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Pleiotrophin Regulates Bone Morphogenetic Protein (BMP)-Induced Ectopic Osteogenesis[1]

Yasuko Sato,* Hiroko Takita,† Noboru Ohata,* Masato Tamura,† and Yoshinori Kuboki†,2

*Department of Oral Functional Science and †Department of Oral Health Science, Graduate School of Dental Medicine, Hokkaido University, North 13 West 7, Kita-ku, Sapporo 060-8586

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We previously isolated pleiotrophin (PTN) from bovine bone as a protein and showed that it stimulated osteoblastic growth and differentiation. Further details of its function, however, have not been fully clarified. The aim of this paper was to elucidate the effects of PTN on bone morphogenetic protein (BMP)-induced ectopic osteogenesis. Recombinant human BMP (rhBMP)-2 (1.2 μg) was combined with a fibrous glass membrane, which had been established as an effective carrier. Various amounts of the purified bovine PTN (5, 10, 50, and 100 μg) or rhPTN (5 and 10 μg) were added to the rhBMP-2/carrier composites and implanted into rats subcutaneously as reported. It was found that the amount of bone induced in the system increased with the addition of 10 μg of either purified PTN or rhPTN. However, the amount of bone decreased with the addition of 50 or 100 μg of purified PTN dose-dependently, as judged by both alkaline phosphatase activity and calcium content in the retrieved implants. It was concluded that purified PTN or rhPTN, at ratios of concentration of 10–100 μg of PTN to 1.2 μg of rhBMP-2 in the carrier, regulated the ectopic bone-inducing activity of rhBMP-2.

Key words: bone morphogenetic protein, osteogenesis, pleiotrophin, purification, synergism.

Elucidation of molecular mechanisms controlling bone formation has been one of the major subjects in bone biology. In the process of bone formation, various extracellular signals including hormones, growth factors, cytokines, and extracellular matrix components as well as their intracellular mediators regulate cell differentiation or expression of phenotypes. To reconstruct these tissues, partially or *de novo* by tissue engineering, we have proposed that five factors must be taken into consideration (*1–3*). They are: (i) cells involved in bone and cartilage formation, (ii) matrices of natural or artificial origin, (iii) body fluid provided by vascularization, (iv) regulators of general cellular activities as well as the calcification process, and (v) biomechanical dynamics (*4–9*).

Pleiotrophin (PTN) was first identified as a heparin-binding protein that possesses mitogenic activity in rat and mouse fibroblasts (*10*) and as a factor that promotes neurite outgrowth in cultures of neonatal rat brain cells (*11*). 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. 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) (*12*), or heparin-binding neurite-promoting factor (HBNF) (*13*). Human, bovine, rat, mouse, chick, zebra fish and *Xenopus* PTN cDNAs have been cloned and sequenced and found to encode protein of 168 amino acids, including a 32-amino-acid signal sequence for secretion. PTN is about 50% identical in amino acid sequence and has 10 cystein residues in common with midkine (MK) (*14*). Nucleotide sequences of miple (midkine/pleiotrophin-like protein) 1 and miple2 were also reported as PTN/MK family protein in *Drosophila melanogaster* (Gen Bank AF149800 and AF215688). Recently structural studies using NMR have revealed that PTN contains two β-sheet domains connected by a flexible linker (*15*). These β-sheet domains correspond to the thrombospondin type I (TSR) repeat.

In 1992, we isolated an 18-kDa lysine-rich protein from a decalcified solution of bovine bone. This protein stimulated proliferation and increased alkaline phosphatase (ALP) activity in osteoblastic MC3T3-E1 cells (*16*), and we hypothesized that it was a new member of growth and differentiation-associated proteins in bone. This bone-derived protein was identical with PTN in its amino acid composition and heparin-binding property. At the mRNA level, Tezuka *et al.* had detected the same mRNA in calvarial osteoblast-enriched cells and MC3T3-E1 cells by differential hybridization screening between osteoblastic and fibroblastic cells, and named it osteoblast stimulating factor (OSF-1) (*17*). Later this protein was detected in significant amounts in cartilage (*18*) and tadpole embryonic tissues (*19*).

PTN has been reported to have a wide range of biological

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[2]To whom correspondence should be addressed. Phone/Fax: +81-11-706-4234, E-mail: kuboki@den.hokudai.ac.jp

Abbreviations; ALP, alkaline phosphatase; BMP, bone morphogenetic protein; PTN, pleiotrophin.

functions. These are classified into (i) mitogenic activity, (ii) angiogenetic activity, (iii) cell motility, (iv) oncogenic activity, (v) synaptic plasticity, and (vi) cell differentiation (*14*). Milner *et al.* (*10*) were the first to report its mitogenic activity for NIH 3T3 fibroblasts. Mitogenic effects of PTN were also reported for cell types such as osteoblasts (*16*), epithelial cells and endothelial cells (*14*). Several groups have reported that PTN promotes neurite cell outgrowth (*20, 21*) and regulates long-term potentiation (*22*). PTN is also an angiogenesis factor. In newly forming blood vessels, PTN gene expression was up-regulated (*14*).

PTN can bind to heparan sulfate proteoglycans of N-syndecan, one of the cell-surface signaling receptors of PTN (*23*). *N*-Syndecan binds a kinase-active protein complex containing src-family kinase c-*src* and *fyn* and the src-substrate cortactin. Binding of N-syndecan by PTN enhances phosphorylation of src and cortactin in neuron cells. Interaction between *N*-syndecan and PTN is important to the development of axonal processes (*24*). In addition to *N*-syndecan, the chondroitin sulfate proteoglycan of RPTP β/ζ (receptor-type tyrosine phosphatase β/ζ) has been identified as a PTN receptor. Recently it was found that PTN binds to and inactivates the phosphatase activity of RPTPβ/ζ, thereby signaling increased tyrosine phosphorylation of the downstream substrate β-catenin through ligand-dependent receptor inactivation of RPTPβ/ζ, (*25*). Ligation of N-syndecan and RPTPβ/ζ of the transmembrane receptor by PTN has been reported to enhance migratory responses in neurons and osteoblasts (*26–28*).

