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13. ABSTRACT *(Maximum 200*

Under goal 1 of this award we studied the mechanisms of regulation of the pleiotrophin (PTN) gene. We discovered a germ line retroviral insertion that contributes to the transcriptional regulation of this gene. Phylogenetic analysis showed that the retroviral insertion happenend approximately 25 million years ago and affects tissue-specific expression of the gene. The same retroviral element was found inserted into the human BRCA-1 gene locus suggesting some overlapping regulation mechanisms between the two genes. Under goal 2 the effects of PTN expression were studied and found to contribute to tumorigenicity of non-expressing cells. Under goal 3 we analyzed the functional domains of the PTN protein and studied its signal transduction. We showed that MAP kinase / PI 3-kinase pathways are used for mitogenesis and identified candidate receptor proteins. Most recently, we identified a PTN receptor candidate and now report data on the effects of targeting and depletion of this receptor from human breast cancer cells that express PTN as well as from cells that respond to PTN. We found that the tumor cells utilize the PTN / PTN-receptor interaction in an autocrine manner. Furthermore, we show that depletion of the receptor mRNA also abolishes the ability of PTN to stimulate growth of receptor positive cells. Under goal 4 we studied the effects of the repression of PTN production on the tumorigenic phenotype and showed that PTN expression is rate-limiting for tumor growth, invasion, angiogenesis and metastasis.

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INTRODUCTION

Under this award we studied the role of the growth factor pleiotrophin (PTN, [1]) in breast cancer. We hypothesized that PTN is an essential, rate-limiting growth factor for PTN-positive breast cancers. Our experiments were designed to address this hypothesis.

PTN belongs to a family of growth factors that includes one other member, midkine [2], and is involved in growth and differentiation processes that are tightly regulated during development [3]. Furthermore, PTN is a mitogen for fibroblasts [4], epithelial cells [4,5] and endothelial cells [4-7]. It stimulates plasminogen activator production [8], can induce tube formation of endothelial cells in vitro [7], and thus can serve as a tumor angiogenesis factor in vivo. PTN is expressed in a variety of tumor cell lines and tumor samples [4] and was found to be oncogenic when over-expressed in NIH3T3 cells [9] and SW-13 human adrenal carcinoma cells [4]. Furthermore, as shown under this award, the growth, invasion, angiogenesis and metastasis of PTN-positive tumor cells was reverted by depleting the tumor cells of their endogenous PTN with specific ribozymes [10-12].

We were the first laboratory to purify PTN from human cancer cells (MDA-MB 231 breast cancer cell line) [13]. We were also the first laboratory to generate biologically active recombinant PTN [4] and to demonstrate its potential role as a tumor growth and angiogenesis factor [4]. Furthermore, we showed its distinct expression in human breast cancer samples [4] and published the genomic structure of the human PTN gene first [14].

With this background knowledge, we had proposed that the secreted polypeptide growth factor pleiotrophin (PTN) plays a major role in the growth and metastasis of breast cancer. This hypothesis is also based on the high levels of PTN expression in 60% of tumor samples from breast cancer patients but not in normal tissues and on the biological effects of PTN in selected tumors models. In particular, the activity of PTN on endothelial cells indicates that PTN can serve as a tumor angiogenesis factor and its expression can thus enhance the ability of breast cancer to metastasize.In summary, our studies quoted above as well as the data published by us and by others show:

a. PTN is a secreted growth factor expressed in a number of human breast cancer cell lines and in the majority of human breast cancer samples [4].

b. PTN stimulates endothelial cells and can act as a tumor angiogenesis factor that promotes tumor invasion and metastasis [9,11,12,15].

OVERVIEW OF THE GOALS PURSUED UNDER THIS AWARD:

In our studies, we elucidated the role of PTN, the regulation of its activity, its functionality and signal transduction with the ultimate goal to develop novel therapeutic strategies.

In particular,

under goal (1) we studied hormonal regulation of PTN and in this context elucidated the underlying molecular mechanisms of the regulation of the gene

under goal (2) we investigated whether PTN expression can support tumor growth

under goal (3) we generated PTN protein for functional and signal transduction studies and

under goal (4) we targeted PTN mRNA to repress its production and study the effect on tumor growth

OVERVIEW OF MAJOR NEW FINDINGS DURING THE PAST REPORT CYCLE:

From the final cycle of this award, I am pleased to present exciting new data on the signal transduction of PTN and a candidate receptor that we discovered. These results conclude our studies on PTN under goal 3. The discovery of a candidate receptor opens up a new area of research on the function of PTN.

