REVIEW ARTICLE

Roles of pleiotrophin in tumor growth and angiogenesis

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ABSTRACT. Pleiotrophin (PTN) is a heparin-binding growth factor with diverse biological activities, the most studied of these being those related to the nervous system, tumor growth and angiogenesis. Although interest in the involvement of PTN in tumor growth is increasing, many questions remain unanswered, particularly concerning the receptors and the signaling pathways involved. In this review, we briefly introduce PTN, and summarize data on its involvement in tumor growth and angiogenesis, and on what is known to date concerning the receptors and pathways involved.

Keywords: angiogenesis, cancer, endothelial cells, pleiotrophin, receptor protein tyrosine phosphatase β/ζ, integrins

Pleiotrophin (PTN) is a secreted heparin-binding growth factor that takes part in many different processes, such as cell growth and survival, cell migration, angiogenesis and neurite outgrowth. It is also known as heparin-binding growth-associated molecule [1, 2], heparin affin regulatory peptide [3], heparin-binding growth factor-8 [4], protein 18 kDa [5], heparin-binding neurotrophic factor [6, 7] and osteoblast-specific factor [8]. PTN is highly homologous to midkine (MK), with which it shares 45-50% sequence identity, forming a family of growth factors [6, 9]. It is highly conserved across different species: more than 90% identity has been observed among the sequences of chicken, rat, mouse, bovine and human [10], while homologues have been also reported in fish, frogs and insects [11].

HUMAN PTN PROTEIN AND GENE/PROMOTER STRUCTURE

PTN consists of 168 amino acids, the mature peptide having 136 amino acids as a result of cleavage of the signal peptide [12, 13]. The calculated mass of the mature protein was determined by plasma desorption time-of-flight mass spectrometry as 15,291 kDa, but in SDS-PAGE it appears as 18 kDa [2], due to the fact that the molecule is rich in cationic amino acids, mainly lysines, that form random coils at both N- and C-terminal ends [12, 14]. The peptide also contains 10 conserved cysteines that participate in the formation of five disulfide bonds [2, 15, 16], and three potential nuclear targeting sequences K-R/K-X-R /K [2]. PTN does not contain any potential sites for N-glycosylation or other post-translational modifications [1, 2] and its binding to heparin is mediated by the two central regions that are homologous to the thrombospondin type I repeat (TSR-1) [14]. More recent data suggest that the carboxyl terminal TSR-1 domain is the main heparin-binding site of PTN [17]. The human ptn gene has been identified as being on chromosome 7 band q33a, having a minimum size of 42 kDa, and containing at least seven exons. The open reading frame is located on four exons and the boundaries between introns and exons seem to be conserved among species in the PTN/MK family [18]. The signal peptide and the first five amino acids of the mature protein are located in exon 2, while the core region is split into exons 2 and 3, containing six and four cysteine residues respectively. Exon 4 comprises the C-terminus of PTN that contains a putative nuclear translocation signal based on its homology with histone H1 [19]. The 5’ untranslated region is unique in the human ptn gene compared with other species [20], while there may be multiple 5’ untranslated regions derived from alternative splicing [21] that may contribute to cell or tissue-specific regulation of PTN expression [19]. From what is known to date, the promoter of the human ptn gene contains sequences for the binding of several transcription factors, as shown in figure 1.

REGULATION OF PTN EXPRESSION

Although PTN seems to have significant biological functions, little is still known on the regulation of its expres-
Figure 1
Schematic representation of the transcription factor binding sites of the ptn promoter. The human ptn promoter contains a CAAT box, four binding sites for myogenic differentiation 1 (MyoD), one for nuclear factors called GT (GT1), two for activator protein 1 (AP1), one for homeobox A5 (HOXA5) and a serum response element (SRE) [19, 29, 32, 33, 36], all marked with filled squares. Putative binding sites for nuclear factor kappa B (NFκB), cAMP response element binding protein (CBP), serum response factor (CArG box) and retinoic acid receptor (RARE/TRE/VDE) are marked with filled circles [19] and need further investigation. There is also a binding site of Sox10 at the proximal site of the promoter; however, its exact location has not been identified [22]. Potential binding sites have been also suggested for early growth response factor-1 and specificity protein-1 transcription factors [23].

