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1α,25-Dihydroxyvitamin D_3 down-regulates pleiotrophin messenger RNA expression in osteoblast-like cells

Masato Tamura[1], Fumihiko Ichikawa[1], R. Paul Guillerman[2], Thomas F. Deuel[2] & Masaki Noda[1]

[1]*Department of Molecular Pharmacology, Division of Functional Disorder Research, Medical Research Institute, Tokyo Medical and Dental University, Tokyo 101 Japan;* [2]*Department of Medicine and Biochemistry and Molecular Biophysics, Washington University School of Medicine, Jewish Hospital, St. Louis, Missouri 63110, USA*

Pleiotrophin (PTN)[heparin-binding-growth-associated molecule (HB-GAM), heparin-binding neurite-promoting factor (HBNF)] is a recently identified polypeptide that stimulates growth of fibroblasts and enhances neurite extension. PTN is expressed in many tissues but relatively high level of expression has been observed in brain and bone. We examined hormonal regulation of PTN mRNA expression in several osteoblast-like cell lines including MC3T3-E1 and ROS17/2.8. The levels of PTN mRNA in these cells was significantly reduced by treatment with 10^{-8} M 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) for 24 h. However, PTN mRNA levels were increased when the non-osteoblastic cell line, ROS 25/1, was treated with 1,25$(OH)_2D_3$. These effects were observed in a dose-dependent manner in a dose range between 10^{-11} M to 10^{-8} M . This effect was specific to 1,25$(OH)_2D_3$, since PTN mRNA levels were not affected by other steroids such as retinoic acid and dexamethasone in MC3T3-E1 or ROS17/2.8 cells. Similar 1,25$(OH)_2D_3$ down-regulation of PTN mRNA was also observed in primary cultures of osteoblast-enriched fetal rat calvaria cells as well as cultures of MC3T3-E1 and ROS17/2.8 cells. These observations suggest that PTN expression in osteoblasts is regulated by the calcitropic hormone, 1,25$(OH)_2D_3$, and that PTN may play a role in vitamin D-dependent regulation of bone metabolism.

Keywords: pleiotrophin; vitamin D_3; osteoblast

Introduction

Pleiotrophin (PTN) was first identified as a heparin-binding protein which possessed mitogenic activity in rat and mouse fibroblasts (Milner *et al.*, 1989) and as a factor that promoted neurite outgrowth in cultures of neonatal rat brain cells (Rauvala, 1989). It was first purified from bovine uterus and neonatal rat brain and subsequently its expression has been observed in many tissues including brain, bone and kidney. The predicted amino acid sequence of PTN indicated that PTN protein is rich in basic amino acids and is particularly lysine rich in both N- and C- terminal domains. The amino acid sequence of PTN is highly conserved among various mammalian species (Li *et al.*, 1990). PTN also has been isolated from rat adult brain as a neurite outgrowth-promoting factor and was named as heparin-binding-growth-associated molecule (HB-GAM) (Merenmies & Rauvala, 1990), or heparin-binding neurite-promoting factor (HBNF) (Kuo *et al.*, 1990). Recently, a PTN-like protein (bone lysine-rich 18 kDa protein) was also purified from bovine bone and was shown to enhance cell adhesion, stimulate proliferation, and increase alkaline phosphatase activity in MC3T3-E1 cells (Zhou *et al.*, 1992). Tezuka *et al.* (1990) used differential cloning to isolate cDNAs that are expressed abundantly in osteoblastic cells relative to fibroblasts and established that these genes were identical to mouse and human PTN. Bone matrix is known to be a storage site for a number of heparin-binding growth factors, called matrikines, which affect osteoblastic functions in an autocrine and/or paracrine manner. PTN thus appears to be a new member of the matrikine family and perhaps is involved in regulation of bone metabolism *in vivo*.

The hormonally active metabolite of vitamin D, 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), is one of the calcitropic hormones which is required for the maintenance of calcium and phosphate homeostasis in the body as well as bone remodeling (Pike, 1991). In osteoblasts, vitamin D regulates expression of a number of genes encoding bone matrix proteins, including type I procollagen, osteocalcin, osteopontin and matrix Gla protein (Rodan & Noda, 1991). Vitamin D also regulates expression of genes encoding enzymes such as alkaline phosphatase, which is one of the phenotypic markers of osteoblasts. In this manuscript, we examined the effects of 1,25$(OH)_2D_3$ on regulation of PTN gene expression in osteoblasts using Northern blot analysis. We now report that PTN mRNA levels in murine osteoblastic MC3T3-E1 cells, rat osteoblastic osteosarcoma ROS17/2.8 cells and osteoblast-enriched fetal rat calvaria cells were down-regulated by treatment with 1,25$(OH)_2D_3$, while PTN mRNA levels were up-regulated by the hormone treatment in non-osteoblastic osteosarcoma ROS25/1 cells.

