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Short Communication

Expression of Pleiotrophin and its Receptors in Human Placenta Suggests Roles in Trophoblast Life Cycle and Angiogenesis

M. Ball [a], M. Carmody [a], F. Wynne [a], P. Dockery [b], A. Aigner [c], I. Cameron [d], J. Higgins [e], S.D. Smith [f], J.D. Aplin [f,*], T. Moore [a,**]

[a] Department of Biochemistry, Biosciences Institute, University College Cork, College Road, Cork, Ireland
[b] Department of Anatomy, National University of Ireland Galway, Galway, Ireland
[c] Department of Pharmacology and Toxicology, Philipps-University Marburg, Marburg, Germany
[d] Developmental Origins of Health and Disease Division, University of Southampton, Princess Anne Hospital, Southampton, UK
[e] Anu Research Centre, Department of Obstetrics and Gynaecology, University College Cork, Cork University Maternity Hospital, Cork, Ireland
[f] Maternal and Fetal Health Research Group, University of Manchester, Manchester, UK

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ABSTRACT

Pleiotrophin (PTN) is a heparin-binding protein with multiple activities in cell growth, migration and differentiation mediated through multiple receptors. In mammals, PTN expression in trophoblast is found exclusively in the human and in some of the apes in which an endogenous retrovirus upstream of the first coding exon generates a phylogenetically new trophoblast-specific promoter associated with exon UV3. To understand the functions of ERV promoter-mediated trophoblastic PTN expression in pregnancy, we correlated the expression of PTN and its receptors anaplastic lymphoma kinase (ALK), receptor protein tyrosine phosphatase beta/zeta (RPTPbeta/zeta), and Syndecan-1 and Syndecan-3 (SDC1 and SDC3) with key developmental processes in first-trimester human placentation. In an extensive survey of cell lines and primary tissues, we found that trophoblastic transcription of PTN is initiated exclusively from the ERV promoter, whereas decidual expression is initiated at the phylogenetically ancient U1 exon-associated promoter. Using immunohistochemistry, we found that different patterns of overlapping expression of PTN and its receptors occur in different trophoblast subtypes. Notably, a role in angiogenesis is supported by expression of PTN and its receptors in villous mesenchyme, fetal macrophages and villus core fetal vessels. PTN staining of extravillous cytotrophoblasts and the syncytial microvillous membrane is consistent with increasing levels of PTN, as measured by ELISA, in the maternal bloodstream as pregnancy progresses.

1. Introduction

Pleiotrophin (PTN) is a low molecular weight heparin-binding protein that is closely related to midkine (MK). PTN interacts with multiple receptors including anaplastic lymphoma kinase (ALK) [1], receptor protein tyrosine phosphatase beta/zeta (RPTPβ/ζ) [2], syndecans (SDC1, SDC3 and SDC4) [3,4] and nucleolin [5]. Its biological activities include promotion of growth, cell migration, tissue morphogenesis and chemokine expression in numerous target cell types. Although a *Ptn* null mouse mutant is viable, a *Ptn*/*Mk* double null mutant exhibits a severe phenotype of runting and female infertility, with a deficit of double mutants at birth [6].

In human and chimpanzee, the insertion of an endogenous retrovirus (ERV) downstream of the PTN promoter-associated U1 exon drives expression specifically in trophoblast [7,8], an expression pattern not found in the mouse. As a step to understanding PTN function during human pregnancy, we report a detailed study of the expression of PTN and its receptors in placenta.

2. Materials and methods

Unless otherwise stated all reagents were obtained from Sigma Aldrich, Dublin, Ireland.

2.1. Cell lines

JEG-3, JAR, BeWo, HeLa and MRC5 were obtained from ATCC. NT2 was obtained from Stratagene. SGHPL4 was a gift from Dr G. Whitley. SHSY5Y was obtained from ECACC. AZ521 was obtained from Japan Health Sciences Foundation. All cells were cultured at 37 °C with 5% CO_2 in the recommended culture media, supplemented with 10% fetal bovine serum.

* Corresponding author. Tel.: +44 (0)161 276 6487; fax: +44 (0)161 276 6134.
** Corresponding author. Tel.: +353 (0)21 490 1339; fax: +353 (0)21 490 1382.
E-mail addresses: john.aplin@manchester.ac.uk (J.D. Aplin); t.moore@ucc.ie (T. Moore).