BIOLOGICAL ACTIVITIES OF PTN

The initial reference involving PTN suggested that it plays a role in the maturation and growth of brain [5]. Since then, the importance of PTN in the nervous system has been well described [13, 27, 28, 33, 40-51]. PTN is over-expressed in neurodegenerative diseases [52] and exhibits a protective or/trophic effect on dopaminergic neurons in vitro and in vivo [45, 46, 48, 53].

Regarding the muscular system, PTN is expressed in developing muscle in vivo [1, 13, 54], is up-regulated during in vitro myogenesis and soleus muscle regeneration, and can be found in newly formed myotubes and perfused activated myoblasts [55]. Moreover, PTN mRNA is present in smooth muscle [56] and cardiac muscle cells [57, 58], is down-regulated during postnatal differentiation of the myocardium [58], up-regulated in heart failure [59], and potentiates cardiomyocyte cell death by apoptosis [60].

PTN is expressed in the fetus liver but its expression gradually decreases [61], although it seems to be involved in liver regeneration [32, 61]. PTN is expressed in the developing kidney mesenchyme [57] and induces formation of branching tubules in an immortalized ureteric bud cell line cultured three-dimensionally in an extracellular matrix gel [62]. PTN is detected during lung development and in embryonic bronchial epithelial cells [57], and regulates lung epithelial cell proliferation and differentiation during fetal lung development [63]. It is also normally expressed in the epithelial ridge of cochlea, suggesting a role in auditory function [64].

In females, PTN is expressed in the uterus, and its expression is dependent on the estrous cycle [56, 65], which it seems to affect [65]. In human mammary gland, PTN is detected in alveolar myoepithelial, epithelial, endothelial and vascular smooth muscle cells [66], and its expression is increased in both terminal end bud and mature ducts in the process of mammary branching morphogenesis [67]. PTN is involved in ectopic endometriosis [68], and female mice deficient in both PTN and MK have shown abnormalities of reproduction [65]. In males, PTN plays a role in normal spermatogenesis. It is expressed in Leydig cells of the testis and is up-regulated in both human Peyronie’s and Dupuytren’s disease [69, 70]. Dominant negative PTN mutant male mice show sterility, atrophic testes and strikingly apoptotic spermatocytes [71].
PTN promotes proliferation, differentiation and proper attachment of osteoblasts [72, 73], and induces chemotaxis, proliferation and differentiation of human osteoprogenitor cells, as well as both bone and cartilage formation in athymic mice [74]. It is also involved in angiogenesis in the growth plate of mice [75], and regulates the ectopic bone-inducing activity of rhBMP-2 [76]. Furthermore, PTN is a vital signaling molecule in regulating periosteal bone formation and resorption in response to four-point bending of right tibias in C57BL/6J mice [77]. Interestingly, mice that over-express PTN tend to have increased bone growth [78], and although PTN-deficient mice seem to have normal bone formation [79], they show growth retardation in the weight-bearing bones by two months of age, and osteopenia during adulthood [80]. PTN is found in developing [81] and adult [82] nasal cartilage, and participates in the proteoglycan synthesis in the developing matrix of fetal cartilage [83]. It is an autocrine growth factor in cartilage [25], is increasingly expressed in the early stages of osteoarthritis [25, 84] and an increase at its mRNA levels is provoked by sclerotic, subchondral osteoblasts in osteoarthritic cartilage [85]. Whether PTN improves or deteriorates osteoarthritis is not known to date.

