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13. ABSTRACT *(Maximum 200 Words)*

The growth factor pleiotrophin is a positive regulator of tumor angiogenesis and is expressed in breast cancer cell lines and in primary tumor specimens and is one of the factors for the malignant phenotypes of human mammary carcinoma. The expression of PTN in normal and pathologic human breast tissue is mediated by co expression of the species specific transcript and the HERV-PTN fusion transcript. The transcription factor YY1 strongly represses HERV-PTN fusion transcript in two breast cancer cell lines. In an effort to understand the biological and biochemical effects of PTN, we recently found that it exerts its action through an orphan membrane tyrosine kinase receptor, anaplastic lymphoma kinase (ALK). We present evidence that ALK is activated by PTN and, sequentially, activates an array of second messengers in various cell lines. Interestingly, human primary mammary fibroblasts express ALK, and Akt and MAPK are activated upon addition of PTN. ALK expression levels are important for the tumorigenicity of cell lines that expresses PTN also. Upregulated levels of ALK in HUVEC render these cells more susceptible to PTN and promote angiogenesis. We conclude that expression of PTN in human mammary cells leads to activation of ALK in stromal cells and promote tumor growth.

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Introduction

In breast cancer there is a direct correlation between blood vessel density in primary tumors and their metastasis and microvessel density is an independent prognostic indicator. Pleiotrophin (PTN), initially known as heparin binding neurite growth factor (1) and secreted by the human cancer cell line MDA-MD 231 (2), is an angiogenic factor (3, 4). PTN has mitogenic activity on endothelial cells (3) as well as on fibroblasts and epithelial cells and therefore it may contribute to the metastatic phenotype as a tumor growth factor. Studies with human tumor samples, serum samples from cancer patients, as well as tumor cells in culture, have shown that PTN is upregulated during tumorigenesis (5) and can function as a growth factor for tumor cells themselves (autocrine stimulation) as well as for the surrounding stroma (paracrine stimulation) (6) As we showed earlier, an endogenous retrovirus-like element (HERV-E.PTN) is located inside of the human PTN gene directly upstream of the first coding exon (7). RT-PCR and in situ hybridization analysis from our lab, showed expression of HERV-PTN fusion transcripts also in some breast cancer cell lines and invasive mammary carcinoma specimens. Moreover, targeting PTN, either by ribozymes (8) or by transfection of mutant *Ptn* cDNA that leads to the formation of inactive PTN dimers (9), inhibited human breast cancer growth. Our initial hypothesis was that utilization of the HERV-derived PTN promoter is one significant mechanism by which breast cancer cells increase their expression level of the angiogenic growth factor pleiotrophin, which potentially supports the transition from the avascular to the vascular stage. Our findings, reported previously, showed that the expression of PTN is not specific for malignant breast specimens. Instead we detected PTN expression in epithelial cells of benign breast cancer specimens as well as in the epithelial cells and myoepithelial cells of normal breast tissue. The biological activity of PTN affects a large palette of cell types but the mechanism of action could not be adequately studied because its receptor was not known. Among the candidates that were proposed are a heparan sulfate proteoglycan N-syndecan, a proteoglycan type of protein tyrosine phosphatase (PTPζ/RPTPβ) and glucosaminoglycans. However, none of the prospective candidates were analyzed from the point of view of signal transduction to underscore a direct relationship between the pleiotrophin stimulation and changes at the receptor level.

In this proposal we investigate the biological and biochemical effects of PTN from the perspective of its interaction with its receptor. Due to unforeseen circumstances (changing of the PI) and, more importantly, the new and more comprehensive possibilities of explaining PTN role in breast cancer we decided to identify and study the PTN receptor and the biochemical and biological effects of its interaction with its ligand, PTN.

Proposal Body

In the approved Statement of Work three tasks were outlined.

Task 1. Expression status of PTN and HERV-PTN fusion transcripts in human mammary carcinoma (month 8 to 12).

Task 2. Characterization of the mechanism(s) of HERV-PTN fusion transcript expression in human breast cancer cell lines (month 1 to 30).

