri A, *et al.*: A heparin-binding growth factor secreted from breast cancer cells homologous to a developmentally regulated cytokine. J Biol Chem 267: 2582–2587, 1992
23. Hampton BS, Marshak DR, Burgess WH: Structural and functional characterization of full-length heparin-binding growth associated molecule. Mol Biol Cell 3: 85–93, 1992
24. Raulo E, Julkunen I, Merenmies J, Pihlaskari R, Rauvala H: Secretion and biological activities of heparin-binding growth-associated molecule. J Biol Chem 267: 11408–11416, 1992
25. Takamatsu H, Itoh M, Kimura M, Gospodarowicz D, Amann E: Expression and purification of biologically active human OSF-1 in *Escherichia coli*. Biochem Biophys Res Commun 185: 224–230, 1992
26. Fang WJ, Hartmann N, Chow D, Riegel AT, Wellstein A: Pleiotrophin stimulates fibroblasts, endothelial and epithelial cells, and is expressed in human cancer. J Biol Chem 267: 25889–25897, 1992
27. Lai SP, Czubayko F, Riegel AT, Wellstein A: Structure of the human heparin-binding growth factor gene pleiotrophin. Biochem Biophys Res Commun 187: 1113–1122, 1992
28. Li YS, Hoffman RM, Le Beau MM, *et al.*: Characterization of the human pleiotrophin gene. J Biol Chem 267: 26011–26016, 1992
29. Milner PG, Shah D, Veile R, Donis-Keller H, Kumar BV: Cloning, nucleotide sequence and chromosome localization of the human pleiotrophin gene. Biochemistry 31: 12023–12028, 1992
30. Li YS, Hoffman RM, Le Beau MM, *et al.*: Characterization of the human pleiotrophin gene. Promoter region and chromosomal localization. J Biol Chem 267: 26011–26016, 1992
31. Kretschmer PJ, Fairhurst JL, Hulmes JD, Popjes ML, Böhlen P, Kovesdi I: Genomic organization of the human HBNF gene and characterization of an HBNF variant protein as a splice mutant. Biochem Biophys Res Commun 192: 420–429, 1993
32. Chauhan AK, Li YS, Deuel TF: Pleiotrophin transforms NIH 3T3 cells and induces tumors in nude mice. Proc Natl Acad Sci USA 90: 679–682, 1993
33. Segrase J, Marks M, Hirschfeld S, *et al.*: Inhibition of estrogen responsive gene activation by the retinoid x receptor beta: evidence for multiple inhibitory pathways. Mol Cell Biol 13: 2258–2268, 1993
34. Gibson DF, Gottardis MM, Jordan VC: Sensitivity and insensitivity of breast cancer to tamoxifen. J Steroid Biochem Mol Biol 37: 765–770, 1990