**BIOLOGICAL ACTIVITIES RELATED TO CANCERS**

**Cancer cells in vitro**

A role for PTN in human cancers was suggested after its detection in conditioned media of the highly malignant breast cancer cell line MDA-MB231 [86]. Since then, screening of various human cell lines and tumor specimens revealed that PTN is expressed as an autocrine or paracrine growth factor by various cancer cells, including human breast [87-89], prostate [29, 87, 90, 91], ovarian [87] and lung [92] cancer, chorionicarcinoma [93], melanoma [87, 94], glioblastoma [95-98] and pancreatic carcinoma cells [99]. Multiple myeloma (MM) cell lines and malignant cells from MM patients’ bone marrow produced and secreted PTN into the cell culture supernatants and ptn gene expression correlated with the patients’ disease status. Inhibition of PTN with a polyclonal anti-PTN antibody reduced growth and enhanced apoptosis of MM cell lines and freshly isolated bone marrow tumor cells from MM patients in vitro [100]. PTN mRNA is also selectively detected in the meningioma cells of meningiomas [101], and PTN expression is up-regulated after loss of the tumor suppressor gene PTEN [39]. Interestingly, it has recently been suggested that decrease of PTN expression in U87MG cells induces tetraploidy and aneuploidy, and arrests cells in the G1 phase of the cell cycle, suggesting that PTN signaling may have a critical role in chromosomal segregation and cell cycle progression [102]. Moreover, PTN disrupts cytoskeletal protein complexes, ablates calcium-dependent homophilic cell-cell adhesion, stimulates ubiquitination and degradation of N-cadherin, reorganizes the actin cytoskeleton and induces a morphological epithelial-mesenchymal transition in PTN-stimulated U373 cells [103]. PTN is also involved in hepatocarcinogenesis and has an apoptotic activity against TGFβ1 in hepatoma cell lines [104]. Finally, in line with the notion that PTN may significantly stimulate tumor progression, independently of its effect on the cancer cells themselves, PTN secretion from MCF-7 breast cancer cells stimulates epithelial island formation, activation of stromal fibroblasts, extensive remodeling of the microenvironment and activation of markers of aggressive breast cancer in co-cultures of PTN-expressing MCF-7 and NIH 3T3 cells [105]. It also affects tumor angiogenesis, as discussed more extensively below.

Conversely, there are cases where PTN has been shown to negatively regulate tumor cell growth. For example, PTN mRNA levels are decreased in colorectal cancers compared with those in normal adjacent mucosa [106]. It has been detected in lysates and conditioned medium from contact-arrested NIH 3T3 fibroblasts, but not in cells transformed by the ras oncogene [107], and its expression is up-regulated in confluent compared with actively proliferating cells [108, 109]. Its expression is low or absent in neuroblastomas with a poor prognosis [110], and negatively affects growth and migration of several glioma cells lines [111, 112].

**Tumor angiogenesis in vitro**

Besides a significant role in the biology of tumor cells themselves, PTN seems also to affect the angiogenic potential of tumor cells. Firstly, PTN stimulates angiogenic functions of endothelial cells in vitro [113-117] and induces embryoid body angiogenesis [118] and trans-differentiation of monocytes into functional endothelial cells [119-121]. In the same line, it increases the in vitro angiogenic potential of several tumor cells, such as multiple myeloma [120], breast [122] and prostate [91] cancer cells.

In contrast to a positive regulation of in vitro angiogenesis by PTN, there are also data that support a negative regulation. First of all, PTN directly binds and inhibits the effect of vascular endothelial growth factor (VEGF) on endothelial cell proliferation, migration and tube formation [123, 124]. It also decreases the expression of the VEGF receptor KDR, another mechanism through which it potentially inhibits VEGF angiogenic activities in vitro [125]. Finally, decrease in the expression of endogenous PTN in C6 glioma cells significantly increased the angiogenic potential of these cells in vitro, partially due to increased availability and activity of VEGF [111].