Results and discussion

We first examined PTN mRNA expression in a mouse osteoblastic cell line, MC3T3-E1. Northern blot analysis of total RNA prepared from MC3T3-E1 cells revealed a single PTN mRNA band of an apparent molecular size of 1.5 kb (Figure 1), which was in accordance with the previously reported size of the PTN transcript in bovine uterus or brain (Li *et al.*, 1990). Among various calcitropic agents tested, 24 h treatment with 1,25$(OH)_2D_3$ (10^{-8} M) was found to decrease the abundance of PTN mRNA in MC3T3-E1 cells by greater than 60% (Figure 1A). As shown in Figure 1A,

Correspondence: Masaki Noda
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the decline of PTN mRNA levels by treatment with $1,25(OH)_2D_3$ was dose-dependent and was detectable at 10^{-11} M and maximal at 10^{-8} M. The $1,25(OH)_2D_3$ dependent decline of PTN mRNA levels was similarly observed in the presence of both 0.3% and 5% FBS, indicating that the $1,25(OH)_2D_3$ effects on steady state levels of PTN mRNA is independent of the serum concentration in the media (Figure 1A). Furthermore, the decline of PTN mRNA levels was already observed within 12 h of the treatment with $1,25(OH)_2D_3$ (10^{-8} M) in MC3T3-E1 cells in the presence of 5% FBS (Figure 2). The effect of $1,25(OH)_2D_3$ was specific to this molecule, since none of other steroids, such as retinoic acid (Figure 1B) or dexamethasone (data not shown) affected PTN mRNA expression in MC3T3-E1 cells.

To examine whether the effects of $1,25(OH)_2D_3$ on PTN mRNA expression would be observed in cells other than MC3T3-E1 cells, Northern blot analysis was conducted using several osteoblast-like cell lines. As shown in Figure 3, treatment of ROS17/2.8 cells with 10^{-8} M $1,25(OH)_2D_3$ decreased the steady state levels of PTN mRNA similarly to its effects observed in MC3T3-E1 cells. On the other hand, in non-osteoblastic ROS25/1 cells that fail to express the osteoblastic phenotypes such as osteocalcin or alkaline phosphatase, PTN mRNA was expressed constitutively at a low level but its level was significantly increased by the treatment with $1,25(OH)_2D_3$ (Figure 3).

We examined whether the effects of $1,25(OH)_2D_3$ on PTN mRNA expression would be similarly observed in primary cultures of normal osteoblastic cells. In fetal rat calvaria-derived osteoblast-enriched cells, PTN mRNA was expressed and the level of PTN mRNA was also declined by the treatment with $1,25(OH)_2D_3$ (Figure 4).

$1,25(OH)_2D_3$ acts via its binding to a vitamin D receptor (VDR) whose structure has been found to be closely related to thyroid and other steroid hormone receptors. After binding to $1,25(OH)_2D_3$, VDR regulates transcriptional activity of its target genes by the binding to vitamin-D responsive element (VDREs) of these genes (Noda *et al.*, 1990). Therefore, it is likely that 5'-flanking region of PTN gene may contain VDRE to which VDR binds in a manner similar to the VDREs of the genes encoding bone matrix proteins such as osteocalcin, osteopontin and type I collagen.

Our results indicate that the levels of expression of the PTN gene in osteoblasts are regulated by $1,25(OH)_2D_3$. The direction of the vitamin D effects on PTN expression appears to depend on the type of cells which are diverse in the levels of differentiation. Although ROS25/1 cells do not express alkaline phosphatase or osteocalcin, these cells express matrix glaprotein and its expression is up-regulated by $1,25(OH)_2D_3$. Therefore, ROS25/1 cells are considered to represent a subtype of relatively immature osteoblastic cells that express differentiation functions in response to $1,25(OH)_2D_3$. The significance of the opposite directions of $1,25(OH)_2D_3$ effects in the two types of osteoblasts remains to be elucidated. Recently, Li *et al.* (1993) demonstrated that platelet-derived growth factor (PDGF) or basic fibroblast growth factor (bFGF) promote PTN mRNA expression in NIH3T3 cells. However, the levels of PTN mRNA in MC3T3-E1 cells were not increased by the treatment with bFGF (data not shown). It may be the case that the steady state levels of the PTN mRNA are regulated in a manner specific to osteoblastic lineage cells by $1,25(OH)_2D_3$.