A. Transcriptional activity of the HERV-derived PTN promoter in breast cancer cell lines (month 1 to 18).

B. Identification of cis-elements in the HERV-derived PTN promoter (month 14 to 30).

C. Examine whether posttranscriptional regulatory mechanisms are involved in the HERV-PTN fusion transcript expression in human breast cancer cell lines (month 28 to 36).

Task 3. Effects of hormones and hormone antagonists on the HERV-PTN fusion transcript expression (month 32 to 42).

In view of the results obtained up to now, we propose to redirect the research, so as to gain a better understanding of the role of PTN, by investigating the PTN receptor. The data obtained will offer a more comprehensive picture of the complex interaction between tumor cells, and stroma and also uncover potential targets for therapy.

Task 1: The goal of our studies regarding Task 1 was to study the expression of PTN and HERV-PTN fusion transcript in human mammary carcinoma.

Progress regarding Task 1 was documented in the first Annual Report (1 Sep 1999 – 31 Aug 2000).

Task 2: The focus of Task 2 was the analysis of the regulatory elements of the HERV-derived PTN promoter. Specifically we are interested in regulatory cis-elements of this promoter and their interaction with the transcription factors that bind to these elements.

Completion of task 2 was documented in the second annual report (1 Sept. 2000- 31 Aug. 2001).

In this report we will document progress towards the redirected

Task 3 Identification and characterization of the PTN receptor (month 28 to 42).

Identification of the receptor for PTN as anaplastic lymphoma kinase.
To identify a receptor for PTN, we used immobilized human PTN protein as a bait to screen a phage-display human fetal brain cDNA library for phage expressing a ligand binding fragment of the putative receptor on its surface. Several rounds of panning against purified, biologically active PTN resulted in the isolation of a phage cDNA insert that coded for a peptide sequence homologous to a region in the ECD of the orphan

tyrosine kinase receptor ALK (aa 396 to 406 in Genbank accession no. U66559). This putative binding site for PTN is contained within a signature sequence patterns typical of the extracellular domains (ECDs) of various transmembrane proteins (MAM domains) (Fig. 1a). The closest homologue of the approximately 220 kDa ALK is LTK (leukocyte tyrosine kinase), a 100 kDa transmembrane protein with a short ECD that lacks the N-terminal 60% of the ALK ECD and thus does not include the putative PTN binding domain. The intracellular domain (ICD) contains the tyrosine kinase (TK) and the translocation site in the juxtamembrane region that is used for fusion with nucleophosmin (t(2,5) that leads to an oncogenic, constitutively activates form of ALK. Also found in the ICD are the consensus binding sites for key signal transduction protein such as insulin receptor substrate–1 (IRS-1), Shc, and phospholipase C-γ (PLC-γ).

PTN Receptor (ALK) binding in intact cells.
To assess receptor binding of PTN in intact cells, we used 32D murine myeloid cells. These cells are dependent on IL-3 for their growth in suspension culture and have been used extensively for the study of tyrosine kinase receptors .The 32D cells do not express ALK mRNA and we stably transfected the ALK cDNA to compare radioligand binding in 32D/control and 32D/ALK transfected cells. PTN radioligand was purified from supernatants of PTN-overexpressing cells that were metabolically labeled with [^{35}S]-cysteine. As shown in Fig. 2c, PTN radioligand binding to 32D/ALK cells was competed either by an excess of cold PTN or by added ECD protein or by antibodies raised against PTN or against a fusion protein containing the putative ligand binding domain in the ALK ECD. FGF-2, as well as control IgGs, did not compete for this binding of the radioligand (not shown). In contrast to the 32D/ALK cells, the respective 32D/control cells showed only non-specific binding that was not competed (not shown). This non-specific binding to 32D/control cells increased linearly with increasing concentrations of the radioligand (Fig. 2b). Parallel binding studies with the 32D/ALK-transfected cells showed a saturation binding isotherm with an equilibrium dissociation constant for PTN of 32 ± 9 pM (=0.5 ng/ml; Fig. 2b). This value is in the range of biologically effective concentrations of PTN. From this series of experiments we concluded that PTN binds to the ALK orphan receptor as a high-affinity ligand.