REFERENCING OF EARLIER REPORTS FOR THIS FINAL REPORT

In addition, for the purpose of this final report, I summarize the findings that were reported previously and reference the respective earlier reports and papers published in the appropriate sections.

BODY

Goal (1): Regulation of pleiotrophin (PTN)

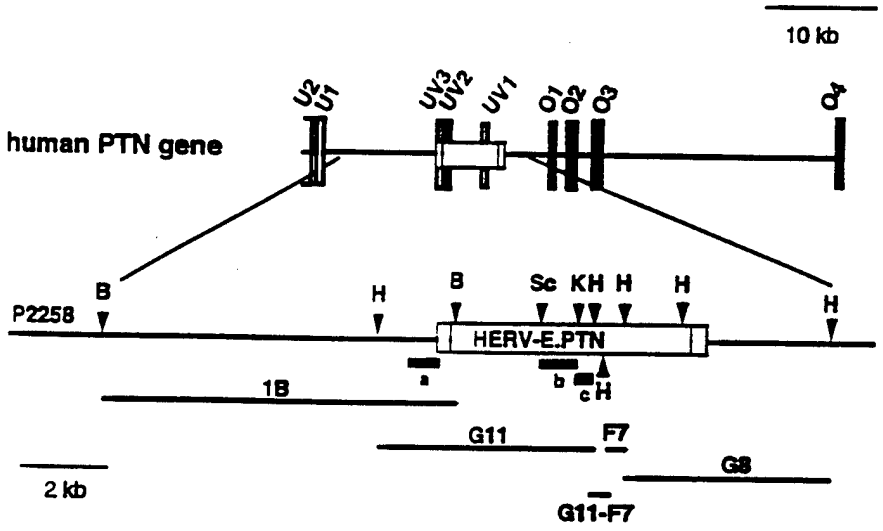
Background:
Hormones and growth factors define the capacity of human breast cancer to grow and metastasize. One of the essential requirements for the development of breast cancer are circulating steroid hormones and one of the most widely used drug therapies of breast cancer with the anti-estrogen tamoxifen is based on this fact. Furthermore, growth factor gene expression can supplement for hormone stimulation and thus contribute to hormone-independent cancer growth as well as to resistance to anti-hormone therapy (reviewed e.g. in [16]).

Work accomplished:
Summary: We discovered that in the human PTN gene a tissue-specific promoter was generated by the germ line insertion of a human endogenous retrovirus (HERV) some 25 million years ago. This is the first report of a retroviral insertion contributing a tissue-specific promoter in a human gene and only the second human gene that was reported to be altered in its expression pattern by retroviral elements. In the last report cycle, I described this discovery in detail [12]). Subsequently, we focused on the evaluation of the precise structure of this retroviral insert, its phylogenesis and parallel insertions in other genes. Surprisingly, the same HERV inserted into the PTN gene was also found in the BRCA-1 gene locus in the intron downstream of exon 2. The potential implications for the regulation of human versus murine BRCA-1 prompted us to plan some preliminary studies and will most likely open up a new area of research interest in the laboratory.

Discovery of a retroviral insertion in the human PTN gene:
To elucidate the mechanisms that regulate expression of the human PTN gene, we examined the 5'regions of mRNAs isolated from different tissues by 5'RACE PCR. To our surprise, 5'RACE PCR clones with mRNA from placenta contained novel 5'UTR that are distinct from the previously described 5'UTR in human placental and brain cDNAs. Sequence comparisons revealed that the novel 5'exons contained in the PTN mRNA from placenta are highly homologous to different regions of human endogenous retrovirus (HERV) type C [17-19]. Based on its Glu-tRNA primer binding site specificity and the location within the PTN gene, we named this element **HERV-E.PTN.**