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2.2. Tissue collection

First-, second- and third-trimester maternal blood from uncomplicated pregnancies was collected at Cork Unified Maternity Services as approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals. Samples (1–5 ml) were collected by venipuncture into Greiner Bio-one EDTA K3 bottles (Cruinn Diagnostics Ltd, Dublin, Ireland). After low speed centrifugation serum was stored at −80 °C.

First-trimester placentas ($n = 9$; age range 8–9.5 weeks post-conception) were collected at elective termination at St. Mary's Hospital, Manchester under approval of the Central Manchester Local Ethics Committee. Term placentas ($n = 4$) were collected at caesarean section or vaginal delivery. Women with essential hypertension and medical complications such as diabetes and renal disease were excluded. Fresh villous tissue was dissected in serum-free DMEM/F12 medium (Biowhittaker, UK), fixed in 4% neutral buffered formalin pH 7.6 for 24 h at 4 °C and stored in phosphate-buffered saline (PBS) before processing into wax.

2.3. Antibody validation, tissue preparation, immunohistochemistry (IHC) and immunofluorescence (IF)

Sections from paraffin-embedded first-trimester and term placental tissues were dewaxed and immunostained using a conventional immunoperoxidase protocol after antigen retrieval. Anti-cytokeratin 7 (mouse monoclonal OV-TL; Dako Cytomation, Dublin, Ireland) was used to detect trophoblast. We predominantly used the PTN N15 Ab (goat polyclonal; Santa Cruz, Fannin, Dublin, Ireland) raised against a 15-amino-acid peptide from the N-terminus, which produced consistent, robust immunostaining, as previously reported for other tissues [9,10]. PTN N15 Ab was used for both IHC and IF. Two other PTN Abs from Santa Cruz (C19 and H75) performed poorly in IHC and WB and were not used further. Because we obtained anomalous results (increased background staining) with N15 peptide in antibody blocking experiments, we undertook additional analyses to validate PTN N15 Ab. First, we observed similar staining using two PTN Abs raised against full-length recombinant human PTN (Abcam, Cambridge, UK; R&D, Abingdon, UK), to that obtained using the PTN N15 Ab. Second, recombinant full-length PTN (R&D Systems, Abingdon, UK) was detected by the N15, Abcam and R&D Abs at 18 kDa on Western blot (WB). In addition, these Abs detected bands of 21 kDa and 18 kDa on WB of HeLa cells transfected with DNA vectors over-expressing His/Streptavidin tagged and untagged PTN, respectively.

Antibodies used in IHC to detect PTN receptors were: anti-RPTPβ (mouse monoclonal; Transduction Laboratories,), anti-ALK (goat polyclonal; Santa Cruz), anti-Syndecan 1 (goat polyclonal; Santa Cruz) and anti-Syndecan-3 (rabbit polyclonal; Santa Cruz,). Secondary antibodies used for IHC were: goat-anti-rabbit IgG, rabbit-anti-goat IgG and horse-anti-mouse IgG (all from Vector Laboratories, all biotinylated). Secondary antibodies used for IF were: FITC conjugated donkey-anti-goat IgG and rhodamine conjugated donkey-anti-mouse IgG (Jackson Laboratories). The Vector Stain Elite ABC system and Vector VIP substrate were used for detection in IHC.

2.4. ELISA measurement of PTN protein in maternal serum

Frozen serum samples (500 μl) were thawed and PTN protein concentration was analysed using an ELISA assay carried out in covered 96-well ELISA plates (Life Technologies, Karlsruhe, Germany) at 4 °C overnight as described [11]. The detection system comprised a mouse anti-PTN monoclonal antibody (4B7) diluted to 1 μg/ml in Tris-buffered saline, a biotinylated affinity-purified goat-anti-human PTN secondary antibody (R&D, Wiesbaden, Germany) at a concentration of 500 ng/ml, and detection using a streptavidin/alkaline phosphatase conjugate (Roche Diagnostics, Mannheim, Germany) with absorbance measured in a plate reader at 405 nm. Recombinant human PTN (R&D Systems) was used as a standard.

