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Identification of the angiogenesis signaling domain in pleiotrophin defines a mechanism of the angiogenic switch ☆

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Abstract

Neoplasms progress through genetic and epigenetic mutations that deregulate pathways in the malignant cell that stimulate more aggressive growth of the malignant cell itself and/or remodel the tumor microenvironment to support the developing tumor mass. The appearance of new blood vessels in malignant tumors is known as the ''angiogenic switch.'' The angiogenic switch triggers a stage of rapid tumor growth supported by extensive tumor angiogenesis and a more aggressive tumor phenotype and its onset is a poor prognostic indicator for host survival. Identification of the factors that stimulate the angiogenic switch thus is of high importance. Pleiotrophin (PTN the protein, *Ptn* the gene) is an angiogenic factor and the *Ptn* gene has been found to be constitutively expressed in many human tumors of different cell types. These studies use a nude mouse model to test if *Ptn* constitutively expressed in premalignant cells is sufficient to trigger an angiogenic switch in vivo. We introduced an ectopic *Ptn* gene into ''premalignant'' SW-13 cells and analyzed the phenotype of SW-13 *Ptn* cell tumor implants in the flanks of nude mice. SW-13 *Ptn* cell subcutaneous tumor implants grew very rapidly and had a striking increase in the density of new blood vessels compared to the SW-13 cell tumor implants, suggesting that constitutive PTN signaling in the premalignant SW-13 cell implants in the nude mouse recapitulates fully the angiogenic switch. It was found also that ectopic expression of the C-terminal domain of PTN in SW-13 cell implants was equally effective in initiating an angiogenic switch as the full-length PTN whereas implants of SW-13 cells in nude mice that express the N-terminal domain of PTN grew rapidly but failed to develop tumor angiogenesis. The data suggest the possibility that mutations that activate *Ptn* in premalignant cells are sufficient to stimulate an angiogenic switch in vivo and, since these mutations are frequently found in human malignancies, that constitutive PTN signaling may be an important contributor to progression of human tumors. The data also suggest that the C-terminal and the N-terminal domains of PTN equally initiate switches in premalignant cells to cells of a more aggressive tumor phenotype but the separate domains of PTN signal different mechanisms and perhaps signal through activation of a separate receptor-like protein.

Keywords: Pleiotrophin; Angiogenic switch; Angiotensin II; RPTPβ/ζ

Pre-malignant cells progress to become more malignant through sequential genetic and epigenetic mutations in individual malignant cells that initiate ''switches'' in signaling pathways to convert the phenotype of the malignant cell to one of higher malignancy [1–3]. The onset of tumor angiogenesis, known as the angiogenic switch [3,4], is essential for nutrients, for adequate oxygenation of the growing tumor mass, and for entry sites for tumors to metastasize to distant organs [5–7]; it is an essential event for premalignant cells to exit dormancy. Deregulation of different factors that are inducers or inhibitors of

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angiogenesis has been postulated to be mechanisms to initiate the "angiogenic switch." The angiogenic factors basic Fibroblast Growth Factor (bFGF) and Vascular Endothelial Growth Factor (VEGF) are expressed in many different human cancers and thus have been viewed as important components of the angiogenic switch [8–10]. However, neither bFGF nor VEGF alone appears to be sufficient for the full picture of the angiogenic switch and thus the pursuit of different regulators of angiogenesis and models to investigate their putative roles in stimulating the angiogenic switch is important not only to better understand tumor progression but to design new therapies to treat aggressive malignancies.

Pleiotrophin (PTN the protein, *Ptn* the gene) is a recently identified and characterized cytokine with established roles in normal and transformed cell growth. It is also an angiogenic factor capable of stimulating new capillary and arteriolar growth in injured tissues [11,12]. Pleiotrophin is a secreted protein of 136 amino acids with lysine rich domains at the N- and C-termini [13] and two separate heparin-binding thrombospondin type 1 repeat domains linked by a short amino acids sequence internally [14]. Pleiotrophin is a highly conserved cytokine [13,15]; it signals proliferation of different cells in culture [13,15] and induces lineage specific differentiation of different progenitor cells [15–17]. The *Ptn* gene also is a protooncogene [18] and expressed in many different malignant cells [13,15,19,20]. The highly malignant phenotype of different malignant cells reverts to the phenotype of the pre-malignant cell when a dominant negative mutant *Ptn* gene [20] is introduced, suggesting that PTN-dependent signaling pathways are responsible for the highly malignant phenotype of aggressive cancers in which the *Ptn* gene is expressed. Furthermore, PTN-dependent signaling pathways appear to stimulate tumor angiogenesis, since, in other experiments, it has been found that the angiogenic phenotype of glioblastoma cell tumor implants in nude mice is entirely reversed when the dominant negative *Ptn* gene was introduced into them, suggesting that PTN in human glioblastoma cells is sufficient alone to initiate the angiogenic switch. These studies collectively suggest that mutations that trigger constitutive expression of the *Ptn* gene in premalignant cells deregulate pathways that provide selective growth advantage, and thus favor selection of clones of *Ptn* expressing malignant cells in the growing tumor mass. In support of this suggestion, *Ptn* expression is found with high frequency in many highly malignant cancer cells [13,15,19,20], perhaps as the result of clonal selection of the cells that express *Ptn* during tumor progression.