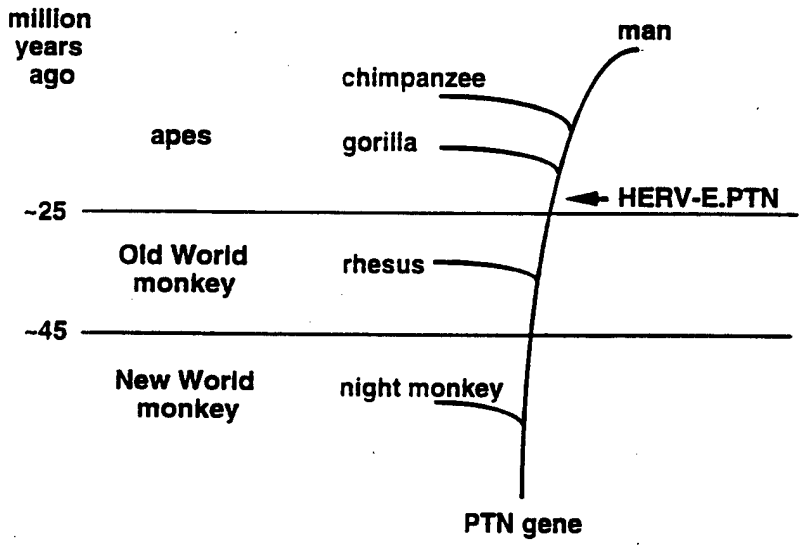
Detailled analysis of the HERV fragment:
Analysis of human genomic DNA revealed that the HERV-E fragment is inserted in sense orientation into the intron region immediately upstream of the ORF of the human PTN gene expanding this region relative to the murine gene. HERV-E.PTN appears to be a recombined viral element based on its high homology (70 to 86 %) in distinct areas to members of two distantly related HERV type C families, HERV-E and RTVL-I. Furthermore, its pseudogene region is organized from 5' to 3' into gag-, pol-, env-, pol, env- similar sequences (Fig. 1). Interestingly, full length and partial HERV-E.PTN homologous sequences were found in the human X chromosome, the human hereditary haemochromatosis region and the BRCA1 pseudogene.

Fig. 1. Organization of the human PTN gene and location of the HERV-E.PTN Cartoon of the structure of HERV-E.PTN based on retroviral pseudogenes.
(Human DNA clones, sequencing and data analysis were reported in the previous cycle)
The genomic organization, restriction enzyme sites (E= EcoRI, H=HindIII, P= PstI) and the size (kb) of restriction fragments are adapted from the GenBank data base and the published literature[20,21]



Finally, Southern analyses indicate that the HERV-E.PTN element is present in the PTN gene of human, chimpanzee and gorilla, but not of rhesus monkey suggesting that genomic insertion occurred after the separation of monkeys and apes about 25 million years ago (Fig. 2).

Fig. 2. Evolutionary tree and proposed time point of the HERV-E.PTN insertion into the PTN gene (most likely also the BRCA-1 locus).



In conclusion, of studies under goal (1):

We discovered a novel regulatory unit in the human PTN gene that was introduced via a retroviral insertion and influences regulation of the human PTN gene. We have initiated studies on the molecular mechanisms the regulate transcription of PTN via this retroviral insertion.

Goal (2):
To study the effect of expression of PTN on the malignant phenotype of PTN-negative breast cancer cells.

Background:
Hormones and growth factors define the capacity of human breast cancer to grow and ultimately to metastasize. One of the essential requirements for the development of breast cancer are circulating steroid hormones and one of the most widely used drug therapies of breast cancer with the anti-estrogen tamoxifen is based on this fact. Furthermore, growth factor gene expression can supplement for hormone stimulation and thus contribute to hormone-independent cancer growth as well as to resistance to anti-hormone therapy (reviewed e.g. in [16]).

Work accomplished:

Transfection of PTN into PTN-negative breast cancer cells:
We have transfected PTN-negative, estrogen-dependent MCF-7 wild-type and T-47D wild-type human breast cancer cells with an expression construct for PTN (see [4]) and have generated a series of different cell lines (mass-transfected and some clonal cell lines) expressing PTN. We have tested the cells *in vitro* for their proliferation and colony forming abilities as well as for expression of PTN mRNA and the secretion of protein.

A wide range of expression levels of PTN was achieved in different MCF-7-derived and T-47D-derived cell lines. No gross difference in the *in vitro* phenotype of the cells was observed. No significantly different proliferation on plastic surface or colony forming ability was found. Based on the current data we conclude that PTN is not utilized by T-47D or by MCF-7 cells as an autocrine growth factor. While this work in my laboratory was ongoing, an abstract was published by Dr. Roy Bicknell's and Adrian Harris's laboratory at Oxford University in the UK [22] suggesting that this group had independently followed the same course of study with the same cell lines and design as proposed by me under this goal.

