PLEIOTROPHIN (PTN) EXPRESSION IN OSTEOBLASTIC CELLS

Byeongyol Kim, Jaesuk Rim, Jongjin Kwon, Hyonseok Jang, Euiseok Lee, Sangho Jun, Youngjin Kim
Department of Oral & Maxillofacial Surgery, School of Medicine, Korea University

Abstract

Pleiotrophin or osteoblast-specific factor 1(OSF-1) is a growth-associated protein present in bone matrix. This study was designed to study pleiotrophin expression in osteoblastic cells. Pleiotrophin was expressed by osteoblast-like cell line. Pleiotrophin expression increased following the proliferative phase and was minimal at the terminal phases of the induced differentiation of cultured MC3T3-E1 cells. Pleiotrophin expression represents another autocrine factor that may contribute to the physiologic control of induced bone formation. In this study, induced osteogenesis will be examined in the context of the osteoblast expression of and regulation by PTN. I hypothesized that PDGF-BB stimulation of PTN expression represents an important paracrine signal during the induced osteogenesis associated with periodontal and implant surgeries. The possible mediation by PTN of anabolic effects attributed to PDGF-BB stimulation was examined in cell culture models of osteoblast differentiation. These studies will contribute fundamental insights to osteoblast biology and insights regarding the potential use of factors such as PTN in the clinical environment.

Key words: Pleiotrophin, Osteoblasts, Cell culture, Differentiation, Osteogenesis, Bone matrix

I. INTRODUCTION

Osteoblast function is controlled by many factors present in the cellular environment[1]. The mineralized extracellular matrix of bone represents a latent source of important regulatory molecules[2]. Many growth factors, including insulin-like growth factors(IGFs), transforming growth factors(TGF-β s) and bone morphogenic proteins (BMPs) are found in extracts of demineralized bone matrix and are known to be products of the osteoblastic cell[3]. These factors released from bone matrix contribute important signals to the cells of bone.

Identifying the repertoire of biologically active components in the extracellular matrix of bone is a current research focus. One abundant protein identified as a result of structural analysis of bone matrix is an 18 kDa protein that was shown to be pleiotrophin(PTN)[4]. Unlike many bone - specific proteins, it has not been shown to display high affinity for hydroxyapatite. PTN was originally isolated based upon its high affinity for heparin matrices. It has been purified from many tissues, most noteably bovine brain as p18[5] and uterus as heparin-binding growth factor(HBGF-8)[6]. The primary structure of this protein has been defined by protein and cDNA sequencing from many species[5-8]. It is a highly conserved 136 amino acid protein that contains 25% lysine and 10 cysteines that are arranged in 5 disulfide bonds. PTN is encoded by a single gene that includes a large exon (11 - 20 kb) separating the 5' - untranslated region from the coding

※본 연구는 보건산업진흥원 보건의료인프라개발사업(A050801)의 지원에 의하여 수행되었음.

sequence[9,10]. The promoter does not contain a TATA box and multiple transcriptional start sites have been identified[11]. This gene is related to a second growth associated molecule, Midkine, whose expression is regulated by retinoic acid[12], but morphogen regulation of the PTN gene has not been observed. Together these genes comprise a small family of growth-associated genes expressed during embryogenesis[13].

PTN is expressed in many tissues of the developing embryo[14,15]. In post-natal tissues, its expression has been observed in developing brain[16]. Recently, PTN has been isolated from extracellular matrix of cartilage[17]. Although PTN expression is not restricted to bone, there may be special significance to the high levels of PTN found in bone matrix.

Several previous observations suggested a role for PTN in bone cell biology. PTN expression was observed at high levels in osteoblasts of developing bone[15]. It is also found in relative abundance in the matrices of bone and cartilage[4,17]. PTN's biologic function includes a growth factor-like role in promoting mitogenesis[7] and a role supporting cellular adhesion as an extracellular matrix protein[4]. In either capacity, PTN may contribute to processes such as tissue repair or remodeling.

PTN expression during *in vitro* differentiation of the MC3T3-E1 cell line was characterized. This descriptive analysis of PTN expression suggests potential role for this growth-associated molecule in osteogenesis and osseous repair. This study was designed to investigate PTN expression in osteoblastic cells.

Ⅱ. MATERIALS AND METHODS

1. Culture of MC3T3-E1 cells

Murine calvarial osteoblast-like cells, MC3T3-E1, which were obtained from the American Type Culture Collection(Rockville, MD, USA) were maintained in alpha MEM media containing 10% fetal calf serum(FCS) and penicillin/streptomycin[18]. To induce differentiation, the growth media was supplemented with 25 ug/ml ascorbic acid and 10 mM glycerophosphate and media was changed every third day[19].

2. RNA extraction and gel electrophoresis

Total RNA was obtained by acid phenol-guanidinium HSCN extraction[20]. The integrity of the RNA was evaluated by denaturing formaldehyde agarose gel electrophoresis and staining with ethidium bromide.