In bone and cartilage tissues, PTN is expressed in developing and regenerating bone as a matrix-bound form; and in culture, it stimulated differentiation of osteoblasts (*16*) and chondrocytes (*29*). In addition, specific localization of PTN was reported within the hypertrophic chondrocytes and their extracellular matrix (ECM), but not in the cells or ECM of the resting zone or the proliferation zone of cartilage, suggesting that PTN acts as a template for endochondral bone formation (*30*). These studies reported that PTN induces osteoblast migration in haptotactic transfilter assays, suggesting that it regulates the migration of osteoblasts and their precursors to the site of bone deposition, though a mechanism similar to that in neurons (*26, 27*). Furthermore, Masuda *et al.* reported that bone mass loss due to estrogen deficiency was compensated in transgenic mice overexpressing the human PTN gene driven by the osteocalcin gene promoter, indicating that PTN increases osteoblastic activity and might play a role in bone formation *in vivo* (*31*).

Previously we have established a procedure for obtaining partially purified BMP from bovine bone. It employs a mixture designated as S300-BMP cocktail and has been used for developing new BMP carriers in our experimental system (*1, 8*). It was observed that the S300-BMP cocktail possesses stronger bone-inducing activity than rhBMP-2 (*32*). We found that 100 μg of S300-BMP contained much less than 1 μg of BMP-2, but that its bone-inducing activity was much higher than that of 1 μg of BMP-2. This result was ascribed to the possibility that S300-BMP contains other bone-inducing proteins than BMP-2 and/or some synergistic factor(s).

In this study, we attempted to analyze the interrelationships between the functions of BMP-2 and PTN, since this protein has been found to be abundant in bone and can be purified in sufficient amount for experimental use (*16*). We showed that S300-BMP contained a significant amount of PTN. Furthermore, we found that the amount of bone induction by rhBMP-2 increased or decreased when we added recombinant human PTN (rhPTN) or purified native PTN to the implant system in a higher or lower concentration, respectively. Thus, we demonstrated a novel function of PTN: a dose-dependent synergistic or inhibitory effect on BMP-induced osteogenesis.

MATERIALS AND METHODS

Purification of Bovine Native PTN—Bovine bone powder, smaller than 60 meshes, was washed with 1.0 M NaCl, 50 mM Tris-HCl, pH 7.4 (including protease inhibitors: 50 mM aminocaproic acid, 5 mM benzamidinehydrochloride, 1 mM benzylsulfonylfluoride), and defatted with $CHCl_3$/CH_3OH (1:1). The processed bovine bone powder was decalcified in dilute HCl, keeping the pH constant at 2.0. The decalcified solution was neutralized with Tris-HCl (pH 7.4) and filtered. The neutralized filtrate was concentrated by ultrafiltration, then with the buffer used in the chromatography. A sample was loaded on a Heparin-Sepharose CL-6B column (3.0 × 30 cm, 190 ml) equilibrated with 0.1 M NaCl, 50 mM Tris-HCl, and 6 M urea, pH 7.0, and eluted with 0.1 M and 1.0 M NaCl in a stepwise manner. PTN was detected in a 1.0 M NaCl elution fraction, which was concentrated, then diluted with the next solvent. Next, the fraction containing PTN was loaded on a Sephacryl S-200 super fine column (2.2 × 140 cm) in 50 mM Tris-HCl containing 4 M urea (pH 7.4) and eluted with the same buffer. Finally, for further purification, the PTN-containing fraction was concentrated by ultrafiltration, applied on a hydroxyapatite column (1.0 × 10 cm) and run with a linear gradient of 0.05–1.0 M KH_2PO_4 and 6 M urea (pH 6.6). The sample was concentrated, then diluted with 0.1% trifluoroacetic acid.

Purification of Bovine BMP Cocktail—The demineralized bone powder described above was filtered. The residue was extracted with 4 M guanidine hydrochloride in 50 mM Tris-HCl, pH 7.4, containing the protease inhibitors described above. The extracts were centrifuged (7,000 ×*g*, 30 min, 4°C) and supernatants were filtered through a Nucleopore membrane (3 μm, Nucleopore, USA). A detailed description of the procedure used for purification of the active fraction of the guanidine extract by an eight-step chromatographic procedure has been reported (*1*), and the final active preparation was shown by internal sequence analysis (residues 410–424) of trypsin digestion to contain a significant amount of BMP-3. In this study, a partially purified BMP fraction, treated by the three-step chromatographic procedure described below, was used.

A column (8.3 × 12 cm, 500 ml volume) packed with hydroxyapatite (FK-1, Sangi, Japan) was equilibrated with 1 mM potassium phosphate buffer containing 6 M urea (pH 6.8) and the applied sample was eluted in a stepwise manner with 0.1 and 0.4 M phosphate ions in a potassium phosphate buffer containing 6 M urea (pH 6.8). An activity was recovered in the 0.4 M phosphate fraction, which was concentrated and with the buffer used in the next chromatography. The solution was loaded on a Heparin-Sepharose CL-6B column (Amersham Biosciences Inc: 3 × 30 cm, 190 ml volume) equilibrated in 0.1 M NaCl, 50 mM Tris-HCl, 6 M urea, pH 7.0, then eluted with 0.1, 0.15 and 0.5 M NaCl

in a stepwise manner. An activity was detected in the 0.5 M NaCl elution fraction, which was concentrated and with the next solvent. The final chromatography employed Sephacryl S-300 HR (Amersham Biosciences Inc: 2.2 × 141 cm, 500 ml volume) equilibrated with 4 M guanidine-HCl/50 mM Tris-HCl, pH 7.4, with elution with the same buffer. The BMP-containing fractions were pooled and designated S300-BMP. The S300-BMP preparation was pooled from at least five extractions in order to keep its quality constant.