The data were published in 1997 in Cancer Research [23] and show an angiogenic role for pleiotrophin in tumorigenesis of MCF-7 cells. PTN was overexpressed in MCF-7 breast carcinoma cells. The group found that expression of PTN had no effect on in vitro growth but conferred a tumor growth advantage in animals. They also showed that enhanced tumor growth correlated with increased vascular density in the tumors. Furthermore, they showed that endothelial proliferation was induced by supernatants from the transfected MCF-7 cells implicating an angiogenic role of PTN.

In conclusion, I was very pleased that another laboratory confirmed the potential implications of the angiogenic activity of PTN for breast cancer using the model that I had also planned to use in the proposal. I decided that it would not be appropriate to duplicate the animal study that was proposed and planned for 1997. Instead I decided to rather quote the published work of the Harris/Bicknell group for futher reference on this point.

Goal (3):
The function of the different domains of the PTN protein, signal transduction and the discovery of a PTN receptor candidate

Background:
The secreted PTN protein contains two distinct cysteine-rich domains (on two separate exons) that contains three and two disulfide bridges respectively. Disulfide bridge formation is required for biological activity of the protein. We hypothesized that defined mutations will generate a protein that can still bind to the receptor but will fail to activate the receptor and can thus serve as an antagonist.

Work accomplished earlier:
We generated point-mutant PTNs that have the N-terminal or the C-terminal cysteine changed to a serine and thus disrupted disulfide bridge formation. We tested the effects of the mutant proteins in transfection assays using expression vectors for the mutant and for wild-type PTN in PTN-responsive SW-13 cells as indicator cells of activity. To our astonishment we found that the N- or C-terminal cysteine mutations affect the activity in transient transfection assays only very little. We concluded from this finding that the N-terminal and the C-terminal disulfide bridge are not essential for stability and activity of the protein when only one of them is destroyed.

A dominant-negative PTN protein:
Late in 1997 the laboratory of Dr. Tom Deuel at Harvard University published a paper [24] that human breast cancer growth is inhibited by a dominant negative pleiotrophin mutant. Dr. Deuel, one of our competitors in this field had told me of this finding earlier on during one of many phone conversations on the progress in the PTN area without revealing the details of the construct. They generated a mutant cDNA that encodes one half of the PTN (a truncated PTN) which they showed to heterodimerize with the endogenous PTN protein. They showed that the mutant gene product blocked PTN-induced transformation of NIH 3T3 cells. Most interestingly the mutant PTN expressed in human breast cancer MDA-MB-231 cells blocked their transformed phenotype. This very aggressive cell line no longer formed tumors in mice. Dr. Deutel concludes in the paper [24] that ".... This finding establishes an important role of PTN in the dysregulated growth of human breast cancer cells and suggests that constitutive expression of PTN may be essential to the malignant phenotype of human breast cancers in vivo.. "

Signal transduction pathway of PTN:
(These data were published in JBC in 1997 [25] and were reported in detail in a previous report). To assess the activity of the differently mutated forms of PTN more easily and more directly, we set up a short-term assay that would detect the induction of phosphorylation of proteins in the PTN signal-transduction pathway. We characterized by immunoprecipitation and by Western-blotting with anti-phosphotyrosine antibodies a **190 kDa and a 215 kDa protein** as well as MAP-Kinase proteins that are tyrosine-phosphorylated within a few minutes (1 to 5 min.) after stimulation of PTN-responsive cells in culture. Amongst other cell lines, we used primary BEL (bovine epithelial lens) cells to investigate the signal transduction pathways involved in the mitogenic activity of recombinant PTN. PTN was purified from conditioned media of SW-13 cells transfected with the human PTN cDNA [4]. We found that inhibitors of tyrosine kinase, MAP kinase or PI3 kinase inhibit DNA synthesis stimulated by PTN. Furthermore, in vitro immunocomplex kinase assay with Akt1, a natural substrate of PI3 kinase, showed an activation of the kinase following PTN stimulation and a reversal by the PI3 kinase inhibitor wortmannin.

