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Dominant Negative Pleiotrophin Induces Tetraploidy and Aneuploidy in U87MG Human Glioblastoma Cells

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Summary

Pleiotrophin (PTN, *Ptn*) is an 18 kD secretory cytokine that is expressed in many human cancers, including glioblastoma. In previous experiments, interruption of the constitutive PTN signaling in human U87MG glioblastoma cells that inappropriately express endogenous *Ptn* reversed their rapid growth *in vitro* and their malignant phenotype *in vivo*. To seek a mechanism for the effect of the dominant negative PTN, flow cytometry was used to compare the profiles of U87MG cells and four clones of U87MG cells that express the dominant negative PTN (U87MG/PTN 1–40 cells); here, we report that the dominant negative PTN in U87MG cells induces tetraploidy and aneuploidy and arrests the tetraploid and aneuploid cells in the G1 phase of the cell cycle. The data suggest that PTN signaling may have a critical role in chromosomal segregation and cell cycle progression; the data suggest induction of tetraploidy and aneuploidy in U87MG glioblastoma cells may be an important mechanism that contributes to the loss of the malignant phenotype of U87MG cells.

Keywords

Pleiotrophin; Glioblastoma; Dominant-negative; Aneuploid; Tetraploid

Introduction

Pleiotrophin (PTN, *Ptn*) [1–3], also known as heparin-binding growth-associated molecule (HB-GAM)[4], is an 18 kDa secreted cytokine that shares over 50% identity in amino acid sequence and high structural homology in two heparin binding domains with midkine (MK, *Mk*), the only other member of the PTN/MK developmental gene family [5,6]. High level expression of the *Ptn* gene has been found in many aggressive human malignancies, including human breast and prostate cancers [7,8], neuroblastomas [9], gliomas [10], melanomas [11], colon cancers [12], pancreatic carcinomas [13], and small cell lung cancers [14,15] and, cell lines derived from these human malignant cancers have been found to inappropriately express the endogenous *Ptn* [7,8]. Different approaches to inhibit *Ptn* expression or constitutive PTN signaling in different malignant cells with inappropriate expression of *Ptn* have effectively

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reversed the malignant phenotype [16–19], thus supporting directly the importance of PTN-signaling in the progression and in the pathogenesis of highly malignant cancers. However, the mechanisms through which PTN stimulates a more malignant phenotype in malignant cells that inappropriately express *Ptn* are only beginning to be unraveled.

Glioblastomas are highly aggressive and highly vascularized tumors [20]; U87MG cells are derived from a human glioblastoma and have been found to inappropriately express high levels of *Ptn* [21]. In previous experiments, introduction of a dominant-negative *Ptn* encoding PTN amino acids 1–40 (which establishes nonfunctional PTN heterodimers during processing) [16] was introduced into human U87MG glioblastoma cells (U87MG/PTN 1–40 cells). The dominant negative PTN effectively reduced cell growth and reversed the malignant phenotype of these highly malignant cells *in vivo* [21], indicating a requirement of PTN-signaling in the malignant phenotype of U87MG glioblastoma cells and raising the question of the mechanism through which the dominant negative PTN reduces growth and reverses the malignant phenotype of U87MG glioblastoma cells.

To seek the (a) mechanism through which the dominant negative PTN effectively reverses the malignant phenotype of U87MG cells, we compared the profiles of U87MG mock cells with U87MG/PTN 1–40 cells using flow cytometry; it is now demonstrated that expression of the dominant negative PTN in U87MG cells induces tetraploidy and aneuploidy and furthermore, that all the tetraploid and aneuploid cells are arrested in the G1 phase of the cell cycle.

Materials and methods

Plasimid construction

The vector pcDNA3.1/PTN1-40 was constructed by inserting the cDNA fragment encoding residues −32 to 40 of human PTN protein into XbaI and B*am*HI of pcDNA3.1/myc-his/hygro vector (Invitrogen).

Cell culture and transfection

Human glioblastoma U87MG cells were maintained in Minimum Essential Medium Egale (ATCC) supplemented with 10% inactivated fetal bovine serum. 4 μg of pcDNA3.1/PTN1-40 or 4 μg of the empty pcDNA3.1/hygro vector were transfected into U87MG cells in 60 mm dishes using the FuGENE 6 transfection reagent (Roche, IN) according to manufacturer's instructions. Forty-eight hours later, the cells were diluted 1:20 into fresh dishes and selected with hygromycin (Sigma) at 200 μg/ml; media were changed every 3 days until foci appeared. Cell lines were expanded from 4 different clones as noted subsequently; cells were shown to express high levels of dominant negative PTN were named (PTN1-40, clone 2, 3, 15 and 16) [21].