2.5. Reverse transcription–polymerase chain reaction (RT–PCR)

Placental tissue RNA was isolated using 1 ml TRI-reagent (Sigma, Dublin, Ireland). cDNA was synthesized using 0.5–2.0 μg total RNA as template in a 20 μl reaction using random hexamer priming and MMLV reverse transcriptase (Invitrogen, Biosciences, Dublin, Ireland). Similarly, cDNA was prepared from cell lines (JEG-3, JAR, BeWo, SHGPL4, MRC5, HeLa, AZ521, NT2, SHSY5Y). To determine PTN promoter usage in placental tissues and cell lines, primers were used that specifically amplify UV3-O2 and U1-O2 PTN transcripts (UV3 PTN primer 5′-CCT GAC TTG CTC AGT CGA TC-3′, U1 PTN primer 5′-GTC AGG GCG TAA TTG AGT C-3′, O2 PTN primer 5′-CTG GGT CTT CAT GGT TTG C-3′). Primers spanning the PTN ORF were also used (PTN ORF-for 5′-ATG CAG GCT CAA CAG TAC CAG-3′ and PTN ORF-rev 5′-TTA ATC CAG CAT CTT CTC CTG TTT C-3′). PCR was carried out in 50 μl reaction using 2.5 U Taq DNA Polymerase with ThermoPol buffer (New England Biolabs, ISIS, Bray, Ireland). Amplification protocol used was: 95 °C 5 min, 35 cycles of 95 °C for 45 s, 55 °C for 45 s, 72 °C for 90 s, and an elongation step of 72 °C for 10 min.

3. Results

3.1. PTN protein is widely expressed in trophoblastic lineages and in the villous core

We carried out an extensive analysis of first-trimester trophoblast tissues ($n = 9$) using IHC and IF. PTN staining was observed in the cytoplasm of extravillous trophoblast cells in columns, with a distal increase of intensity (Fig. 1A). In villous cytotrophoblast, PTN localisation was perinuclear and near the basement membrane (Fig. 1B). Not all villous cytotrophoblast was stained (Fig. 1B). Intense staining was also observed on the syncytial microvillous membrane (Fig. 1A,B), and in syncytial sprouts (data not shown). Fainter cytoplasmic staining was observed in the syncytiotrophoblast (Fig. 1B), and staining of variable intensity was found in the mesenchyme and fetal macrophages (Fig. 1B), and in villous core fetal vessels (data not shown). Double immunofluorescence for PTN and cytokeratin 7 (CK) was used to confirm staining in cytotrophoblast lineages (data not shown).

Analysis of first-trimester decidua basalis ($n = 3$) was facilitated by staining serial sections with anti-CK antibody. Moderate cytoplasmic staining was observed in CK-negative decidual cells, presumably arising from U1 promoter transcription (Fig. 1C–F). Interstitially migrating cytotrophoblast sometimes showed moderate staining (Fig. 1C–F). Staining of varying intensity was observed in endovascular trophoblast and cytotrophoblast plugs in arterial lumens were variably immunopositive (Fig. 1E,F). This pattern of staining was confirmed using double immunofluorescence for PTN and CK (data not shown). Vascular endothelium of untransformed blood vessels did not stain for PTN or CK (Fig. 1C,D).

In normal term placental samples ($n = 4$), PTN staining was predominantly observed on the syncytial microvillous membrane (Fig. 1G). Some punctate staining was also observed in the villous core, including in intermediate villi close to blood vessels; however, the vessels themselves were not always stained (Fig. 1G).

To confirm trophoblastic expression of PTN from the ERV promoter, we used promoter-specific primers and RT–PCR to examine expression in cell lines and placental samples. Primers spanning the PTN open reading frame (ORF) amplified the expected product in all cell lines examined and in all placental samples (data not shown). Primers specific for the ERV promoter transcript (UV3 exon) amplified a product predominantly in trophoblastic tissues and the placental cell lines JEG-3, JAR, BeWo, SGHPL4. UV3 (in addition to U1) transcripts were also found in cDNA from fetal and adult thymus (AMS Biotechnology, Abingdon, UK). Primers specific for the phylogenetically ancient promoter (U1) amplified a product in a subset of samples comprising non-trophoblastic cell lines (HeLa, SHSY5Y, NT2, MRC5), and in maternal decidual tissue.

3.2. PTN receptors co-localise with PTN in trophoblastic tissues

IHC was used to determine the pattern of expression of PTN receptors in placental tissues. ALK exhibited cytoplasmic staining in cytotrophoblast columns, which increased in intensity in distal portions (Fig. 2A). Intense punctate perinuclear staining in villous cytotrophoblast was also observed (Fig. 2A–C). ALK staining was also observed in the mesenchyme and villus core fetal vessels (Fig. 2B,C). Limited areas of the syncytial microvillous membrane stained for ALK (Fig. 2B,C).

Intense staining for SDC1 was observed distally in cytotrophoblast columns and on the syncytial microvillous membrane (Fig. 2D,E). SDC1 staining was also observed in some villous