One unit of BMP activity was defined in this study as follows: the amount of S300 or any other BMP preparation which induced a calcium content of at least 20% in a dry implant that was implanted with 20 mg of insoluble bone matrix and harvested after 2 weeks.

Preparation for rhPTN and rhBMP-2—rhPTN was purchased from Genzyme/Techne, USA. rhBMP-2 was a gift from Yamanouchi, Japan.

Polyacrylamide-Gel Electrophoresis and Western Blotting Analysis—Samples were separated by 15% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The polyclonal antibody against PTN was from Oncogene Research Products. Immunoblotting was done using alkaline phosphatase–conjugated secondary antibodies (Vector Laboratories, USA), which were detected by staining with nitro blue tetrazolium (5-bromo-4-chloro-3-indolylphospha-*p*-toluidine salt).

Implantation of PTN—To examine the effect of PTN on osteogenesis, BMP-induced ectopic bone formation using a carrier of fibrous glass membrane (FGM) was established. FGM made of unwoven glass fibrils (GA-100) with a diameter of 1.0 μm and an exclusion size of 1.0 μm was obtained from Advantec, Tokyo. The membrane was cut into uniform rectangular pieces (10 × 5 mm, 6 mg), which were used as carriers the implant. The carriers were impregnated with 1.2 μg of rhBMP-2 and various amounts of native PTN or rhPTN. Four-week-old rats (Wistar strain, male, n = 11) were anesthetized with pentobarbital sodium (3.6 mg/100 g body weight) and the implants were placed subcutaneously in their backs for ectopic osteogenesis. For a specific experimental condition, triplicate implantation was planned each on the different animal individual, in order to minimize individual variation. After 3 weeks, the animals were killed and the implants were removed for the analyses described below.

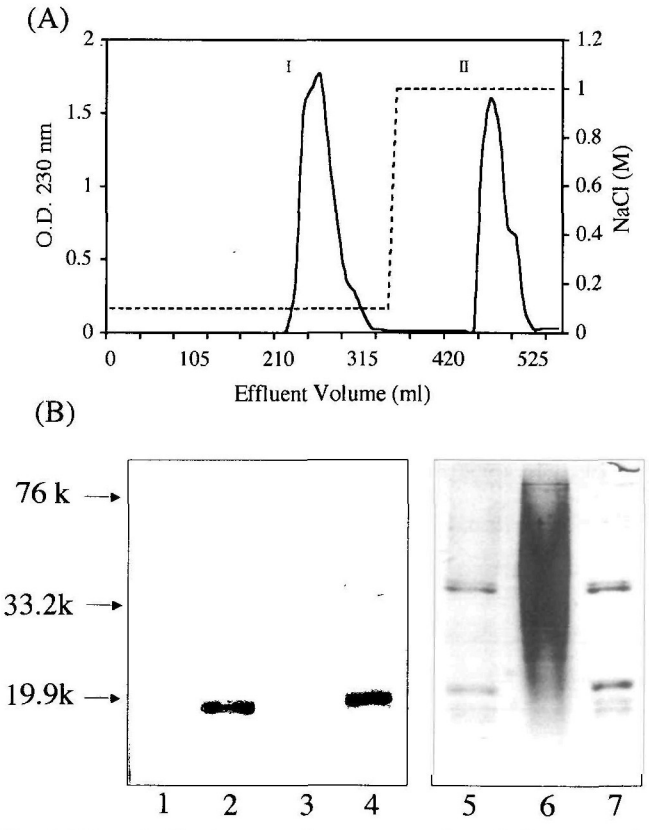

Fig. 1. **Heparin-Sepharose chromatography of the neutralized filtrate of decalcified solution of bovine bone.** (A) The bovine bone powder was decalcified with HCl at the constant pH of 2.0. The decalcified solution was neutralized with Tris-HCl (pH 7.4) and filtered. The filtrate was concentrated by ultrafiltration and chromatographed on a Heparin-Sepharose CL-6B column by stepwise elution with 0.1 and 1.0 M NaCl/50 mM Tris-HCl/6 M urea at pH 7.0. Fractions I and II were obtained. (B) SDS-PAGE of fractions I and II as detected by anti-PTN antiserum and CBB. Lane 1, standard marker (prestained SDS-PAGE standard, Amersham Biosciences Inc); lanes 2 and 5, neutralized supernatant before separating by Heparin-Sepharose chromatography; lanes 3 and 6, fraction I; lanes 4 and 7, fraction II. Lanes 1–4 were visualized by Western blotting, and lanes 5–7 were stained with CBB.