We concluded from these studies that the mitogenic activity of PTN is dependent on tyrosine kinase activation and utilizes the MAP kinase and the PI3 kinase pathways to transduce a mitogenic signal. Furthermore, we concluded that **the 190 or the 215 kDa phosphoproteins could be potential signal transducing receptors** for the ligand. Details of the methods and the findings were outlined in earlier reports.

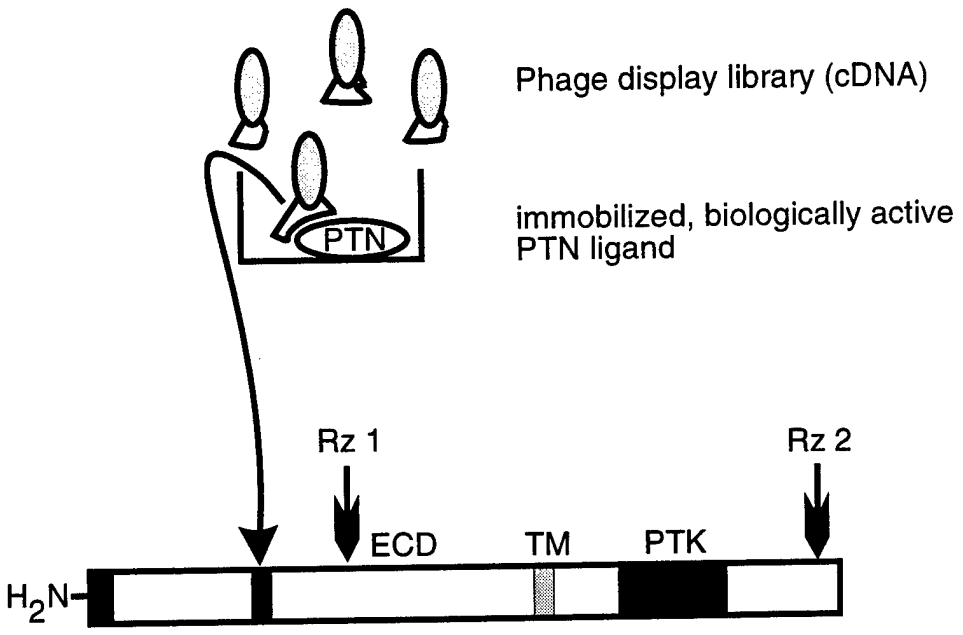
Identification of a PTN-receptor candidate.

In the most recent work we focussed on the identification of the receptor for PTN, since that would allow to analyze the growth factor signalling at the molecular level.

After several attempts, we identified a candidate PTN receptor fragments using a phage display library of human fetal brain cDNAs. This system is commercially available and is utilized in our laboratory for a number of other projects to study protein / protein interactions. Candidate phage were purified by several rounds of panning against immobilized PTN (Fig. 3). We found that the fragment identified was homologous to a sequence stretch in the ECD of a transmembrane tyrosine kinase receptor with a predicted apparent molecular mass of close to 200 kDa (without significant extent of glycosylation). As mentioned above, we [25] and others [26,27] found that upon stimulation of different cell types with PTN, 190 to 220 kDa proteins were tyrosine-phosphorylated and hypothesized that these could be part of the PTN-receptor complex.

Fig. 3. Identification of a receptor for PTN.

Panning of a human fetal brain cDNA phage-display library against immobilized PTN protein generated clones that bind to PTN. One of the selected clones contained an insert homologous to a sequence in the extracellular domain (ECD) of a novel transmembrane (TM) protein tyrosine kinase (PTK). The functional (predicted) regions in this candidate tyrosine kinase receptor are indicated. Rz1 and Rz2 indicate ribozymes targeted to cleave at different sites.



As assessed by RT-PCR (reverse-transcription polymerase chain reaction), the candidate PTN receptor mRNA was expressed in SW-13 cells. These cells do not express the PTN growth factor protein but respond to the added protein by forming colonies in soft agar [4,13]. The PTN-receptor candidate mRNA was also expressed in Hs578T human breast cancer cells. These cells express high levels of PTN mRNA [4].

