OsteoArthritis and Cartilage (2007) **15,** 155–162

doi:10.1016/j.joca.2006.07.005

Osteoarthritis and Cartilage

International Cartilage Repair Society

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Effects of pleiotrophin, a heparin-binding growth factor, on human primary and immortalized chondrocytes[1]

Dr. T. Pufe Ph.D.†, G. Groth†, M. B. Goldring Ph.D., Professor‡, Dr. B. Tillmann M.D., Professor† and R. Mentlein Ph.D.†*

† *Department of Anatomy, University of Kiel, D-24098 Kiel, Germany*

‡ *Beth Israel Deaconess Medical Center, Department of Medicine/Rheumatology, Harvard Institutes of Medicine, Boston, MA 02115, USA*

Summary

Objective: Pleiotrophin (PTN) is a secreted heparin-binding peptide expressed in mesodermal and neuroectodermal cells during development, but rarely in adult tissues. In fetal and juvenile, but not in mature cartilage, PTN is abundant. Furthermore, PTN is re-expressed in chondrocytes in early stages of osteoarthritis (OA). Since little is known about the functions of PTN in cartilage, we investigated the occurrence of PTN receptors in human articular cartilage *in situ* and PTN effects on human primary and immortalized chondrocytes *in vitro*.

Methods: Receptor expression and regulation was monitored by quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) and immunohistochemistry. PTN effects and signal transduction were studied by electrophoretic mobility shift, Boyden chamber cell migration and proliferation assays, effects on gene expression by real time RT-PCR and that on nitric oxide (NO) by the Griess reaction.

Results: Of the putative PTN signaling receptors, immortalized and primary chondrocytes (pc) expressed the anaplastic lymphoma kinase (ALK), less the receptor-type protein tyrosine phosphatase ζ/β (PTPζ). ALK expression was upregulated upon ligand exposure. PTN stimulation activated the AP-1 (activator protein-1) transcription factor and altered gene expression. Prolonged stimulation induced PTN mRNA expression slightly, reduced vascular endothelial growth factor (VEGF) mRNA as well as NO production. Whereas mRNA expression of matrix metalloproteinases (MMPs) MMP-1 and MMP-13 was reduced, their inhibitors TIMP-1 and TIMP-2 were induced. Furthermore, PTN stimulated chondrocyte migration and proliferation.

Conclusions: These results show that PTN is an autocrine growth factor in cartilage. We suggest that PTN may be involved in the clustering and proliferation of chondrocytes observed in the early stages of OA.

Key words: Pleiotrophin, Growth factor, Chondrocytes, Cartilage, Signal transduction, Gene expression, Chemotaxis, Osteoarthritis.

Abbreviations: ELISA enzyme-linked immunoassay, OA osteoarthritis, PTN pleiotrophin, RT-PCR reverse transcriptase-polymerase chain reaction.

Introduction

Pleiotrophin (PTN), also known as heparin-binding growth-associated molecule (HB-GAM), heparin-binding growth factor 8, heparin-binding neurotrophic factor (HBNF) and osteoblast-specific protein-1 (OSF-1), is an 136-amino acid (15.3 kDa) secreted growth/differentiation cytokine that is developmentally regulated (for reviews, see Refs. 1–4). Mature PTN is a non-glycosylated, lysine-rich peptide that is derived from an 168 residue precursor with a 32-amino acid signal sequence. PTN is a differentiation or growth factor for various cell types (therefore named pleiotrophin): it has mitogenic, anti-apoptotic, transforming, angiogenic and chemotactic biological activities that can differ between its target cells. Together with midkine (MK) it forms a family of heparin-binding proteins that are normally expressed during embryogenesis, but only at low levels in healthy adult tissues.

Both, PTN and MK bind with high-affinity to extracellular heparan-sulfate proteoglycans as well as to cell surface syndecans, LDL receptor-related protein (LRP), anaplastic lymphoma kinase (ALK), and the receptor-type protein tyrosine phosphatase ζ/β (PTPζ). However, signaling effects appear to be mediated only by ALK and PTPζ and include phosphorylation of phosphatidylinositol-3-kinase (PI3-kinase), extracellular-signal related kinases (Erk) and further intracellular proteins like β-catenin that is involved in the transcription and the transformation of the cytoskeletal architecture[5–8].