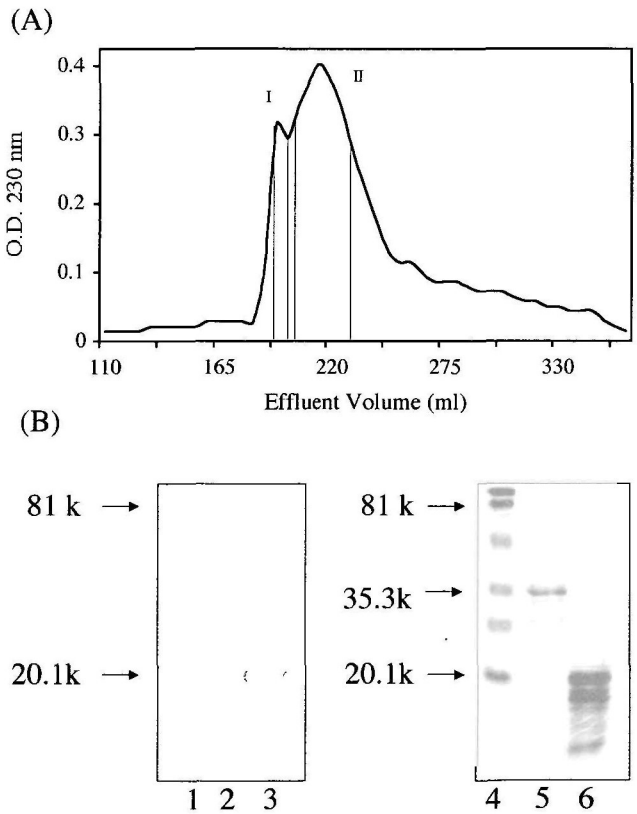

Fig. 2. **Sephacryl S-200 chromatography of Heparin-Sepharose chromatography fraction II.** (A) Sephacryl S-200 chromatogram eluted with 50 mM Tris-HCl containing 4 M urea, pH 7.4, of fraction II from Heparin-Sepharose chromatography. (B) SDS-PAGE of fractions I and II from Sephacryl S-200 as detected by anti-PTN antiserum and CBB. Lanes 1 and 4, standard marker (prestained SDS-PAGE standard, Amersham Biosciences); lanes 2 and 5, fraction I; lanes 3 and 6, fraction II. Lanes 1–3 were visualized by Western blotting, and lanes 4–6 were stained with CBB.

Biochemical Analyses—ALP activity and calcium content were measured. Lyophilized samples were cut with scissors into a fine powder. They were mixed in 1 ml of 0.2% igepal, 10 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.5. ALP activities of the suspensions were measured by the Kind-King phenylphosphate method (*33*). Calcium contents of powders were measured by the orthocresolphthalene-complexone method (*34*).

Histological Observations—A part of each sample was fixed in 10% neutral formaldehyde, decalcified in 10% formic acid, processed for paraffin embedding and cut into sections 5 μm thick. The sections were stained with hematoxylin and eosin routinely and alcian blue for confirmation of chondrogenesis.

Statistical Analyses—All data were analyzed by ANOVA with the multiple-comparison Lyan test. P values of less than 0.05 were considered significant.

RESULTS

Purification of PTN from Bovine Bone—Figure 1A shows the result of Heparin-Sepharose chromatography by stepwise elution with 0.1 and 1.0 M NaCl buffer, pH 7.0. PTN was defected by Western blotting and Commassie brilliant blue (CBB) staining of SDS-PAGE gels as a band of 18 kDa in the fraction eluted with 1.0 M NaCl, but not in the 0.1 M NaCl fraction (Fig. 1B). The 1.0 M NaCl effluent contained contaminant bands of about 30 kDa (Fig. 1B, lane 7), which were removed by Sephacryl S-200 chromatography into fraction I (Fig. 2, A and B). PTN was eluted in fraction II, which also contained lower molecular weight bands as seen by CBB staining (Fig. 2B, lane 6). Fraction II of S-200 chromatography was further purified by hydroxyapatite chromatography (Fig. 3A). A single sharp peak was eluted at 0.55 M phosphate, which appeared as single band in Western blotting, although traces of low molecular weight bands were revealed by CBB staining (Fig. 3B, lane 4). Figure 3 indicates that PTN was eluted at the phosphate concentration of 0.55 M in the gradient elution system, which is higher than the concentrations for the elution of BMPs. Bovine BMPs are known to be eluted at around 0.2–3 M phosphate at pH 6.8 from the hydroxyapatite column under the same conditions, for which reason we purified the S300-BMP by stepwise elution at 0.4 M phosphate. Thus, the discrepancy arose that the eluted BMP-containing 0.4 M phosphate fraction still contained PTN that eluted at 0.55 M