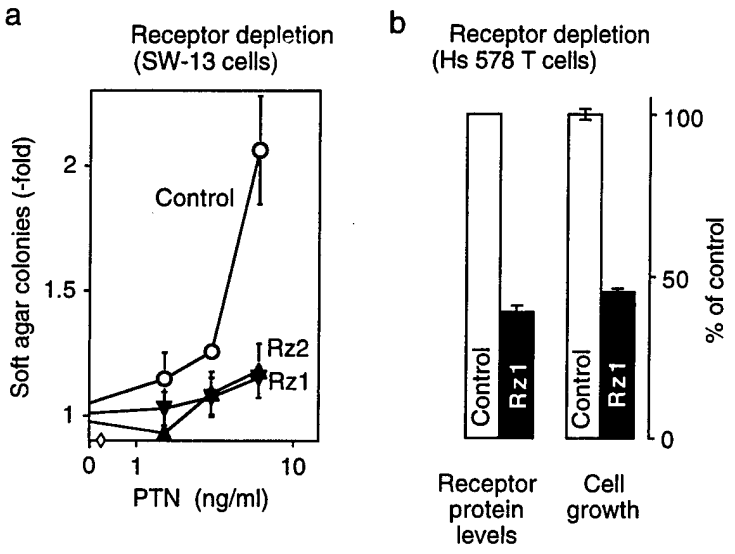
To prove that the gene identified by the phage display is indeed a candidate PTN-receptor, we depleted the gene product from SW-13 cells and from Hs578T cells by stable transfection with hammerhead ribozymes as done for a number of different genes by my laboratory (for a review see reference [28]). The target sites are indicated in Fig. 3 (above) Expression of the PTN-receptor-targeted ribozymes did not affect proliferation of SW-13 cells in tissue culture dishes and we were able to select stably transfected cells. However, when the ribozyme-transfected cells were placed in soft agar, they lost the ability to respond to PTN. Obviously depletion of the candidate PTN receptor mRNA from SW-13 cells attenuates PTN-stimulated soft agar colony formation (Fig. 4 a).

To assess whether the PTN-receptor is a limiting factor for the growth of tumor cells that also express PTN, we reduced the endogenous receptor in the PTN-positive Hs578T human breast cancer cells [4] by stable expression of PTN-receptor-targeted ribozymes. In these cells the reduction of the receptor protein by 60% also reduces the proliferation of the tumor cells (Fig. 4 b). This finding supports the notion that the PTN/PTN-receptor axis can function in an autocrine loop [29] of growth stimulation.

Fig. 4. Effect of the depletion of the PTN-receptor using ribozymes.

a, PTN-stimulated colony formation of SW-13 cells stably transfected with PTN-receptor targeted ribozymes (Rz1 and Rz2) versus control cells.

b, Proliferation of PTN-receptor depleted versus control Hs578T human breast cancer cells. In this PTN-positive cell line [4], the endogenous PTN-receptor mRNA was depleted by stable transfection with ribozyme expression vectors. Western blot for the PTN-receptor (see Methods (below) were used to quantitate the receptor protein and cell proliferation (day 5) of control and of ribozyme-transfected cells is shown relative to each other.



Methods

Phage Display Cloning.

Commercially available human placental and fetal brain M13 phage display libraries from Clontech (EasyMATCH Phage Display; Palo Alto, CA) were used according to the protocol of the manufacturer. In these libraries human cDNA fragments are inserted downstream of the gene III leader sequence to generate gene III fusion proteins exposed on the phage surface. Phage containing candidate PTN receptor fragments as inserts were selected by repeated panning of the libraries against purified PTN [13,25] (approximately 1 μg / well) that had been immobilized in a 96-well plate.

Purification of PTN from Conditioned Media

Conditioned media from approximately 10^9 SW-13/PTN cells grown for 4 to 5 days in 1.5 l of DMEM/2% FCS was adjusted to 50 mM TrisHCl pH 7.5/0.5 M NaCl and was passed through a 2 ml heparin-Sepharose column (Pharmacia, Piscataway). The column was then washed with 40 ml of 50 mM TrisHCl pH 7.5/0.5 M NaCl and heparin-bound proteins were eluted with 10 ml of 50 mM TrisHCl pH 7.5/1 M NaCl. The eluate was diluted to 50 mM TrisHCl pH 7.5/0.25 M NaCl and passed through a Mono S column using an FPLC system (Pharmacia). The column was washed extensively in the same buffer containing 0.45 M NaCl and the bound proteins eluted using a gradient from 0.45 to 2 M NaCl. Fractions of 1 ml were collected, quickly aliquoted and stored at - 80 °C.

