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PLEIOTROPHIN GENE EXPRESSION IS HIGHLY RESTRICTED AND IS REGULATED BY PLATELET-DERIVED GROWTH FACTOR

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SUMMARY: Pleiotrophin (PTN) is a growth and neurite extension promoting polypeptide which is highly expressed in brain and in tissues derived from mesenchyme. The PTN gene is developmentally regulated and is closely related to the MK and RI-HB genes, both of which are developmentally regulated and induced by retinoic acid. We now have screened 17 cell lines and report that expression of the PTN gene in these cells is restricted to embryo fibroblasts and intestinal smooth muscle cells. However, NIH 3T3 cells stimulated by the platelet-derived growth factor (PDGF) express a marked increase in levels of PTN mRNA whereas retinoic acid failed to increase levels of PTN mRNA in NIH 3T3 cells or in F9 embryonal carcinoma cells within 72 hours of exposure. The results suggest that expression of the PTN gene is highly restricted and that the PTN gene is a new member of the PDGF-induced cytokine family.

Pleiotrophin (PTN) is an 18 kD heparin-binding protein which is a weak mitogen for mouse fibroblasts (1, 2) and a neurite outgrowth promoting activity in mixed neonatal rat brain cultures (2, 3). Its gene is highly conserved among human, rat, bovine, and mouse species, and is developmentally regulated (2, 4). PTN shares nearly 55% amino acid sequence identity with midkine (MK), the product of a retinoic acid responsive gene (5), which also mitogenically stimulates 3T3 fibroblasts and promotes neurite extension from PC-12 cells (6) and with RI-HB, also a retinoic acid induced, heparin-binding protein and a weak mitogen for PC-12 cells (7). Because of the highly conserved sequences and similar functions shared by PTN, MK, and RI-HB, we proposed that these related genes of heparin-binding cytokines be called the PTN family of developmentally regulated genes (2).

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We now have analyzed 17 representative cell lines by Northern analysis with the PTN cDNA. Of the 17 cell lines tested, PTN was observed to be significantly expressed in embryo fibroblasts and in intestinal smooth muscle cells. NIH 3T3 cells expressed low levels of the transcripts of PTN. NIH 3T3 cells also were analyzed after stimulation with growth regulatory molecules. Both PDGF and basic fibroblast growth factor (bFGF) sharply increased the levels of PTN mRNA in NIH 3T3 cells, whereas retinoic acid failed to increase levels of PTN transcripts in embryonal carcinoma (F9) cells or in NIH 3T3 cells within 72 hours of exposure. In contrast, transcripts of the MK gene were sharply upregulated in response to retinoic acid in F9 cells, in agreement with previous work (8).

MATERIALS AND METHODS

Cell lines and cell culture. Fetal bovine heart endothelial (FBHE), human intestinal smooth muscle (HISM), human medulloblastoma (TE 671), human neuroblastoma (SK-N-MC), human neuroblastoma (IMR-32), rat aorta smooth muscle (A-10), normal rat kidney fibroblasts (NRK-49F), simian sarcoma virus-transformed (SSV)-NRK, rat intestinal epithelial (IEC-6), rat adrenal pheochromocytoma (PC-12), rhabdomyosarcoma (RD), mouse neuroblastoma (NB41 A3), mouse squamous cell carcinoma (KLN 205), mouse embryonal carcinoma (EC, F9), mouse embryo fibroblasts (NIH 3T3), and mouse embryo fibroblasts (C3HT10 1/2) cells were obtained from American Tissue Culture Collection (ATCC) (Maryland). The human neuroblastoma (NLF) cell line was a kind gift of Dr. Garrett Brodeur (9). All cell lines were cultured and maintained in media according to ATCC recommendations. Fresh media was used one day prior to each experiment unless otherwise noted.