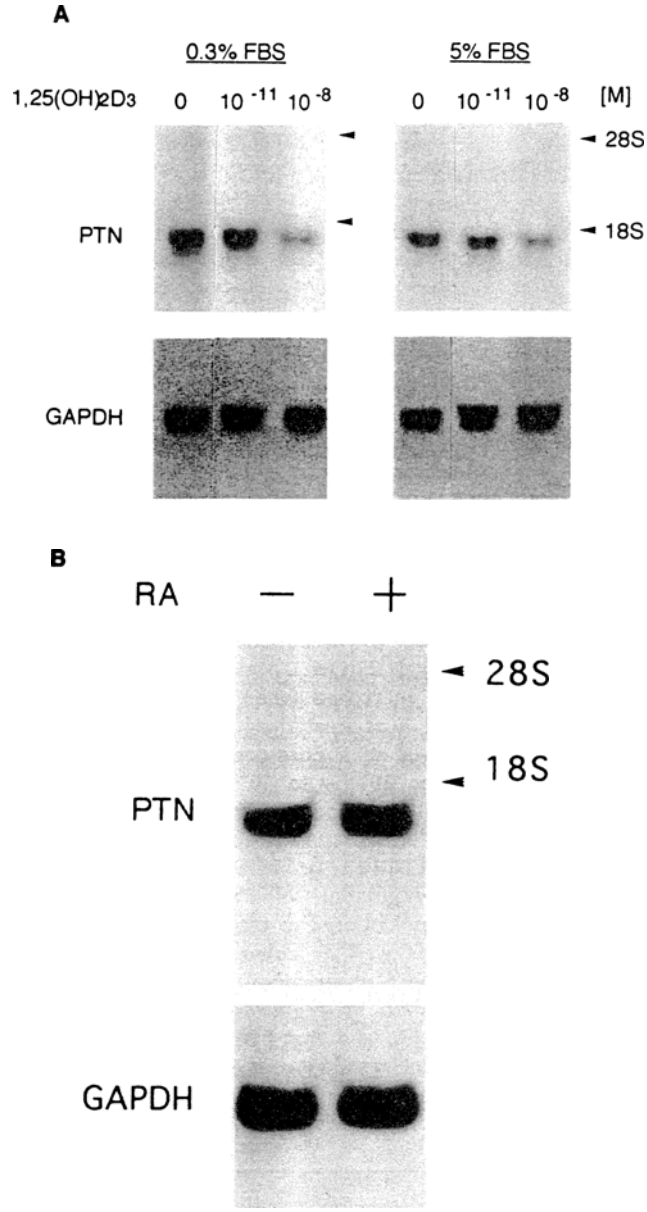

Figure 1 Effects of $1,25(OH)_2D_3$ or retinoic acid on the levels of PTN mRNA in MC3T3-E1 cells. The cells were plated at 2000 cells per cm^2 and cultured in α-MEM supplemented with 5% FBS. (**A**) Subconfluent MC3T3-E1 cells were treated with vehicle or indicated doses of $1,25(OH)_2D_3$ for 24 h in α-MEM supplemented with 0.3% or 5% FBS. (**B**) Subconfluent MC3T3-E1 cells were treated with vehicle (−) or 10^{-8}M of retinoic acid (+) for 24 h in α-MEM supplemented with 5% FBS. Total cellular RNA (10 μg per lane, **A**; 15 μg per lane, **B**) extracted from the cells was fractionated by electrophoresis and blotted onto a nylon filter as described in Materials and methods. PTN mRNA level was determined by Northern blot analysis using ^{32}P-labeled mouse PTN cDNA as a probe (upper). Equal loading in lanes was checked by re-hybridization with ^{32}P-labeled GAPDH cDNA as a probe (lower). Arrows indicate the positions of 28S and 18S ribosomal RNA. The data represents one of two experiments with similar results

The predicted amino acid sequence of PTN showed approximately 55% homology with that of a secreted heparin binding protein or MK/midkine, whose expression is induced by retinoic acid treatment in