These studies were designed to test whether activation of the endogenous *Ptn* gene is sufficient in the premalignant cell to initiate an angiogenic switch. To test this hypothesis, premalignant SW-13 cells into which an activated *Ptn* gene was introduced were implanted in flanks of nude mice and examined for growth and tumor angiogenesis. We also tested the separate N- and C-terminal domains of PTN to attempt to identify which domain in PTN may initiate the different phenotypes in premalignant cells that express the endogenous *Ptn*.

Materials and methods

Expression constructs. The full-length human *Ptn* cDNA (GenBank Accession No. NM_002825) encodes a 168 amino acid protein that includes a 32 amino acid signal peptide. The cDNAs encoding the N-terminal (amino acid residues 1–64) and C-terminal (amino acid residues 69–136) domains of PTN and the full-length PTN (amino acid residues 1–136) were coupled with the endogenous signal peptide and cloned into the PAGE 103 vector as previously described [21].

Cells, cell culture, and DNA transfection. Human adrenal carcinoma (SW-13) was obtained from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. DNA transfections were performed by calcium phosphate co-precipitation as described [18]. The transfectants were selected with G418 for three weeks. In each case, colonies were clonally selected, and the clonal cell lines were established, expanded, and confirmed by Northern blot (Fig. 1A) and Western blot (Fig. 1B) analyses. Clones with high-level expression of the exogenous PTN protein or its variants' gene were retained for further study.

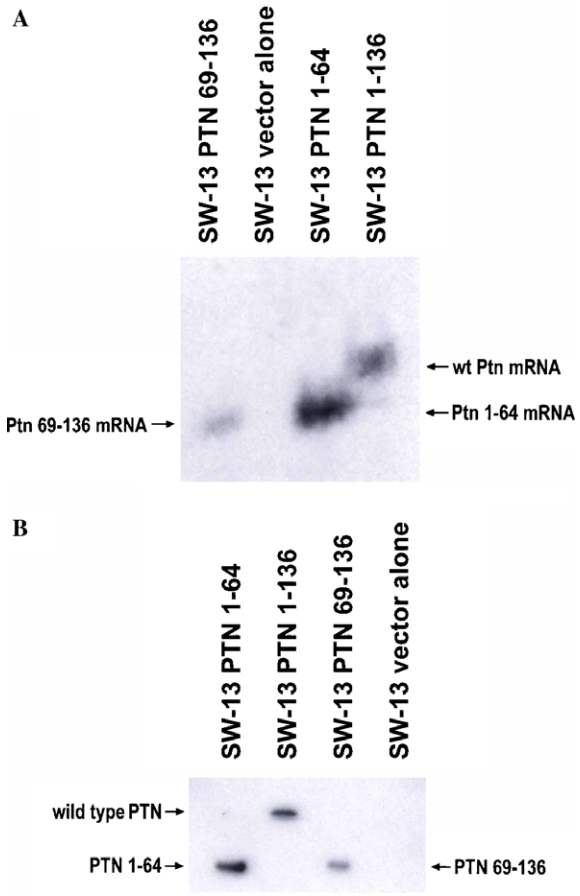

Fig. 1. SW-13 cells with stable expression of an exogenous full-length *Ptn* gene (SW-13 PTN 1–136) or an exogenous truncated *Ptn* gene encoding the N-terminal 1–64 amino acids (SW-13 PTN 1–64) or the C-terminal 69-136 amino acids (SW-13 PTN 69–136) were analyzed in Northern blots (A) and Western blots (B), and found to be expressed in high level; the clones with high level expression of PTN and the truncated PTNs were selected for these experiments.