3. Northern blot analysis of PTN Expression

Tissue and MC3T3-E1 cellular expression of PTN mRNA was examined by Northern blot analysis of 15 ug aliquots of total RNA separated in formaldehyde agarose (1.0%) gels, transferred to nylon and fixed by baking at 80℃. A [^{32}P] labeled probe was prepared by random primer labeling[21]. After overnight hybridization at 65℃ using 5.0 × 10^6 cpm/ml labeled probe, blots were washed to high stringency (1× SSC, 65℃) and exposed to Kodak XAR film at -86℃.

After subconfluent cultures were grown for 24 hours in serum - free media (0.5% BSA and ITS supplement), PDGF(0, 0.1, 10 ng/ml) was added, and cultures were grown for an additional 24 hours. Total RNA was prepared at 2, 4, 18 hours and evaluated by Northern blot analysis[21] using a [^{32}P] dCTP random primer-labeled PTN cDNA probe.

Ⅲ. RESULTS

Analysis of PTN mRNA abundance during *in vitro* differentiation of MC3T3-E1 cells indicated increased abundance of PTN preceeding terminal differentiation as reflected by osteocalcin expression (Fig. 1). PTN mRNA abundance increased from day 4 - 14, attaining a 3-fold increase prior to reductions at day 20 in culture. Northern blot analysis of PTN mRNA abundance by MC3T3-E1 cells revealed that physiologic concentrations (10 ng/ml) of PDGF-BB induced rapid increases in PTN mRNA abundance (Fig. 2).

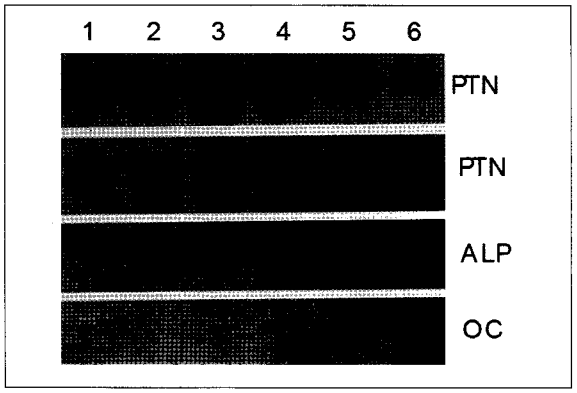

Fig. 1. Northern analysis of PTN, alkaline phosphatase (ALP) and osteocalcin (OC) mRNA abundance in MC3T3-E1 cell induced differentiation at days 2 (lane 1), day 4 (lane 2), day 7 (lane 3), day 10, (lane 4), day 14 (lane 5) and day 20(lane 6). First panel: 10 ug total RNA separated, transferred to nitrocellulose and probed with [^{32}P] dCTP - labeled PTN cDNA. Lower three panels: Duplicate differentiation experiment;10 ug total RNA separated, transferred to nitrocellulose and probed with [^{32}P] dCTP - labeled PTN cDNA (PTN) and stripped and reprobed with [^{32}P] dCTP - labeled ALP and OC cDNAs.

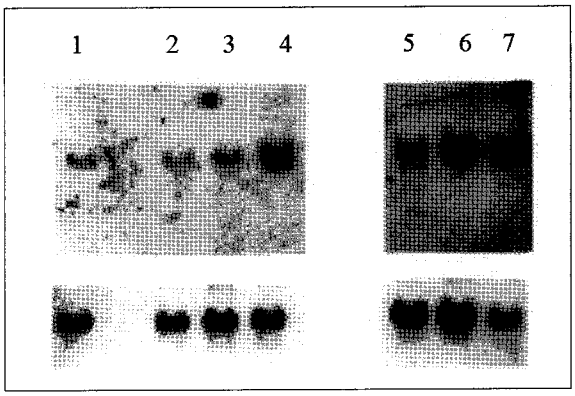

Fig. 2. Northern analysis of PTN mRNA abundance in osteoblasts treated with PDGF. 15mg total RNA from MC3T3-E1 cells treated with 0.0ng/ml PDGF (lane 1) or 0.1ng/ml PDGF for 2 hours (lane 2), 4 hours (lane 3) or 18 hours (lane 4) or treated with 10 ng/ml PDGF for 2hours (lane 5), 4 hours (lane 6) or 18 hours (lane 7). Top panel: blot probed with [^{32}P] dCTP - labeled PTN cDNA. Bottom panel: blot reprobed with [^{32}P] dCTP - labeled rat actin cDNA.

Ⅳ. DISCUSSION

The processes of bone formation, modeling and remodeling are controlled by multiple factors provided by serum as well as factors released from the matrix of bone[2,3]. The expression of matrix - included regulatory factors and their subsequent contribution to the regulation of osteoblastic cells represents a critical aspect of osteoblast physiology. A role for PTN in the post-natal regulation of osteoblast physiology has been suggested by the abundance of this heparin binding growth - associated molecule in bone and cartilage[4,17].