Previous studies have mostly focused on PTN/MK effects in neurons and neurite outgrowth, osteoblasts and bone formation, fibroblast and neutrophil activation in chronic inflammatory diseases like rheumatoid arthritis, cancer cells and cancer angiogenesis[1–4,9,10]. However, less is known about their functions in cartilage, despite PTN is an abundant protein in fetal and juvenile, but not in adult cartilage except in early stages of osteoarthritis (OA)[11–14]. Therefore, with immortalized and primary human chondrocytes as

[1]Financial support: This work was supported by a grant from the Deutsche Forschungsgemeinschaft DFG (PU 214/3-1). Dr Goldring's research is supported by NIH grants AG22021 and AR45378.

*Address correspondence and reprint requests to: Prof Dr Rolf Mentlein, Ph.D., Department of Anatomy, Institute of Anatomy, University of Kiel, Olshausenstraße 40, D-24098 Kiel, Germany. Tel: 49-431-880-2460; Fax: 49-431-880-1557; E-mail: rment@anat.uni-kiel.de

Received 28 October 2005; revision accepted 13 July 2006.

models, we analyzed the expression of PTN signaling receptors, PTN-mediated signal transduction pathways, and PTN effects on chondrocytes. Prolonged (24 h) PTN exposure regulated the gene expression of PTN, vascular endothelial growth factor-A (VEGF-A, in the subsequent text simply termed VEGF), matrix metalloproteinases (MMPs) and their endogenous tissue inhibitors (TIMPs) in diverse ways. Furthermore, PTN reduced nitric oxide (NO) production and induced chondrocyte chemotaxis as well as proliferation. Thus, PTN appears to be a novel autocrine factor in cartilage with mainly protective functions.

Materials and methods

CELL CULTURES AND PEPTIDES

For most experiments we used immortalized human costal chondrocytes (C28/I2) which have been previously characterized to express collagen type II and aggrecan strongly, lesser collagen X. Furthermore, they produce MMPs MMP-1, MMP-3 and MMP-13 that can be induced by interleukin-1β (IL-1β), and VEGF. These differentiated chondrocytes were cultured as described[15–18]. Primary articular chondrocytes for verification of the C28/I2 experiments were derived from elderly patients (67–80 years) undergoing joint replacement due to fracture. These "normal" chondrocytes produce strong amounts of collagen type II as well as slight amounts of collagen X indicating early OA changes. They were obtained from Oligene® (Berlin, Germany). Cells were shipped proliferating and were expanded into five subcultures at densities of 500,000 cells per flask for further experiments. The company vouches for given consent of the patient. The glioma cell line U343 (Deutsches Krebsforschungszentrum, Heidelberg, Germany) served as positive control[19]. Recombinant human PTN and other cytokines/growth factors were from PepproTech (Rocky Hill, NJ, USA).