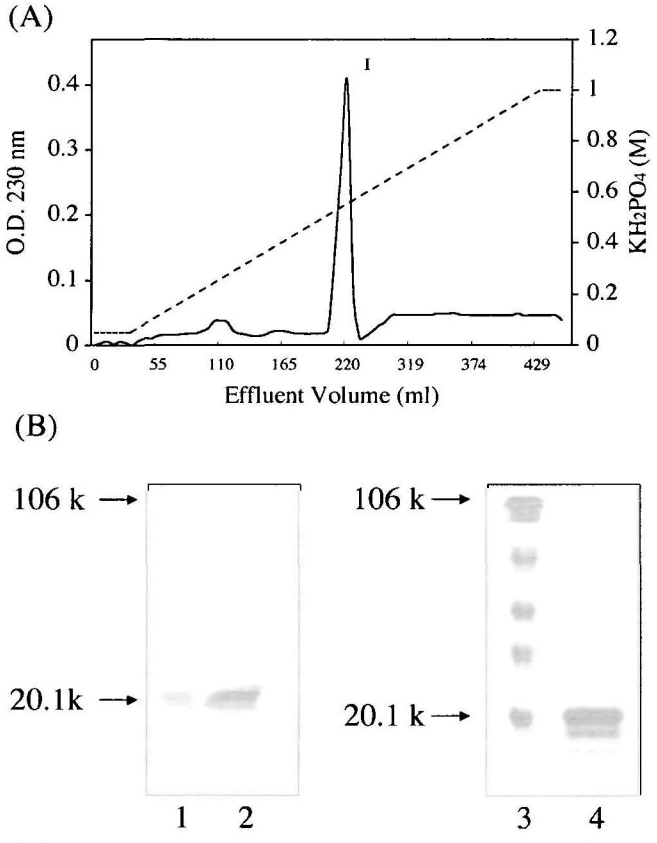

Fig. 3. **Hydroxyapatite column chromatography of Sephacryl S-200 fraction II.** (A) Hydroxyapatite chromatography of fraction II from Sephacryl S-200, eluted with a linear gradient between 0.05 and 1.0 M KH_2PO_4/6 M urea at pH 6.6 with a total volume of 200 ml. (B) SDS-PAGE of fraction I of hydroxyapatite chromatography as detected by anti-PTN antiserum and Coomassie brilliant blue (CBB). Lanes 1 and 3, standard marker (prestained SDS-PAGE standard, Amersham Biosciences Inc); lanes 2 and 4, fraction I. Lanes 1 and 2 were visualized by Western blotting, and lanes 3 and 4 were stained with CBB.

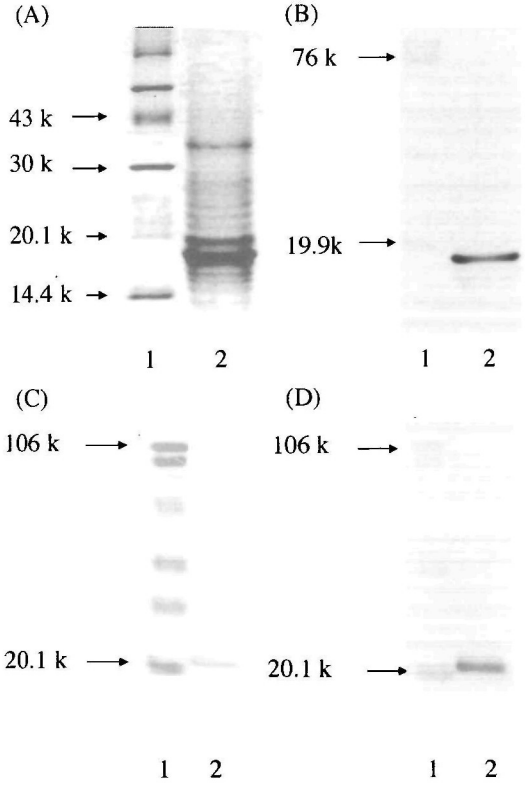

Fig. 4. **Detection of PTN in BMP-containing fraction from bovine bone.** A minor part (approximately 20% of the total) of the PTN in bone was extracted in the 4 M guanidine extract, while the major part (90%) was extracted in the acidic decalcifying solution. PTN was detected in the S300 fraction, which was the BMP-containing product of a three-stage chromatographic purification. (A) S300 fraction, stained by CBB (lane 2). (B) S300 fraction, detected by Western blotting using anti-PTN antiserum (lane 2). (C) rhPTN, stained by CBB (lane 2). (D) rhPTN detected by Western blotting using anti-PTN antiserum (lane 2). Lanes 1 of (A), (B), (C), and (D) contained standard markers (A, LMW electrophoresis calibration kit; B, C, and D, prestained SDS-PAGE standard, Amersham Biosciences).

phosphate in the gradient system from the hydroxyapatite column. Our interpretation was that the initial gross separation by stepwise elution with 0.4 M phosphate involved PTN that eluted at a lower concentration of phosphate than it should have. Whatever the case, the finding that PTN has significantly higher affinity to hydroxyapatite than natural BMPs indicates that it may be a mineral-associated protein in bone and play an important role in bone formation. About 1.6 mg of PTN was obtained per 5 kg of dry bovine bone powder by this method.

Detection of PTN in the Partially Purified BMP (S300-BMP)—Figure 4 shows the result of Western blotting of the guanidine extract and partially purified bovine BMP fraction, S300 BMP cocktail. The single band in lane 2 of Fig. 4B clearly indicates that this fraction contained PTN. It was found that a minor part (approximately 20% of the total) of the PTN in bone was extracted in the 4 M guanidine extract, while the major part (80%) was extracted in the acidic decalcifying solution. Considering the close similarity of the chromatographic procedures for PTN and BMP, which employed Heparin-Sepharose, Sephacryl S200 or S300 HR and hydroxyapatite columns, it is reasonable to expect that PTN might be co-purified with the S300-BMP.

Effect of PTN on BMP-Induced Bone Formation—We have developed and tested more than ten BMP carriers and found that BMP-induced osteo- and chondrogenesis are highly carrier-dependent (*4–9*). Among these carriers, FGM is a highly reproducible material of inorganic origin that induces both chondrogenesis and osteogenesis at 3 weeks after implantation with rhBMP-2 (*8*). Thus, it was considered that the rhBMP-2/FGM system of osteogenesis at 3 weeks after implantation was adequate to examine the