RNA preparation and Northern analysis. RNA was extracted by the guanidium-thiocyanate method (10) and quantitated by absorption at 260 nm, denatured in 1x MOPS buffer with 50% formamide, 12.5% formaldehyde at 80°C for 10 min., separated by electrophoresis in 1.5% agarose gels containing 6% formaldehyde in 1x MOPS buffer, and transferred to a nylon membrane (MSI). Membranes were probed with ^{32}P-labeled human or mouse cDNA probes (see figure legends) after labeling with random primers (Boehringer Mannheim GmbH). Prehybridizations and hybridizations were done at 42°C in 50% formamide, 5XSSC, 5x Denhart's solution, 0.1 mg/ml denatured salmon sperm DNA, and 0.1% SDS (pre-hybridization solution only) for 16-18 hours. After a wash in 0.2x SSC, 0.1% SDS (42°C) and two washes (same buffer) for 30 min. at 65°C, the filters were exposed to Kodak X-Omat film for 48-72 hours with intensifying screens and analyzed.

Materials. Human recombinant platelet-derived growth factor (PDGF-AB) was obtained from Upstate Biotechnology, Inc. (New York). Actinomycin D, cycloheximide, and retinoic acid were purchased from Sigma Chemical Co. (St. Louis, MO), and were of the highest grade available.

RESULTS

The patterns of expression of the PTN gene were distinct and reproducible. Transcripts of the PTN gene were strongly expressed in the positive control (whole neonatal rat brain RNA) and were considerably more weakly expressed in human intestinal smooth muscle cell lines (HISM) and in mouse embryo fibroblast cell lines (NIH 3T3, C3Ht10 1/2) (Fig. 1). Under conditions of analysis, PTN gene transcripts were not detected in the other

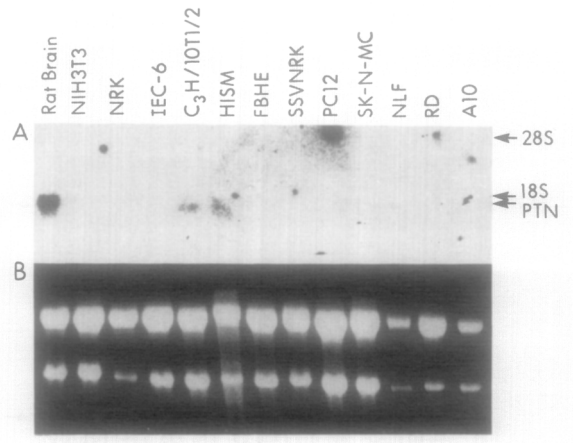

Fig. 1. Expression of PTN in different cell types. All cell lines were cultured and maintained in media according to recommendations of ATCC and changed to fresh media one day prior to use. Total cellular RNA was extracted by the guanidium-thiocyanate method and subjected to Northern blot analysis (20 µg of RNA/lane). (Upper) Northern blot probed with human PTN cDNA probe. (Lower) Ethidium bromide staining of RNA. Arrows indicate 28S and 18S rRNA subunits and the PTN mRNA (1.5 kb). The filter was exposed at -70°C for 3 days.

cell lines examined, including NRK, SSV NRK, IEC-6, FBHE, RD, A-10, NLF, SK-N-MC, PC-12 NLF cells (Fig. 1). In addition, transcripts were not seen at NB41, TE671, IMR32, and KLN205 cells (data not shown). Twenty µg whole cell RNA was tested in each Northern analysis. Thus, the patterns of expression of the PTN gene seen agreed with previous results (2) of whole organ analysis in which it was found that PTN transcripts were highly expressed in brain and to a lesser degree in intestine.

Because PTN transcripts are expressed in a highly cell type restricted pattern, and thus were tightly regulated, we explored other mechanisms which might regulate expression of the PTN gene. PDGF is known to regulate expression of normally quiescent genes some of which themselves encode cytokines (11-13). Because NIH 3T3 cells express limited levels of the transcripts of PTN and, because NIH 3T3 cells are strongly responsive to PDGF (14), serum starved (36 hours) NIH 3T3 were stimulated with PDGF. The levels of PTN mRNA were compared in Northern analyses of control cells and cells serially after the addition of PDGF. As seen in Fig. 2, the addition of PDGF resulted in a transient but highly significant increase in levels of PTN mRNA; transcripts of the expected size (~ 1.5 kb) were significantly increased 2, 4, and 12 hours after stimulation of NIH 3T3 cells with 100 ng/ml PDGF. Peak levels of the PTN gene transcripts were found at 4 hours (Fig. 2). The overall increases were estimated by gel scanning as 3.5-fold at 2 hours, 6-fold at 4 hours, and 4-fold at 12 hours (Fig. 2, n=3). PTN expression thus