PTN-induced signal transduction through ALK.
To assess how ALK participates in PTN signal transduction and affects the growth response to PTN, we generated SW-13/ALK-overexpressing cells because SW-13 cells express very low levels of endogenous ALK. Tyrosine-phosphorylated proteins, apparent at 2-5 minutes after PTN stimulation, were immunoprecipitated from SW-13/ALK cell extracts and further analyzed by α-PY immunoblotting (Fig. 3a and b). The anti-phosphotyrosine antibody precipitated several distinct phosphoproteins. To evaluate the specificity of the PTN / receptor interaction in the SW-13/ALK cells, we included an α-PTN antibody and an excess of the ALK-ECD protein in the phosphorylation studies. The PTN-stimulated tyrosine phosphorylation of proteins in SW-13/ALK cells was inhibited significantly by pre-incubation of the ligand with the recombinant ALK-ECD protein or with the α-PTN antibody. The ligand-induced protein phosphorylation in intact cells occurs rapidly and is specific to the PTN ligand as well as the ALK receptor.

Next we analyzed signaling proteins for which a consensus binding site was found on the ICD of ALK (see Fig. 1a); i.e. Shc, PLC-γ. Furthermore, our previous studies had shown that PI 3-kinase was a target for PTN-induced phosphorylation and we included this in our analysis. Immunoprecipitation with an α-PY antibody and subsequent Western blots revealed that PLC-γ, PI3-kinase and Shc are also phosphorylated after PTN stimulation of SW-13/ALK cells (Fig. 3b). These findings in conjunction with our earlier studies, suggest that PTN induces the phosphorylation of ALK and subsequent signal propagation via the adaptor molecules Shc as well as the enzymes PLC-γ, ERK and PI3-kinase.

ALK is expressed in stroma of the breast and is activated by PTN

An important finding is constituted by detection of ALK in normal fibroblasts breast fibroblasts figure 4. Primary fibroblasts from reduction mammoplasties were grown in culture and subsequently serum starved to assess their response to PTN stimulation. The treatment showed that PTN is activating ALK and leads to activation of important signaling molecules as Akt and MAPK. Moreover, we detected a two to four fold increase of the level of ALK in endothelial cells (HUVEC) upon stimulation with conditioned media from PTN expressing MDA-MB 231 cells or FGF-2, respectively. Taken together, these two crucial findings offer a new paradigm for the role of PTN in breast cancer.

Key Research Accomplishments

- We identified the receptor for PTN as Anaplastic lymphoma kinase
- We characterized the binding properties of ALK
- We detected crucial signaling molecules that are activated by PTN
- We demonstrated that ALK is present in the stroma of breast and its level of expression can be modulated.

Reportable Outcomes

- Generation of ALK mutants (ATP, Shc and IRS-1 binding defective).
- Detection of ALK in various stromal cells
- Gerald E. Stoica, Angera Kuo, John T. Lahuisen, Anton Wellstein, "Expression and modulation of ALK in stroma of PTN expressing tumors" manuscript in preparation.

Conclusions

In this proposal we wanted to investigate whether utilization of the HERV-derived PTN promoter is one significant mechanism by which breast cancer cells increase the expression level of the angiogenic growth factor pleiotrophin. Specifically, we want to study PTN expression patterns in invasive and noninvasive primary human mammary carcinomas and characterize the regulatory mechanism(s) responsible for the expression

of HERV-PTN fusion transcripts in human breast cancer cells. However, our findings, reported previously, showed that the expression of PTN is not specific for malignant breast specimens. Instead we detected PTN expression in epithelial cells of benign breast cancer specimens as well as in the epithelial cells and myoepithelial cells of normal breast tissue. The biological activity of PTN affects a large palette of cell types and now the mechanism of action can studied because we uncovered the PTN receptor. The two crucial findings that ALK is expressed in stroma of the breast and is activated by PTN offer a new paradigm for the role of PTN in breast cancer. We expect that the completion of our initial experiments give us valuable insights into the role of the PTN and its interaction with ALK. The PTN/ALK axis may have a pivotal role in the progression and metastatic phase of breast cancer.