Proliferation and Soft Agar Colony Formation Assay

A commercially available assay (MTT; Promega) was used to assay for the proliferation of cells plated in 96-well dishes. The proliferation rate was read at day 5. For soft agar colony formation cells in 0.45% agar containing growth media and growth factor as appropriate were placed on top of a 0.6% agar containing layer in 35 mm dishes, incubated for 10 days and colonies larger than 80 micrometers were counted.

Generation of PTN-Receptor -Targeted Ribozyme-transfected Cell Lines

Different PTN-receptor-targeted ribozymes were generated as described in previous reports using the cDNA sequence of the receptor candidate. As in previous studies, these ribozymes were expressed in a CMV-driven expression vector and stably transfected cells selected by additon of G-418 as a selection medium.

Western Blot

After separation in SDS-PAGE gels, proteins were transferred to a nitrocellulose membrane (Biorad, Hercules, CA) for 2 hrs at 150 mAmps/gel unless indicated otherwise in 25 mM Tris pH8.3/200 mM glycine/20% methanol. The membrane was blocked in PBS (phospate-buffered saline)/0.1% Tween 20/5% powdered milk and probed with the peptide antibodies raised against the PTN-receptor at appropriate dilutions for 1 hr at room temperature. The blots were then washed in PBS/0.1% Tween 20 and incubated with the appropriate secondary antibody coupled to horseradish peroxidase (Amersham) for 1 hr. After additional washing in PBS/0.1% Tween 20, bound antibody was visualized using the enhanced chemiluminescence reagents system from Amersham.

Statistical analysis

Unless stated otherwise data points were run in triplicate and experiments repeated at least

twice. Typically the mean ±SE from a representative experiment is presented.

In conclusion, from the work under goal (3), the identification of a receptor for PTN will be the basis for studies into the pathological and physiological functions attributed to PTN as well as the molecular definition of the ligand/receptor interacting domains. Our studies with the Hs578T cells, though only in cell culture (to date), suggest that therapeutic targeting of the receptor has the potential to disrupt malignant progression of receptor- and ligand-positive tumors.

Other uses of these findings beyond the field of cancer can be the role of PTN / PTN-receptor interactions in the induction of neurite outgrowth during neonatal brain development and maintenance of neuronal function [30]. In addition, dysregulation of PTN has been described in neurodegenerative disease processes [31] and it is conceivable that parallel studies of the receptor will generate new insight into the mechanisms underlying the disease process.

Goal 4:
To inhibit production of PTN

Background
We planned to use three independent approaches to target PTN mRNA and thus reduce the amount of PTN produced by PTN-positive breast cancer cells:

1. antisense oligonucleotides
2. antisense constructs
3. ribozyme constructs

Data from the first two approaches were discussed in earlier reports. In more recent reportw we presented work that demonstrates that PTN drives tumor invasion, angiogenesis and metastasis. This proof was achieved by depleting tumor cells of their endogenously produced PTN using targeting with different ribozyme constructs.

Work accomplished using the ribozyme approach (described in detail in earlier reports)

1. "Gene-dose-response" of PTN for tumor angiogenesis and metastasis

To assess whether PTN is a rate-limiting factor for tumor growth in vivo, angiogenesis and metastasis, we took advantage of a human cell line that produces high levels of PTN mRNA, grows into subcutaneous tumors in mice and metastasizes from the subcutaneous site as hematogenous metastases into the lung. These metastases can be observed 6 to 9 weeks after inoculation of the primary tumor cells. The cell line (named 1205LU) was generated by Dr. M. Herlyn (Wistar Institute, Philadelphia, PA) who kindly provided them to us.

By transfection with ribozyme expression vectors, we generated a panel of derivative clonal and mass-transfected 1205LU cells that expressed different residual levels of PTN mRNA and protein. We separated the cell lines accordingly into high (= close to control levels), medium (= 60 to 75% of control) and low (= <30% of control) PTN expressors and analyzed the in vitro and animal tumor growth data accordingly.

In vitro growth on plastic surface as well as colony formation in soft agar by the 1205LU cells expressing different levels of PTN was unaffected by the changes in PTN levels (not shown).