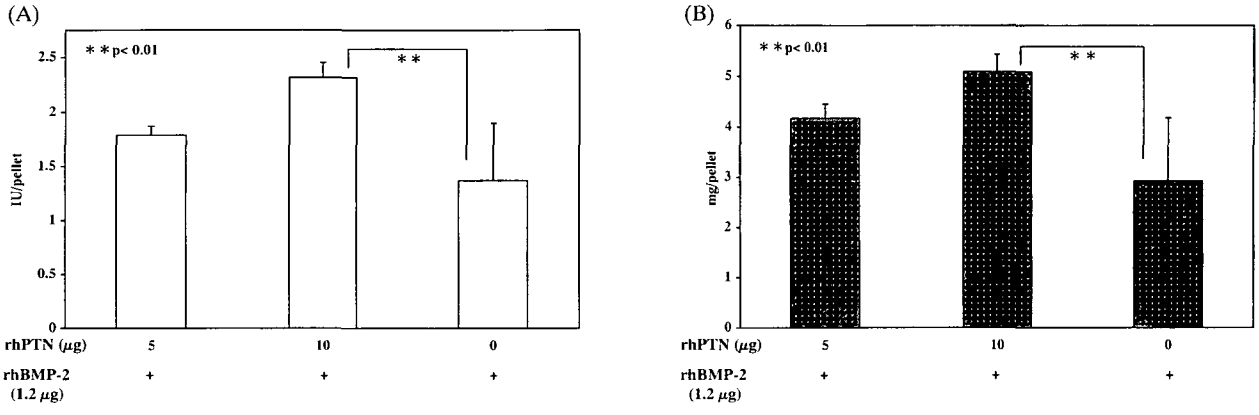

Fig. 5. **ALP activity and calcium content of BMP-2–induced ectopic bone on addition of rhPTN to the rhBMP-2/FGM composites.** FGM pieces were impregnated with various amounts of rhPTN or vehicle together with rhBMP-2 (1.2 μg), and implanted subcutaneously into the backs of four-week-old rats. After 3 weeks, the animals were killed and the implants were removed. Lyophilized implants were cut with scissors into a fine powder and were mixed in 1 ml of 0.2% igepal, 10 mM Tris-HCl, and 1 mM $MgCl_2$, pH 7.5. ALP activity of the suspensions (A) and calcium content of powders (B) were measured as described in "MATERIALS AND METHODS." On addition of 10 μg of rhPTN to the rhBMP-2/FGM composites, both ALP activity and calcium content increased, compared with the addition of 5 μg of rhPTN to the rhBMP-2/FGM composites and rhBMP-2/FGM alone as a control. (Double asterisk indicates $p < 0.01$.)

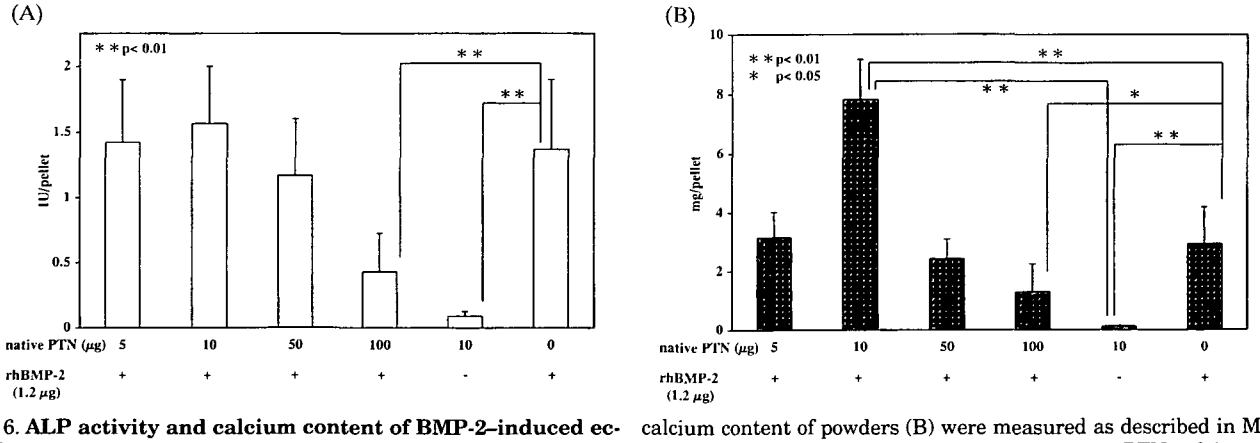

Fig. 6. **ALP activity and calcium content of BMP-2–induced ectopic bone on addition of native PTN to the rhBMP-2/FGM composites.** FGM pieces were impregnated with various amounts of native PTN in addition to rhBMP-2 (1.2 μg) or vehicle as indicated in the figure, then implanted subcutaneously into the backs of 4-week-old rats. After 3 weeks, the implants were removed, lyophilized and cut into a fine powder, then mixed in 1 ml of 0.2% igepal, 10 mM Tris-HCl and 1 mM $MgCl_2$, pH 7.5. ALP activity of the suspensions (A) and calcium content of powders (B) were measured as described in Materials and Methods. On addition of 10 μg of native PTN, calcium content increased to twice as much as that with 1.2 μg of rhBMP-2/FGM, although ALP activity did not change. On addition of native PTN in amounts of more than 10 μg, calcium contents and ALP activity decreased. (Asterisk and double asterisk indicate $p < 0.05$ and $p < 0.01$, respectively.)

effect of addition of PTN.

Figure 5 shows ALP activity and calcium content of implants containing 5 and 10 μg of rhPTN in addition to the rhBMP-2/FGM system. Addition of 10 μg of rhPTN increased both ALP activity and calcium content by 60–70% compared with rhBMP-2 alone.

On the other hand, as shown in Fig. 6, when 10 μg of native PTN was added to the rhBMP-2/FGM system, cal-