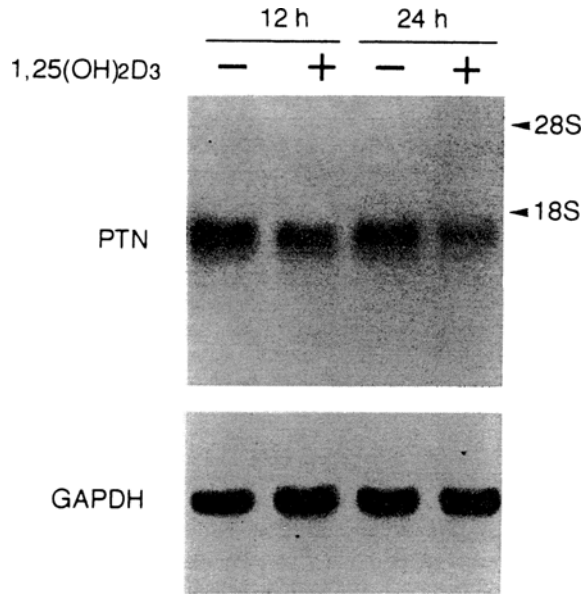

Figure 2 Time course of PTN mRNA expression by the treatment with $1,25(OH)_2D_3$ in MC3T3-E1 cells. The cells were plated at 2000 cells per cm^2 and cultured in α-MEM supplemented with 5% FBS. Subconfluent MC3T3-E1 cells were treated with vehicle (−) or $1,25(OH)_2D_3$ (10^{-8}M) (+) for the indicated periods (in hours) in α-MEM supplemented with 5% FBS. Northern blot analysis using 10 μg of total RNA per lane was carried out as described in the legend to Figure 1. Arrows indicate the positions of 28S and 18S ribosomal RNA

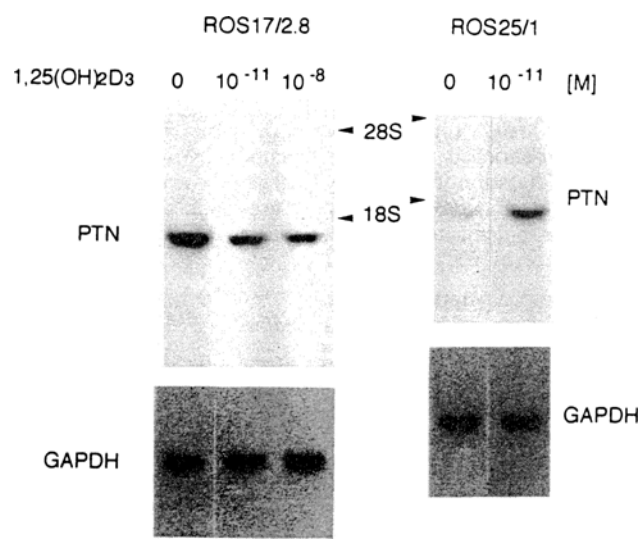

Figure 3 Effects of $1,25(OH)_2D_3$ on the levels of PTN mRNA in ROS17/2.8 and ROS25/1 cells. The cells were plated at 2000 cells per cm^2 and cultured in modified F-12 medium supplemented with 5% FBS. Subconfluent cells were treated with vehicle or the indicated doses of $1,25(OH)_2D_3$ for 24 h. Total cellular RNA was extracted and was subjected to Northern blot analysis (10 μg per lane) using ^{32}P-labeled mouse PTN cDNA as a probe (upper). Equal loading in lanes was checked by re-hybridization with ^{32}P-labeled GAPDH cDNA as a probe (lower). Arrows indicate the positions of 28S and 18S ribosomal RNA

differentiating mouse embryonic carcinoma cells (Tomomura *et al.*, 1990). However, the levels of PTN mRNA are not affected by the treatment of retinoic acid in NIH3T3 cells or F9 embryonic carcinoma cells as reported by Li *et al.* (1993). In the osteoblast-like cells which were used in this report, PTN mRNA expression was not influenced by retinoic acid treatment (Figure 1B). These observations suggest that in osteoblastic cells the responses to reinoic acid of the two genes that encode the heparin-binding growth factors, PTN and MK, both of which appear to be important in early development, could be different.

Bone metabolism and calcium homeostasis are under the concerted control of both systemic calcitropic hormones and various local regulators. The results of this work suggest a possibility that $1,25(OH)_2D_3$ may regulate PTN expression in osteoblasts in the local environment in bone, but its physiologic role *in vivo* still remains to be determined.

Materials and methods

Materials

[α-^{32}P]dCTP (1111 TBq mmol^{-1}) was purchased from Du Pont-New England Nuclear (Boston, MA). Alpha modified Eagle's minimum essential medium (α-MEM), F-12 medium, retinoic acid and dexamethasone were from Sigma Chemical Co. (St Louis, MO). Fetal bovine serum (FBS) was from Gibco Laboratories (Grand Island, NY). 1,25 $(OH)_2D_3$ was from Chugai Phamaceutical Co. (Tokyo, Japan). A cDNA fragment for human glyceroaldehyde-3-phosphate-dehydrogenase (GAPDH) was provided by S Sakiyama (Chiba Cancer Center Research Institute and Hospital, Chiba, Japan).