However, subcutaneous tumor growth was slowed dramatically even in the medium group that had a less than 50% reduction of endogenous PTN. The low group took several months to catch up in their growth with the control group. A direct relationship between tumor growth and residual "gene dose" of PTN was observed.

Angiogenesis was quantitated in the subcutaneous tumors after they had been allowed to grow to the same size (i.e. after different time periods depending on the subgroup). A significant reduction of the number of blood vessels per sqmm due to the reduction of PTN in the tumor cells was observed. This effect was "gene-dose-dependent".

Finally, upon analysis of macroscopic and microscopic sections of the lungs, in all of the animals carrying the PTN-depleted tumors, no macroscopic metastases were seen. After microscopic metastases were tested for by serial sections of the lungs, each animal with any sign of a metastasis was counted as positive and the incidence of metastasis arrived.

We conclude from this data that PTN can be a rate-limiting factor for tumor growth, angiogenesis and metastasis. No threshold effect with regard to a minimum level of reduction of PTN was observed.

2. Depletion of the PTN gene product generated due to the retroviral insertion (=HERV-PTN mRNA).

Cells in which **only** the promoter generated by the retroviral insertion was actively driving expression of PTN (JEG-3 cells) were used to screen for PTN protein secretion and then for its potential biological role in tumor cell growth in vitro as well as invasion, growth and angiogenesis in animal models. A vector (pTET/Rz261) with high transcriptional activity in these cells (unpublished data) was used to express PTN-targeted ribozymes.

Northern blot analysis revealed that ribozyme expression reduced the amount of HERV-PTN mRNA in JEG-3 cells to background levels. No difference in the proliferation rate of PTN-depleted versus control cells was apparent in vitro, suggesting that the cells do not require PTN as an autocrine growth factor even though they secrete the protein in a biologically active form (see above). However, a marked difference in the growth phenotype of PTN-depleted versus control cells was observed after xenografting tumor cells into athymic nude mice. After subcutaneous injection of tumor cells control cells grew rapidly into highly angiogenic tumors but no tumor growth was observed with PTN-depleted cells. The data were highly statistically significant ($p<0.001$).

We conclude that the HERV-driven PTN transcript can be an essential and rate-limiting factor for tumor growth, invasion, and angiogenesis *in vivo*. Since the PTN protein is the same irrespective of the promoter driving gene transcription this biological function should be the same for the protein produced by different types of tumor cells.

Methods:

Depletion of PTN mRNA using Ribozyme Targeting.

The PTN-targeted ribozyme Rz261 [10] was expressed under the control of the tTA / heptameric operator binding site and a CMV minimal promoter [32]. For this purpose, the major portion of the luciferase gene and the SV40 polyadenylation site in the pUHC13-3 plasmid [32] were deleted by HindIII / HpaI cut and replaced with the Rz261 / bovine growth hormone polyadenylation HindIII / PvuII fragment from the pRc/Rz261 expression vector [10]. The remaining luciferase start codon was replaced by a SalI / ClaI / HindIII cassette to yield the construct pTET/Rz261. This ribozyme is designed to cleave PTN mRNA 3' of nucleotide 261 of the ORF [10]. In JEG-3 cells the ribozyme expression vector (pTET/Rz261 , 0.5 µg), was co-transfected with the tTA expression vector (pUHG15-1 [32], 0.5 µg) and pRc/CMV (0.1 µg) to provide G-418 resistance. After selection for stable integrants in the presence of 1 mg/ml of G-418, the cells were tested for PTN expression by Northern analysis as described in previous reports.

Further details of the methodologies used were described in previous reports. Methods have also been published in the papers listed under REFERENCES and in the BIBLIOGRAPHY.

For a review see e.g. Ref. [28].

OVERALL CONCLUSIONS FROM THIS FINAL REPORT

Under this award, we discovered a novel retrovirally-derived promoter in the human PTN gene, elucidated the structure of this insertion and will -in the future- study the molecular mechanisms regulating this gene. We have expanded PTN functional and signal transduction studies and have recently identified a receptor candidate for PTN. Finally, we have run extensive studies repressing or deleting PTN from tumor cells and by this shown its rate-limiting role, when it is expressed in human tumor cells.

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APPENDIX

no APPENDIX material is added