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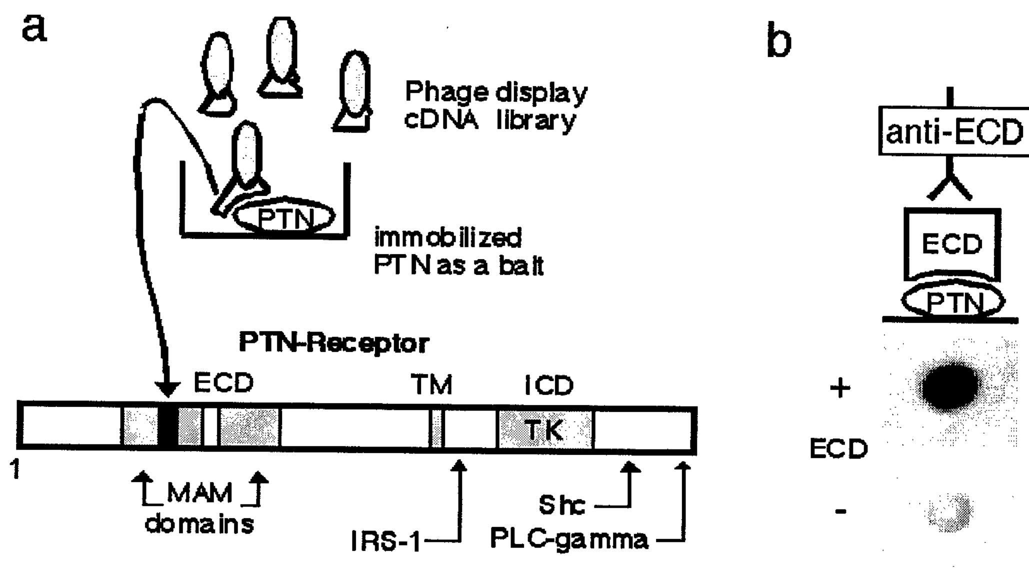

Figure 1. Identification of ALK as the PTN receptor

a) Organization of ALK. The PTN binding site was identified by phage display and located in the extracellular domain (ECD) of ALK(aa 391-401). The intracellular domain (ICD) containing the tyrosine kinase (TK) region is also depicted. Consensus binding sites for insulin receptor substrate –1 (IRS-1), Shc, and phospholipase C-γ (PLC-γ), are indicated by the arrows.**b)** Binding of the ALK ECD to PTN. The purified PTN protein used as a bait for phage display was immobilized on a nitrocellulose membrane, incubated without (-) or with (+) ECD-Fc fusion protein (ECD of ALK produced as an Fc fusion protein in CHO cells) and bound ECD was visualized by immunodetection.

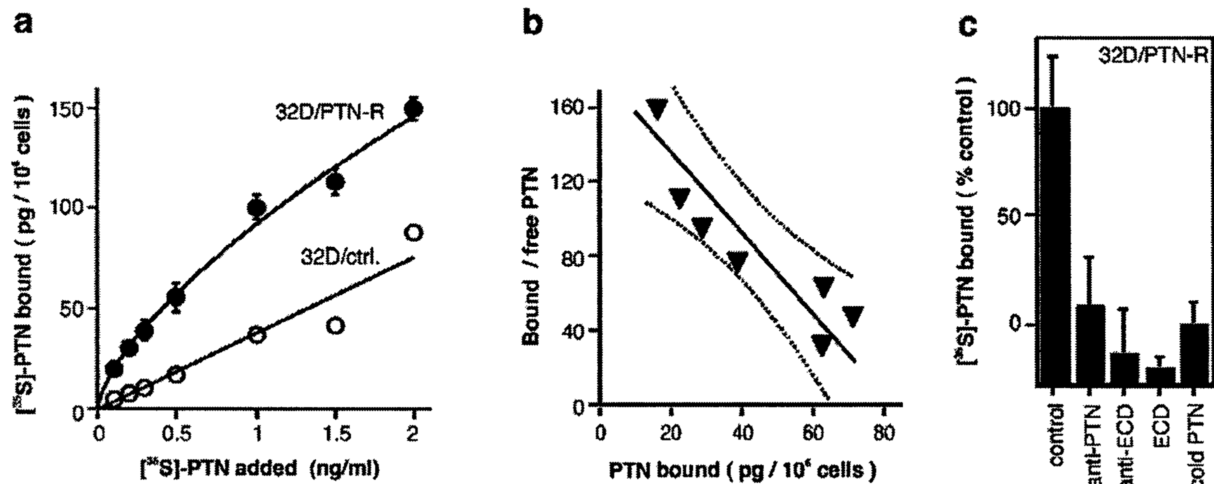

Figure 2 ALK binding studies of PTN in intact cells.