**Tumor growth and angiogenesis in vivo**

Much in vivo data suggest that PTN plays a role in angiogenesis of tumors that grow in nude mice. This was initially shown in NIH-3T3 cells that constitutively over-expressed PTN. When these cells were implanted into the flanks of nude mice, they tended to form tumors with significant neovascularization compared with the mock-transfected cells [126]. In the same line, over-expression of PTN in a human adrenal carcinoma cell line SW13 promotes not only in vivo tumor growth, but also tumor-induced angiogenesis, suggesting that constitutive PTN signaling fully regulates the angiogenic switch [127]. Expression of PTN in breast cancer MCF-
7 cells stimulates tumor growth, remodeling of the microenvironment and tumor-induced angiogenesis in vivo [105, 122]. Ribozyme-mediated depletion of HERV-PTN mRNA in human chorionicarcinoma suggests that PTN is an essential and rate-limiting factor for chorionicarcinoma growth, invasion, and angiogenesis in vivo [93]. Moreover, RNA interference-mediated gene silencing of PTN suppresses glioblastoma growth and angiogenesis in vivo [128]. Finally, PTN antisense expression in human prostate cancer LNCaP cells inhibits LNCaP cell-induced angiogenesis in vivo in the chicken embryo chorioallantoic membrane [91].

On the other hand, there is also evidence that PTN can act as an angiostatic factor. For example, vascularization was significantly decreased in neuroblastoma xenografts that over-express PTN and that are resistant to the DNA-topoisomerase I inhibitor irinotecan [110]. Similarly, PTN antisense expression in rat glioma C6 cells, increased C6 glioma cell-induced angiogenesis in vivo in the chicken embryo chorioallantoic membrane [111]. In both cases, direct binding of PTN to VEGF has been discussed as the possible reason, although other mechanisms may be also involved.

**STRUCTURE-FUNCTION DATA**

Many studies have been undertaken in order to determine which regions of PTN are responsible for its diverse functions, in an effort to identify the molecular mechanisms involved and to identify possible therapeutic targets or/and agents. Kilpelaiinen et al. suggested that the two TSR-1 motifs are responsible for the interaction of PTN with heparin, an interaction associated with many of the biological activities of PTN [14]. More recent data suggest the involvement of only the carboxy terminal TSR-1 motif in heparin binding [17, 129], and the mitogenic, transforming and angiogenic activities of PTN in vitro and in vivo in nude mice [129]. It has been shown that PTN exists in two naturally occurring forms, PTN15 and PTN18, with differential interactions with its receptors anaplastic lymphoma kinase (ALK) and receptor protein tyrosine phosphatase β/ζ (RPTPβ/ζ), and different activities. PTN18 interacts with RPTPβ/ζ and induces glioma cell migration, while PTN15 interacts with ALK and induces glioma cell proliferation [97]. The two forms of PTN differ in their carboxy terminus, which is being investigated for its role in tumor growth and angiogenesis. It has long been shown that the C-terminal lysine-rich domain of PTN (amino acids 111-136) is not involved in neurite outgrowth activity, but it seems to play a key role in the mitogenic and tumor formation activities of PTN [130]. A truncated PTN lacking the C-terminal 111-136 portion inhibits tumor development by inhibition of both endothelial and breast cancer cells [131]. The exact mechanism of action of the C-terminal lysine-rich domain of PTN is not known. It has been suggested that it acts through binding to ALK [132], or RPTPβ/ζ [133] and antagonizes PTN binding and activity [132, 133]. Since this domain of PTN seems to play an important role in its biological activities related to tumor growth and angiogenesis, more work is needed to identify the molecule(s) with which it interacts, the result(s) of such interactions, as well as its possible therapeutic potential.

**RECEPTORS, MOLECULAR MECHANISMS AND INTERACTIONS WITH OTHER MOLECULES INVOLVED IN PTN ACTIONS RELATED TO ANGIOGENESIS AND CANCER**

**Syndecans**

The first identified receptor for PTN has been N-syndecan [134]. The interaction of PTN with N-syndecan takes place via its heparan sulfate side chains [134, 135] and is mediated by both TSR-1 domains of PTN [136, 137]. Binding of PTN to N-syndecan promotes several PTN-induced actions in the nervous system [135, 138-140].