Osteoblastic culture models are well defined with respect to the temporal segregation of proliferation and differentiation[25]. When the MC3T3-E1 cell was used to investigate the association of PTN expression during *in vitro* osetoblast differentiation, PTN expression was increased after the early, proliferative phase of the culture period[19].

Other studies have indicated that PTN expression increased during post - confluent culture conditions[13,26] and our observations indicated that PTN expression increased during the multilayering phases of osteoblastic cell culture[4,13]. PTN expression in the MC3T3-E1 model continued at high levels until osteocalcin expression in multilayered cultures was evident at the molecular level. The role of PTN in the adult may be specific to certain tissue types such as bone and cartilage of the craniofacial region and may be developmentally regulated in different ways for each cell or tissue type[27]. The present cell culture studies support a role for PTN in osteoblasts committed to differentiation. The fact that the highest levels of PTN were observed following the proliferative phase of this culture model is not consistent with a mitogenic role for PTN.

This concordance of PTN expression with the onset of osteoblast-specific gene expression[19] suggests a potential role for PTN in supporting events related to osteoblast differentiation. The function of PTN in the adult are presently unknown. A possible link of PTN to cellular differentiation was suggested by studies of cultured neural cells and PC12 cells where PTN promoted neurite outgrowth of cultured cells[28]. PTN's possible contribution to the process of angiogenesis is also aligned with this protein's association with tissue formation[29]. The temporal pattern of PTN

expression observed in the MC3T3-E1 model of osteoblast differentiation supports these possible functions, and it was thought that PTN could act to promote the further differentiation of a functionally defined cell population[30].

Another possible roles for PTN in adult osteogenesis and repair of bone may include mitogenic and morphogenic stimuli. Early reports suggested its mitogenic role[8]. This has proven to be controversial[26-28]. Although the present description does not directly test a mitogenic role for PTN, the present findings temporally connect elevated PTN expression with molecular events associated with matrix elaboration, not with the earlier period of proliferation. As a matrix component, PTN may also have a role in cell adhesion. PTN purified from bone matrix was shown to be an efficient cell attachment molecule, specifically comparable to two RGD -containing matrix proteins, osteopontin and bone sialoprotein[4]. Gieffers et al[29] later confirmed that PTN binding to osteoblastic cells was heparin-sulfate sensitive and supported cell attachment. Raulo et al[30] have demonstrated that a PTN receptor is the syndecan 3 molecule. Given the role of syndecans in development, this interaction is consistent with the ubiquitous and prominent expression of PTN during tissue formation. However, the co-localization of syndecan 3 with PTN that is observed in many cell types expressing abundant PTN has not been observed for osteoblasts[15].

We have demonstrated that PDGF administration increased PTN mRNA steady state abundance in cultured osteoblasts. PDGF' s effect on bone mass is indicated by the increased bone mass resulted from chronic administration of PDGF in rodents[22]. While it is still not known what impact PDGF has on osteoclasts, PDGF represents an anabolic cytokine for bone[22,23]. In vitro data also suggests that PDGF directly increases osteoblast activity. PDGF stimulation induces biochemical responses including endogenous tyrosine kinase activity, increased turnover of phosphatidyl inositol, increased intracellular calcium concentrations, changes in intracellular pH, alterations of the cytoskeleton, and increased transcription as many as thirty early response genes, such as c-fos, c-myc, junB, and JE[23,24]. These previous observations[22-24] and our preliminary findings suggest that the potential relationship between PDGF treatment and PTN expression by osteoblasts is an essential part of the complex process for induced osseous regeneration. The proposed experiments may contribute to be a integral part of a major initiative to define PTN' s role in osseous bone formation.

V. CONCLUSION

In conclusion, *in vitro* data indicated that PTN was expressed by differentiating osteoblastic cells *in vitro*. The induction of osteoblastic activity leading to bone formation may be associated with abundant pleiotrophin expression in cells associated with newly formed bone matrix. These results will provide a set of tools for further investigation of the expression and function of PTN in bone formation and repair.

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저자 연락처
우편번호 425-707
경기도 안산시 단원구 고잔동 516번지
고려대학교 안산병원 치과, 구강악안면외과
장 현 석

원고 접수일 2007년 7월 13일
게재 확정일 2007년 10월 30일

Reprint Requests
Hyonseok Jang
Dept. of Oral & Maxillofacial Surgery, School of Medicine, Korea Univ.
516, Gozan-dong, Danwon-gu, Ansan, Gyeonggi, 425-707, Korea
Tel: 82-31-412-5370, 5956 Fax: 82-31-401-7125
E-mail: omfs1109@korea.ac.kr

Paper received 13 July 2007
Paper accepted 30 October 2007