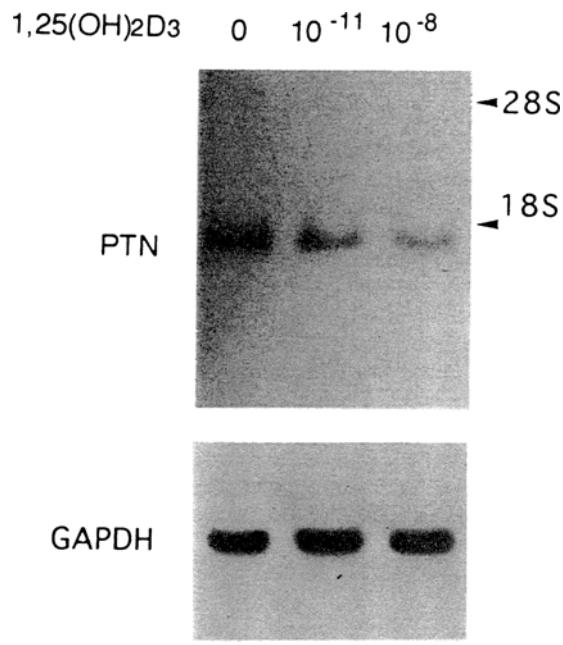

Figure 4 Effects of $1,25(OH)_2D_3$ on the levels of PTN mRNA in primary cultures of osteoblast-enriched fatal rat calvaria cells. Primary osteoblast-enriched cells from rat calvaria were prepared as described in Materials and methods. The cells were treated with vehicle or the indicated doses of $1,25(OH)_2D_3$ for 24 h. Northern blot analysis using 15 μg of total RNA per lane was carried out as described in the legend to Figure 1. Arrows indicate the positions of 28S and 18S ribosomal RNA

Cell Culture

Mouse calvaria derived MC3T3-E1 cells (Kodama *et al.*, 1981) were kindly provided by Dr Kodama (Ohu University, Koriyama, Japan), and were cultured in α-MEM supp-

lemented with 5% FBS. Rat osteosarcoma derived ROS17/2.8 cells (Majeska & Rodan, 1980) were kindly provided by Dr G Rodan (Merck Research Laboratories, West Point, PA), and were cultured in modified F-12 medium (Majeska & Rodan, 1980) supplemented with 5% FBS. Primary osteoblast-enriched cells were obtained by sequential collagenase digestion from 18-day fetal rat calvaria as described (Rodan *et al.*, 1987), and were grown in modified F-12 medium supplemented with 5% FBS at 37°C in a humidified atmosphere of 5% CO_2 in air.

RNA isolation and Northern blot analysis

Total cellular RNA was prepared at the indicated time points according to the acid guanidium thiocyanate-phenol-chloroform method as described by Chomczynski & Sacchi (1987). Ten or fifteen microgram of total RNA was fractionated by electrophoresis in 1% agarose gels containing 0.66 M formaldehyde and were transfered to a nylon filter (Hybond-N; Amersham Corp., Arlington Heights, IL) overnight by electroblotting (Thomas, 1980). Filters were u.v. cross-linked and prehybridized overnight at room temperature in a buffer containing 50% formamide, 5 × SSC (1 × SSC consists of 0.15 M NaCl, 10 mM sodium citrate), 5 × Denhardt's solution, 0.1% SDS, and 50 $\mu g\ ml^{-1}$ sheared and denatured herring sperm DNA. A complementary DNA fragment for mouse PTN (Li *et al.*, 1990) was excised with EcoRI and XhoI, and cDNA fragment for GAPDH was by EcoRI. They were separated from vector DNA by SeaPlaque (FMC Co., Philadelphia, PA) gel electrophoresis and then radiolabelled by random primer method using Klenow fragment (Pharmacia, Sweden) and [α-^{32}P]dCTP as described by Feinberg and Vogelstein (1983). Specific radioactivity of the radiolabelled cDNA was $1-5 \times 10^8$ c.p.m. per μg DNA. Filters were hybridized with ^{32}P-labelled cDNA probes at 42°C for 18 h in a solution containing all ingredients described above. Filters were washed in 0.1 × SSC containing 0.1% SDS for 15 min at 65°C, and were exposed to X-ray film using intensifying screens (Du Pont, New England Nuclear) at −80°C for several days. Bands on the autoradiograms were quantificated by densitometric scanning using laser densitometer (LKB 2222-010 UltroScan XL). Equal loading of RNA in each lane was checked by re-hybridization with ^{32}P-labelled GAPDH cDNA.

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