Flow Cytometry

To analyze cell cycle profiles, cells were digested by 0.25% typsin containing 1mM EDTA, washed with phosphate-buffered saline (PBS), and fixed in 70% methanol at 20°C for 10 min. Cells were then pelleted and resuspended in PBS with RNase A (100 □g/ml) and incubated for 30 min at 37 °C. Cells were then stained with propidium iodide (20 μg/ml) for 30 min and analyzed by flow cytometry using linear amplification. Data were collected using a FACScan flow cytometer (Becton Dickinson). For each sample, 20,000 events were collected, and aggregated cells were gated out. Data were analyzed by an auto analysis function of ModFitLT3.0 software (Verity Software, Topsham, ME).

Results

In previous studies, introduction of a dominant negative PTN into U87MG glioblastoma cells was found to reverse their malignant phenotype *in vitro* and *in vivo* [21]; U87MG glioblastoma cells that express the dominant negative PTN (U87MG/PTN 1–40 cells) grew more slowly than U87MG/vector (control) cells in monolayer culture, they formed fewer colonies in soft agar, and they grew more slowly than U87MG/vector cells as U87MG/PTN 1–40 xenografts in flanks of nude mice. U87MG cells inappropriately express *Ptn*; thus, the reversal of the malignant phenotype of U87MG cells with the dominant negative PTN supports the critical importance of constitutive PTN signaling in determining the malignant phenotype of U87MG glioblastoma cells. In the present studies, the goal was to uncover a mechanism potentially able to explain the reversal of the malignant phenotype of U87MG glioblastoma cells by the dominant negative PTN was sought by comparing the profiles of 4 clonal U87MG/PTN 1–40 cell lines with U87MG/vector cells using flow cytometry.

The profile of the control U87MG/vector cells depicted in Figure 1 established that these cells were 100% diploid. The relatively high level of U87MG/vector cells in G1 (Figure 1) is consistent with levels of G1 in different malignant cells characterized by a high proliferation rate. The modest increase in cells in the S phase of the cell cycle also is consistent with a rapid doubling time, a speculation supported by the high rate of growth of U87MG cells that were observed *in vitro* and of U87MG glioblastoma xenografts in nude mice. In contrast, each of the U87MG/PTN 1–40 clonal cell lines examined exhibited a significant fraction of cells either tetraploid or, in one case, both tetraploid and aneuploid (Figure 1); it was found that 6.38 % of U87MG/PTN 1–40-clone-2, 28.95 % of U87MG/PTN 1–40-clone-3, 5.8 % of U87MG/PTN 1–40-clone-15, and 17.01 % of U87MG/PTN 1–40-clone-16 cells were tetraploid. Furthermore, 12.75 % of U87MG/PTN 1-40-clone-16 cells also were aneuploid (summarized in Table 1). The data thus demonstrate directly that the block in U87MG/PTN 1–40 cells effectively blocks normal chromosomal segregation, leading to tetraploidy and aneuploidy. Fuethermore, the profiles of the U87MG/PTN 1–40 glioblastoma cells demonstrated that 100% of the tetraploid and aneuploid cells in each of the four clonal U87MG/PTN 1–40 cell lines were arrested in the G1 phase of the cell cycle (Figure 1), suggesting that the tetraploid and aneuploid cells arrested in G1 are likely to be targeted for apoptosis [22].

The data thus demonstrate that each of the four U87MG/PTN 1–40 cell lines contain significant numbers of cells either tetraploid or aneuploid and each of the tetraploid or aneuploid cells is arrested in the G1 phase of the cell cycle. Expression of the dominant negative PTN thus profoundly disrupts normal chromosomal separation to induce tetraploidy and aneuploidy and a G1 arrest of the tetraploid and aneuploid cells. The data suggest the possibility that induction of tetraploidy and aneuploidy and the G1 arrest of these cells may be a mechanism to slow rate of growth and reverses the malignant phenotype of U87MG glioblastoma cells and, to the best of our knowledge, these data are the first data to suggest that PTN-signaling has an important role in cell cycle progression.

Discussion

Tetraploidy can arise by exit of a cell from mitosis following a failure of spindle assembly, chromosome segregation, or cytokinesis [23]. Aneuploidy frequently follows an intermediate state of tetraploidy and is commonly found in malignant cells when tetraploid cells respond to activation of other genes through additional mutations in the cancer cells during tumor progression [24,25]. These data in this manuscript demonstrate that expression of the dominant negative PTN in U87MG cells is associated with tetraploidy and aneuploidy and with a G1 arrest of the tetraploid and aneuploid U87MG cells; thus, interruption of constitutive PTN-

signaling initiates a failure of chromosomes to segregate and a G1 arrest of the tetraploid and aneuploid cells potentially leading to apoptotic cell death.

The data suggest several conclusions; they suggest that PTN-signaling is required for a critical step in chromosomal segregation and perhaps in regulation of the cell cycle; they suggest the possibility that inappropriate *Ptn* expression through de-regulation of a critical step in cell cycle progression and/or perhaps separation of chromosomes during mitosis may contribute to the pathogenesis of glioblastomas; they suggest that interruption of constitutive PTN signaling in U87MG glioblastoma cells may contribute to the reversal of their highly malignant phenotype through induction of tetraploidy and aneuploidy.