Besides the nervous system, possible involvement of N-syndecan in PTN activities has been mentioned in osteoblasts [73, 78], and in parenchymal cells in adult and embryonic liver [61]. No involvement of N-syndecan in PTN-induced activities related to angiogenesis and cancer has been mentioned, nor is it known whether other syndecans interact and mediate PTN-induced actions.

**Anaplastic lymphoma kinase (ALK)**

ALK is a 220-kDa receptor tyrosine kinase (RTK) encoded by the alk gene on chromosome 2p23. ALK was first identified as part of the NPM-ALK oncogenic fusion protein, resulting from the (2;5)(p23;q35) translocation that is frequently associated with anaplastic large-cell lymphoma [141]. Full-length ALK has the typical structure of an RTK, with a large extracellular domain, a lipophilic transmembrane segment, a cytoplasmic tyrosine kinase domain, and belongs to the insulin receptor superfamily. It was initially described as an orphan RTK that shows restricted tissue distribution and is regulated during organ development [142, 143]. PTN was initially identified as a potential ligand of ALK, based on a genetic screen by peptide phage display [144]. Different groups have since suggested ALK to be a functional PTN receptor [144-147]. In support of this, the expression pattern of PTN partially overlaps with that of ALK in the rodent developing nervous system [144].

Beyond ALK expression in the nervous system, cultured fibroblasts and endothelial cells, it has also been detected in osteoblastic cells [148] and chondrocytes [25], in pancreatic and breast carcinoma [144, 149], melanoma [150], neuroblastoma [151] glioblastoma [145, 146] and non-Hodgkin’s lymphoma [152].

On the other hand, ALK expression is low in a wide variety of soft tumors [153], and is characterized by limited tissue distribution [142]. Moreover, recent studies performed by different groups argue against PTN as a specific ALK ligand, since binding or activation of ALK by PTN cannot be detected [150, 154-158]. A possible explanation to the confusion in the literature may be
the differential activation of ALK by the two naturally occurring forms of PTN [97], or the indirect PTN-induced ALK activation through PTN-dependent inactivation of the RPTPβζ [159].

**Receptor protein tyrosine phosphatase βζ (RPTPβζ)**

RPTPβζ was initially isolated from neural tissue as a transmembrane protein-tyrosine-phosphatase (PTPase) that consists of a putative signal peptide, a very large extracellular domain containing an N-terminal sequence homologous to carboxy anhydrase, a transmembrane region and a cytoplasmic portion that contains two repeated PTPase-like domains [160]. A shorter transmembrane and two secreted isoforms corresponding to the extracellular portions of the long and short transmembrane isoforms have been described, all considered splice variants of RPTPβζ [161-163]. The short transmembrane isoform lacks 859 amino acids from the extracellular domain [161] and also interacts with PTN. Phosphacan, the secreted isoform that corresponds to the extracellular portion of the long RPTPβζ is also able to bind PTN [162], and is considered to modulate cell interactions and developmental processes in the nervous system [164]. Changes in chondroitin sulfate on phosphacan are developmentally regulated and regulate phosphacan’s affinity for PTN [165]. Phosphacan short isoform that corresponds to the extracellular portion of the short RPTPβζ is not a proteoglycan [163] and has not been shown to interact with PTN. Apart from the RPTPβζ splicing variants that are normally expressed, under physiological conditions, RPTPβζ is cleaved by matrix metalloproteinase 9, tumor necrosis factor-α converting enzyme, presenilin/γ-secretase [166] and plasmin [167], leading to secreted, transmembrane, or cytoplasmic forms of, not yet, fully identified biological significance.