a) Saturation binding of radiolabeled PTN to 32D/ALK (filled symbols) and 32D/control cells (open symbols). The fitted curves were obtained from non-linear regression analysis for saturation binding studies and a K_d -value of 32 ± 9 pM was calculated.

b) Scatchard transformation of the binding data. Bound represents specific binding, i.e.the difference between binding to 32D/ALK and 32D/control cells; "free' refers to the free concentration of PTN.

c) Competition for the binding of radiolabeled PTN (1 ng/ml) to 32D/ALK-transfected cells by: cold PTN (30X), ALK ECD protein (0.7 μg/ml), an affinity-purified anti-PTN antibody (2.5 μg/ml)or an IgG raised against an ECD fragment containing the ligand binding domain (6 μg/ml).

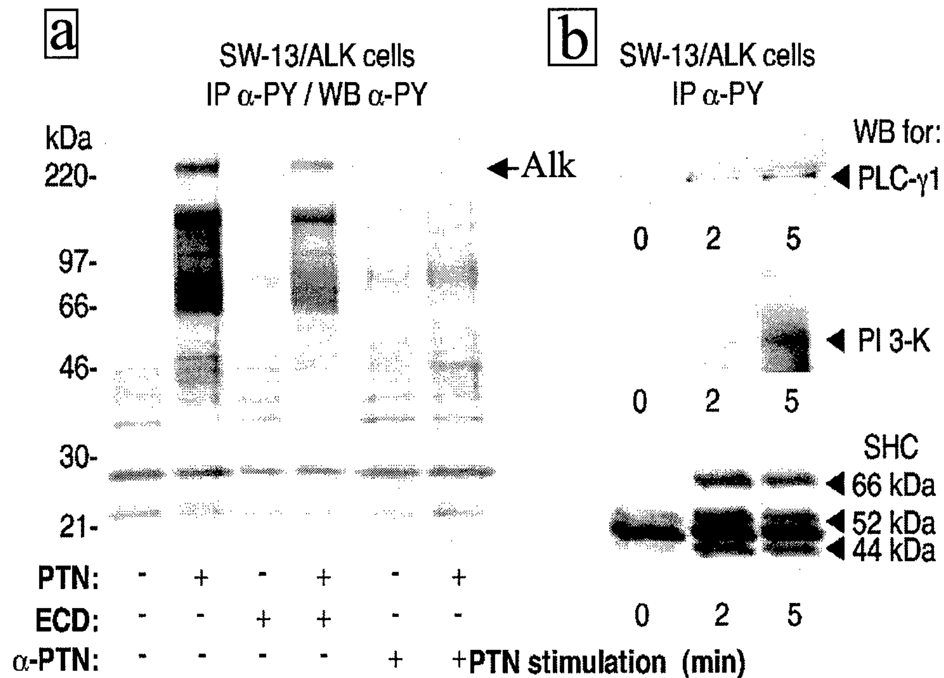

Fig. 3. Signal transduction of PTN through ALK

a) Effect of addition of the ALK ECD-Fc fusion protein (0.7 μg/ml) or an affinity-purified α-PTN antibody (2.5 μg/ml). SW-13/ALK cells were stimulated for 5 minutes with PTN that had been preincubated with α-PTN or with the ALK ECD. Lysates were prepared from PTN-induced SW-13/ALK cells and immunoprecipitated with anti phosphotyrosine antibodies (α-PY). The resulting precipitates were analyzed by SDS polyacrylamide gel electrophoresis. An α-phosphotyrosine Western blot (WB α-PY) is shown. **b)** Identification of phosphoproteins after PTN-stimulation of SW-13/ALK cells. Cells were stimulated with PTN for different times and extracts subjected to immunoprecipitation with an α-PY antibody and subsequent Western blots with the antibodies indicated. PI3-K refers to the p85 subunit of PI3-kinase.