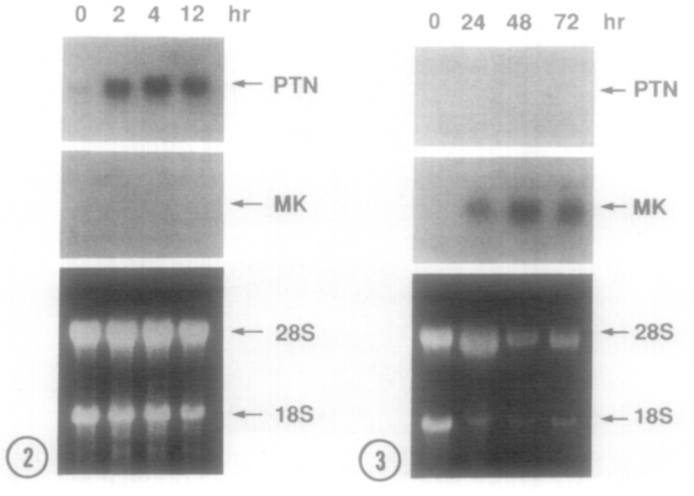

Fig. 2. Effect of PDGF on the levels of PTN and MK mRNA in NIH 3T3 cells as determined by Northern blot hybridization. NIH 3T3 were rendered stationary by 36 hours of serum-free growth in DME media with high glucose (4.5 g/l). The cells were stimulated with 100 ng/ml PDGF for different time periods. Total RNA samples (20 µg/lane) were separated on 1.5% formaldehyde containing agarose gels, blotted onto nylon membranes, and hybridized to a ^{32}P-labeled mouse PTN cDNA (isolated from a mouse brain cDNA library [Stratagene #937301], 16) and an MK cDNA probe (isolated from mouse teratocarcinoma [PCC4] cDNA library [Stratagene #936301], 16), respectively. (Upper) Northern blot probed with mouse PTN cDNA: filter exposed at -70°C for 2.5 days. (Middle) Northern blot probed with mouse MK cDNA: filter exposed at -70°C for 4 days. (Lower) Ethidium bromide staining of the RNA gel. Positions of the 28S and 18S rRNA subunits and of the PTN and MK mRNAs are indicated.

Fig. 3. Effect of retinoic acid on the level of PTN and MK mRNA levels in F9 cells as determined by Northern blot analysis. F9 cells were maintained in DME media with high glucose (4.5 g/l), 15% FBS, and retinoic acid (1 µM) for 24, 48, 72 hours, respectively. The samples of total RNA (20 µg/lane) were analyzed by Northern hybridization with mouse PTN and MK cDNA probes separately as described in Fig. 2. (Upper) Northern blot probed with mouse PTN cDNA: filter exposed at -70°C for 4 days. (Middle) Northern blot probed with mouse MK cDNA: filter exposed at -70°C for 2 days. (Lower) Ethidium bromide staining of the RNA gel. Positions of the 28S and 18S rRNA subunits and of the PTN and MK mRNAs are indicated.

may be regulated *in vivo* by PDGF and perhaps its transcripts may be expressed at different times in the cell cycle. The increase in levels of PTN mRNA also was observed when NIH 3T3 cells were stimulated with bFGF; the temporal pattern of increase of PTN transcripts induced by bFGF is similar to that seen when these cells were stimulated by PDGF (data not shown). The results thus indicate that the PTN gene is a new member of the family of PDGF inducible cytokine genes.