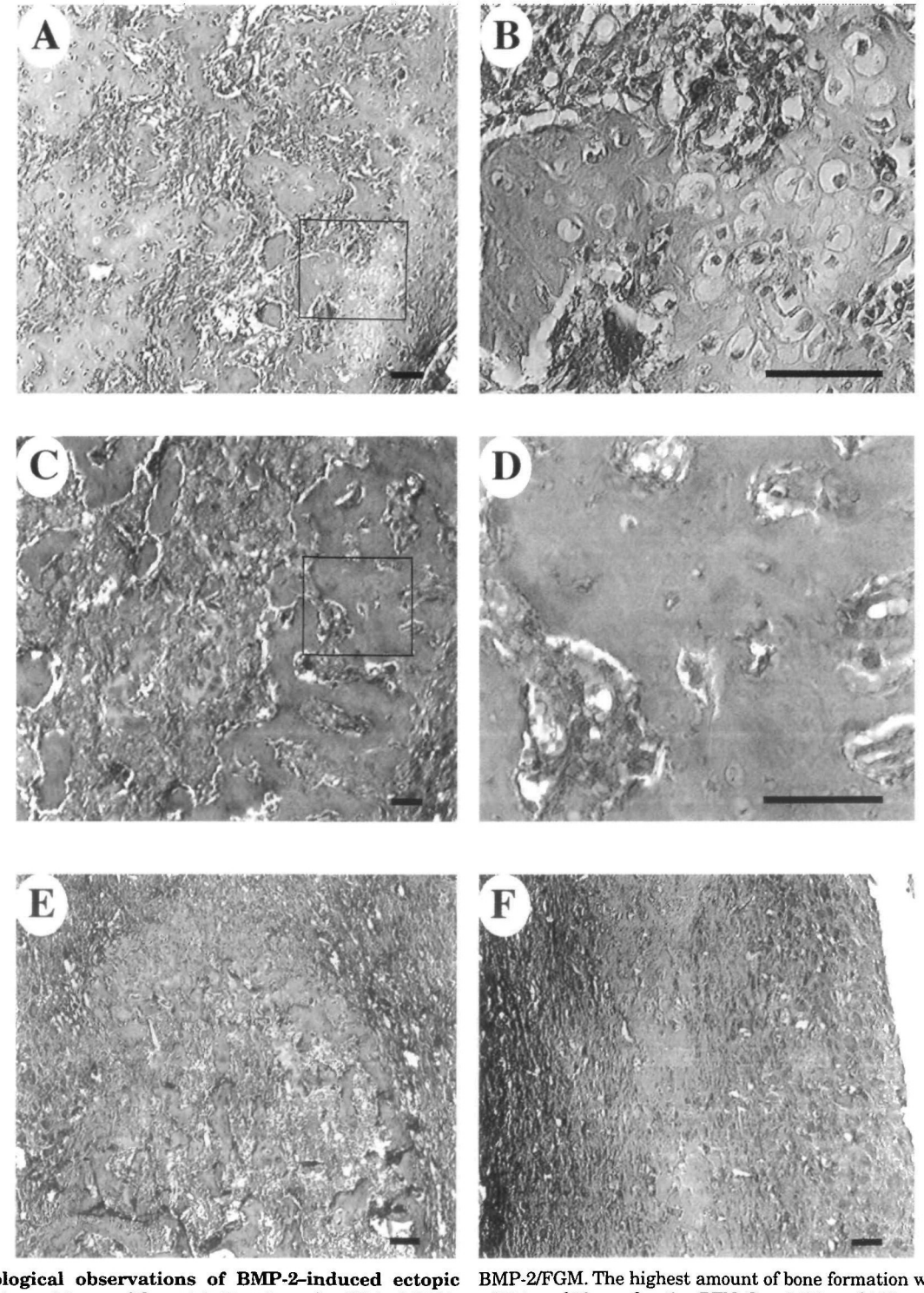

Fig. 7. **Histological observations of BMP-2–induced ectopic bone formation with or without PTN at 3 weeks.** With rhBMP-2/FGM alone, typical endochondral-type osteogenesis was observed (A, B). Trabecular bone was formed around the outer region and as islands in inner areas of the implants. Similar patterns were observed on addition of 10 μg of native PTN (C, D) or 10 μg of rhPTN (E) to rhBMP-2/FGM. The highest amount of bone formation was observed on addition of 10 μg of native PTN. On addition of 100 μg of native PTN, bone and cartilage were not observed (F). These results are consistent with the biochemical data shown in Fig. 6. Specimens were stained with hematoxylin and eosin. Bars indicate 100 μm.

cium content increased to a level twice that of rhBMP-2 alone, but ALP activity did not change significantly. However, when a higher amount of native PTN (100 μg) was added, both ALP activity and calcium content clearly decreased, compared with those of rhBMP-2 alone. These results indicate that osteogenesis decreased when more than 50 μg of native PTN was added under these experimental conditions. When 10 μg of native PTN alone was added, both ALP activity and calcium content were extremely low, indicating that native PTN by itself did not induce osteo- or chondrogenesis in our system.