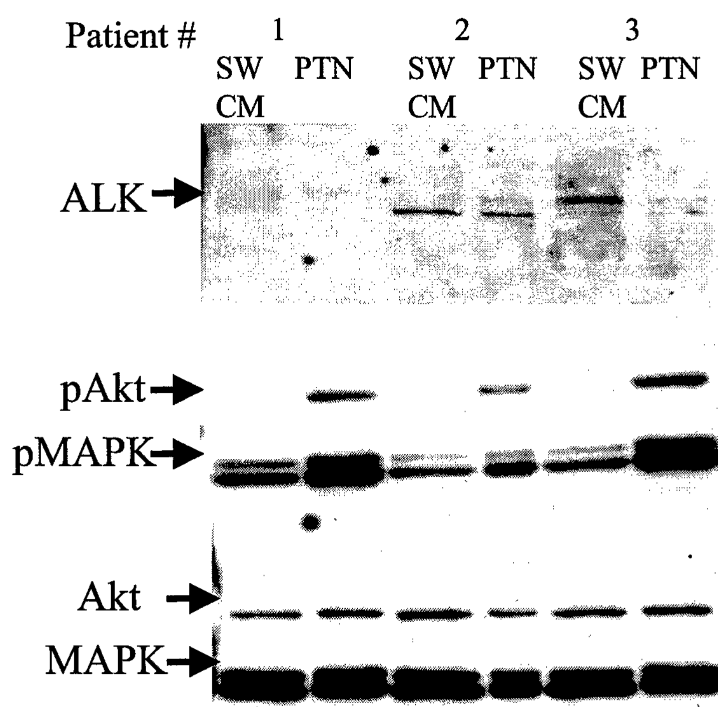

Figure 4. ALK is expressed in primary human mammary fibroblast cells from three reduction mammoplasties and is activated by PTN, as detected by S/T phosphorylation of Akt and MAPK. SW 13 conditioned medium was used as control. The position of the molecule immunobloted for is indicated by arrows. The last blot represents a loading control for the experiment.

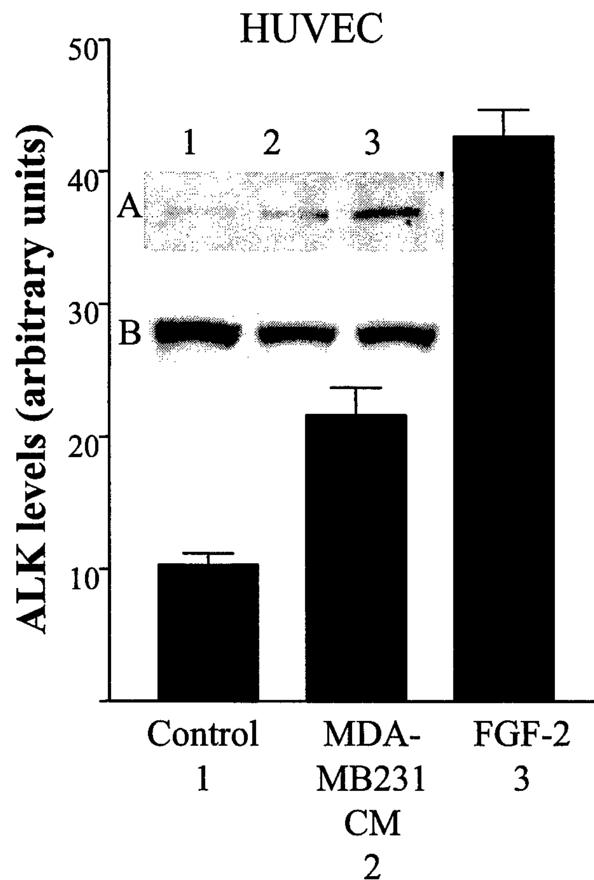

Figure 5 A In HUVECs, the levels of ALK can be upregulated within 8 hours by various growth present in the condition medium of MDA-MB 231 breast cancer cell line or by 10 ng/ml of FGF-2. **B.** A loading control for the experiment.

ALK expression level quantitation of the immunoblots is presented