The site in progression of the cell cycle at which PTN may be required is unknown; however, different studies suggest the possibility that PTN regulates the cytoskeleton reorganization required for the cell division or for spindle assembly, chromosome segregation, or cytokinesis. In different studies, PTN has been shown to increase tyrosine phosphorylation of β-catenin, to disrupt the association of N-cadherin and β-catenin, and to disrupt adherens junction complexes and homophilic cell-cell adhesion in PTN-stimulated cells [26,27]. Pleiotrophin also has been found to activate protein kinase C (PKC) in PTN-stimulated cells; in PTN-stimulated cells, PTN stimulates the PTN-dependent PKC catalysed phosphorylation of serine residues 713 and 726 of β-adducin, which, when phosphorylated, reduces the affinity of β-adducin for actin and spectrin and disrupts β-adducin/actin/spectrin cytoskeletal complexes; the β-adducin/actin/spectrin complex is required for cytoskeleton stability and disruption of these complexes leads to distabilization of the cytoskeleton needed for cell division [28]. Perhaps the most likely site at which PTN signaling regulates cell cycle progression may be in centrioles of the mitotic spindle during mitosis; previous studies found that PTN stimulates serine phosphorylation of β-adducin at serines 713, 726 that is localized in centrioles of the mitotic spindle [28], suggesting that PTN may regulate the association of β-adducin with mitotic spindle protein complexes. The failure to disrupt these complexes in U87MG/PTN 1–40 cells through failure to activate PKC and thus to phosphorylate serines 713 and 726 of β-adducin in the U87MG/PTN 1–40 cells with a block in PTN signaling may lead to failure of chromosomal separation and tetraploidy and aneuploidy. Since the tetraploid and aneuploid cells fail to exit G1, it is postulated that the successive cell divisions will fail to progress beyond G1 and likely initiate apoptotic cell death, suggesting a potential mechanism to account for the loss of aggressive growth of U87Mg cells that express the dominant negative PTN. To the best of our knowledge, this study is the first to provide evidence that PTN signaling may be required for the orderly progression of cell cycle.

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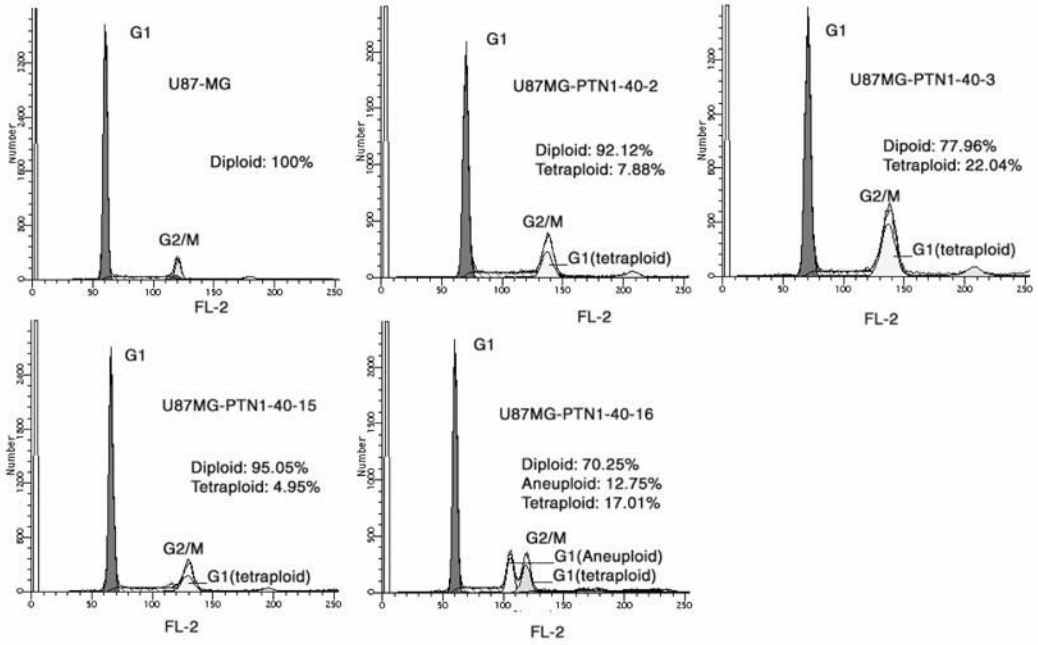

Figure 1.

Cell cycle of U87MG glioblastoma cells expressing PTN 1–40. Representative histograms are shown. Data was obtained and analyzed as described in Materials and Methods.

Table 1

Cell Cycle Profile of different U87MG Cell lines that express exogenous dominant negative PTN gene*.

Cell line	Diploid (%)	Tetraploid (%)	Aneuploid (%)
U87MG/vector	99.83	0.17	0
U87MG/PTN 1-40-2	93.62	6.38	0
U87MG/PTN 1-40-3	71.15	28.95	0
U87MG/PTN 1-40-15	94.2	5.8	0
U87MG/PTN 1-40-16	71.3	17.01	12.75

* One representative results from three repeat experiments.