TISSUES

Tibial plateaus were obtained from patients with OA who underwent joint replacement (Clinic for Orthopedic Surgery, University of Kiel, Germany), and normal cartilage was obtained from body donors (Department of Anatomy). From the center of each side, a biopsy of normal cartilage and subchondral bone was taken and the tissue was fixed in 4% formaldehyde in phosphate-buffered saline (PBS), pH 7.4, for histological and immunohistochemical analyses. Total RNA was extracted from crushed cartilage with the RNeasy Total RNA Kit (Qiagen, Hilden, Germany) as described[20]. The glioblastoma multiforme samples were obtained from patients who underwent neurosurgery (Department of Neurosurgery, University of Kiel, Germany). All human samples were obtained with the patients' or donors' consent, the permission of the responsible committee of the University of Kiel and in accordance with the Helsinki Declaration of 1975.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Specimens were decalcified in 10% ethylenediaminetetraacetic acid (EDTA) at 40°C for several days until X-ray examination revealed complete decalcification. Then, sections were dehydrated and embedded in paraffin. Deparaffinized 8 μm sections were stained with toluidine blue and examined by light microscopy. The stage of OA was classified using the score according to Mankin. For immunohistochemistry, sections were dewaxed, incubated with testicular hyaluronidase (2 mg/ml in PBS, pH 5.0, for 30 min at room temperature) and pronase (1 mg/ml in PBS, pH 7.4, 30 min at room temperature)[20]. For chromogenic immunostaining, sections were incubated with anti-ALK (1:40; rabbit polyclonal, 51-3900 from Zymed, San Francisco, CA, USA) followed by biotinylated secondary antibodies and a peroxidase-labeled streptavidin–biotin staining technique; nuclei were counterstained with hemalum[20]. Controls were performed either by omitting the primary antibody or by absorption of the primary antibody to recombinant human ALK (2 μg/500 μl) overnight at 4°C.

RNA PREPARATION, cDNA synthesis, and real time PCR

Cells cultivated in medium containing 10% fetal calf serum (FCS) for 3 days (80% confluence) were exposed to stimulators for 24 h in serum-free medium after 4 h preincubation with only serum-free medium. Total RNA was extracted with the RNeasy Total RNA Kit (Qiagen, Hilden, Germany), contaminating DNA destroyed by digestion with RNase-free DNase-I (20 min at 25°C; Boehringer, Mannheim, Germany), and cDNA generated from 100 ng RNA with 1 μl (20 pmol) of oligo(dT)15 primer (Amersham Biosciences, Amersham, UK) and 0.8 μl of superscript RNase H-reverse transcriptase (Gibco, Paisley, UK) in 50 μl for 60 min at 37°C[21]. For each sample, a control without reverse transcriptase was run parallel to allow assessment of genomic DNA contamination.

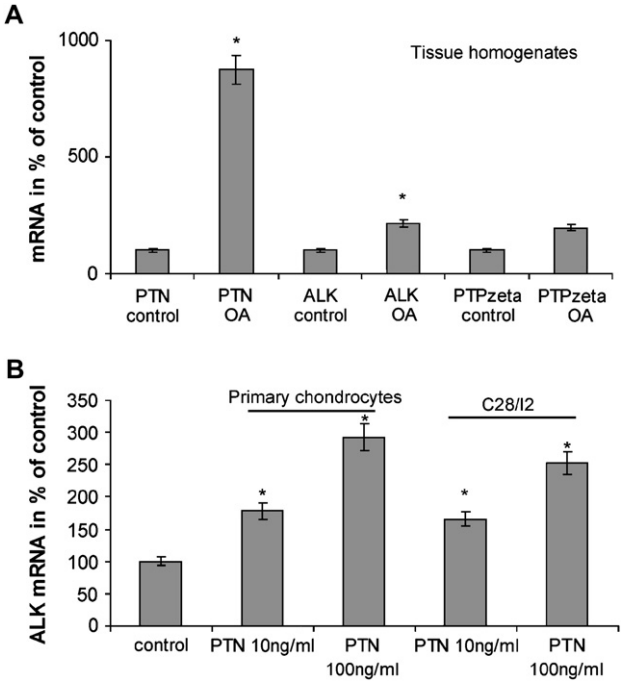

Fig. 1. Expression of mRNA for PTN and its putative signaling receptors ALK and receptor-type PTPζ in human cartilage tissue samples (A) and their regulation in cultivated chondrocytes (B) as determined by real time RT-PCR. (A) Samples of healthy articular cartilage (control) show a low expression of PTN, whereas in surgical sample from OA patients PTN and ALK are considerably higher. Donors: $n = 6 \pm$ SD. (B) Immortalized human chondrocytes and pc were stimulated for 24 h with/without PTN at increasing concentrations, and ALK mRNA expression was monitored by quantitative RT-PCR. $n = 6 \pm$SD; $P \leq 0.01$ control vs PTN.