The MK gene product is strongly induced by retinoic acid in F9 embryonal carcinoma cells (8). F9 cells form parietal endoderm-like cells in the presence of retinoic acid (15). Since the amino acid sequence of PTN is 55% identical with the predicted product of the MK gene, levels of PTN transcripts were estimated in F9 embryo carcinoma cells treated continuously with retinoic

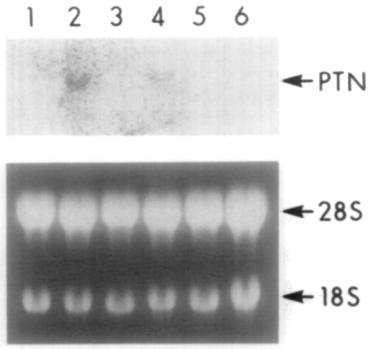

Fig. 4. Effect of cycloheximide and actinomycin D on PTN mRNA levels in NIH 3T3 cells stimulated by PDGF. Serum-starved cells (lane 1) were treated for 4 hours with PDGF (100 ng/ml, lanes 2, 4, 6) and/or cycloheximide (CHX, 10 µg/ml, lanes 3, 4) or actinomycin D (Act-D, 2 µg/ml, lanes 5, 6). Total RNA samples of 20 µg/lane were used. (Upper) Northern blot with mouse PTN cDNA probe. (Lower) Ethidium bromide staining of the RNA gel. Positions of the 28S and 18S rRNA subunits and of PTN are indicated. The filter was exposed at -70°C for 3 days.

acid (1.0 µM) for 24, 48, and 72 hours. Levels of the 1 kb transcripts of the MK gene rose in precise agreement with results of Kadomatsu et al. (5). However, transcripts of the PTN gene were not detected at any time point tested in this experiment (Fig. 3). Conversely, when MK expression was tested in NIH 3T3 cells stimulated with PDGF, transcripts of the MK gene were not detected (Fig. 2) under conditions where PTN transcripts were shown to be significantly induced (Fig. 2). This result indicates that PTN does not detectably respond under conditions that MK readily responds to retinoic acid; furthermore, MK is not detectably responsive to PDGF under conditions that PTN expression is sharply increased. Both cycloheximide (10 µg/ml) and actinomycin D (2 µg/ml) blunted significantly the increase in levels of expression of the PTN gene in PDGF stimulated cells, suggesting that the induction of PTN mRNA by PDGF requires both RNA and protein synthesis (Fig. 4).

DISCUSSION

PTN was first observed as a weak mitogen for murine fibroblasts (1), and as a neurite outgrowth promoting factor from rat brain (3). The deduced amino acid sequence of the PTN cDNA is related to midkine (MK) and RI-HB, both of which also stimulate neurite outgrowth and which are weak mitogens. MK mRNA has a widespread distribution during mouse midgestation but becomes restricted to the kidney in adulthood (5). The RI-HB protein is widely expressed in basement membranes during early chick embryogenesis but later is restricted to the lens capsule (7). The restricted, cell specific pattern of expression of

the PTN gene observed here thus is consistent with the restricted pattern of expression of the other members of the PTN gene family and suggests that the PTN family of genes are highly regulated. The increases in levels of PTN gene expression in fibroblasts stimulated with PDGF and bFGF further suggest a complex regulation of this gene and also raise the possibility that the PTN gene product may play a role in early-mid phases of the cell cycle. However, the function of the PTN family of proteins in vivo remains unknown.

This work establishes that of 17 cell lines examined, expression of the PTN gene is restricted to embryonic fibroblasts and smooth muscle cell lines. It is also strongly expressed in neonatal rat brain. This data and earlier work (2) which indicated a significant degree of developmental regulation of PTN gene expression supports the general proposal that PTN may be important in developmental programs. Additional experiments are needed to identify the precise sites and cell types expressing PTN in vivo and to provide additional clues to its possible functions in development. This work also establishes that PDGF and bFGF sharply upregulate expression of the PTN gene and thus PTN may have a role related to the cell cycle and/or a role at sites where cytokines are released such as in injury or in association with abnormal cell proliferation.

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