It has been suggested that PTN binding to RPTPβζ leads to dimerization of the receptor and inhibition of the PTPase activity. The PTN-dependent RPTPβζ inactivation was shown to lead to increased phosphorylation of β-catenin [168], β-aducin [169] and Fyn [170], thus regulating cytoskeletal stability, cell plasticity and cell-cell adhesion mechanisms [169]. In U373 cells, PTN induced increased tyrosine phosphorylation of different RPTPβζ substrates required for epithelial-mesenchymal transition [103]. On the other hand, PTN binding to RPTPβζ in endothelial cells leads to dephosphorylation and thus activation of c-src, focal adhesion kinase, phosphatidylinositol-3-kinase and mitogen-activated protein kinases, all participating in PTN-induced endothelial cell migration and tube formation on matrigel [116]. We have more recently shown that in order for RPTPβζ to induce cell migration, the presence of αβζ, integrin is required. RPTPβζ and αβζ form a functional complex on the surface of endothelial and glioma cell lines, and RPTPβζ seems to be responsible for βζ tyrosine phosphorylation through the activation of c-src [112]. PTN inhibits migration of cells that do not express αβζ, even if these cells express RPTPβζ [112], however, the exact mechanism(s) involved are not known.

**Other possible (co-)receptors**

It has long been shown that PTN interacts with several proteoglycans (PGs) with different affinities [171], interactions that seem to contribute to PTN dimerization [172] or storage into the extracellular matrix [113]. Among PGs, many reports have implicated a role for chondroitin sulfate (CS) PGs in the PTN-mediated signaling pathway. It has been shown by several studies that PTN interacts especially with over-sulfated CSs [162, 165, 173-175], an interaction important for the development of the nervous system [173, 176, 177] and for growth and/or progression of tumors [178]. Versican, a CS-PG with a high content of the E disaccharide units, was found to bind strongly PTN, an interaction abolished by chondroitinase ABC digestion [179]. Similarly, the appican CS chain from rat C6 glioma cells, but not that from SH-SY5Y neuroblastoma cells that contained no E disaccharide, was found to bind specifically PTN [180]. These findings indicate that the E motif is essential for the interaction of the CS chains with PTN. Analysis of the oligosaccharides isolated from embryonic CS/dermatan sulfate (DS) chains revealed that octasaccharide is the minimal size capable of interacting with PTN at a physiological salt concentration, and that PTN binds to multiple sequences in embryonic CS/DS chains with distinct affinity [181].

PTN also binds αβζ, but not αβζ integrin, an interaction that is responsible for PTN-induced cell migration in both endothelial and glioma cell lines [112]. Integrin αβζ forms a functional complex with RPTPβζ on the cell surface, both components of which are required for the stimulatory effect of PTN on cell migration. Activation of βζ through phosphorylation of its cytoplasmic tyrosine 773, is required, but is not sufficient to transduce the stimulatory effect of PTN [112]. Further studies are being conducted to elucidate the signaling pathway involved. Interestingly, αβζ is not a receptor for MK [112], in contrast to all other PTN receptors discussed to date.

Finally, PTN binds nucleolin [182], a 100 kDa multifunctional protein present in the nucleus, cytoplasm, and on the surface of some types of cells, including endothelial [183] and cancer [184, 185] cells. HB-19 pseudopeptide, a specific antagonist that binds the C-terminal tail of nucleolin, has been shown to suppress the growth of tumor cells and angiogenesis in various in vitro and in vivo experimental models [184]. Nucleolin is considered to be a low affinity receptor for PTN, and has been suggested to possibly import PTN into the nucleus [182]. PTN binds nucleolin through its C-TSR-1 domain with a Kd value of 1.3-1.4 x 10-6 M [186] in the absence of heparin. This binding is strongly inhibited by heparin even though it has not been clarified whether the inhibition was caused by the binding of heparin to PTN or nucleolin [182]. Following its binding to cell surface nucleolin, PTN is internalized in a temperature-sensitive manner, which is independent of heparin and CS PGs [186]. What is the role of the interaction of PTN with nucleolin and of the subsequent PTN internalization remains unknown, but is under further investigation for its possible implication in the effects of PTN on tumor growth and angiogenesis.
CONCLUSION

Although many aspects remain obscure, PTN seems to be significant for tumor growth and angiogenesis, possibly through diverse mechanisms. Clarification of the receptors, as well as the signaling pathways involved, is of great importance, both for increasing our knowledge concerning cancer growth, and for developing new therapeutic strategies.

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