Histological Observations—As shown in Fig. 7, A and B,

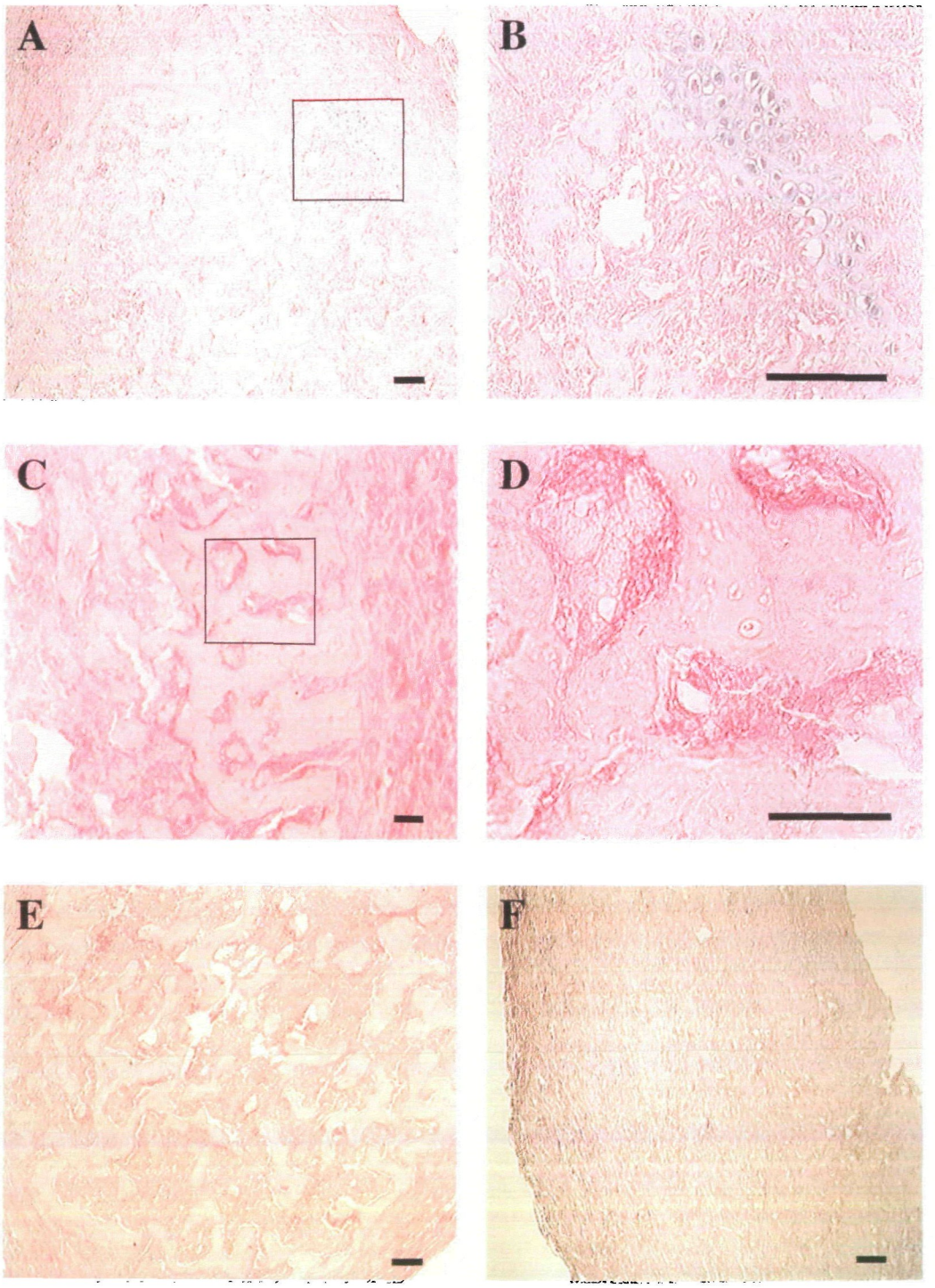

Fig. 8. **Histological observations of BMP-2–induced ectopic bone formation with or without PTN at 3 weeks.** In rhBMP-2/FGM alone, chondrogenesis was observed in ectopic bone (A, B). On addition of 10 μg of native PTN (C, D) or rhPTN (E) to the rhBMP-2/FGM, chondrogenesis was not observed. On addition of 100 μg of native PTN, bone and cartilage were not observed (F). Specimens were stained with alcian blue. Bars indicate 100 μm.

the histological profile at 3 weeks after the implantation of rhBMP-2/FGM had a typical features of the later stage of endochondral osteogenesis, consisting of bone and cartilage as previously reported (*8, 9*). Generally, bone tissues were distributed in the region around the implants and also inside in island form. Cartilage was also observed accompanied by adjacent bone formation (Fig. 8, A and B). Fibrous tissues were not observed in the inner region. The highest amount of bone formation was seen on addition of 10 μg of native PTN to rhBMP-2/FGM implants (Fig. 7, C and D), among implants with 5, 50, and 100 μg of native PTN (data not shown). In this implant, bone grew continuously, whereas there was no cartilage (Fig. 8, C and D). On addition of 10 μg of rhPTN to rhBMP-2/FGM implants, the pattern of bone formation was similar to that described above (Figs. 7E and 8E). In contrast, with 100 μg native of PTN, there was no bone or cartilage (Figs. 7F and 8F). These results were consistent with the biochemical data shown in Fig. 6.

DISCUSSION

In this paper we found that PTN from bovine bone was co-purified with BMPs from the same tissue into the partially purified fraction after a three-step chromatographic procedure. Furthermore, the amount of ectopic bone formation induced by rhBMP-2 increased with the addition of 10 μg of rhPTN or native PTN, but decreased with the addition of 50 or 100 μg of native PTN. Thus the enhancing and inhibitory effects of PTN on BMP-2–induced osteogenesis were regarded as being highly dose-dependent.

Although no detailed evidence is available on the mechanism by which osteogenesis is increased by PTN, recent reports have shown that vascular capillary cells and endothelial cells (*14*) are stimulated by angiogenetic activity of PTN. Our previous observations on BMP-induced osteogenesis indicated that vascular development is crucial for efficacy of osteogenesis. When the vasculature is inhibited by the geometry of the BMP carrier, osteogenesis decreases and chondrogenesis becomes overwhelming instead (*4–8*). On the other hand, chondrogenesis is reduced when the BMP carrier, namely, artificial ECM, provides "vasculature-inducing geometry," and this leads to direct bone formation (*6–8*). In the present study, FGM was used as a carrier. This carrier is considered to be a "cartilage-inducing carrier" in that it induces six times more cartilage than insoluble bone matrix, a conventional carrier, at 2 weeks after implantation (*8, 9*). If vascular invasion or angiogenesis is stimulated by the addition 10 μg of PTN, the decreased cartilage formation may be understandable.

Another possible mechanism by which PTN may enhance bone formation is through its effects on chondrocytes. Tapp *et al.* reported that PTN inhibits chondrocyte proliferation, while stimulating the synthesis of proteoglycan (*29*). They also reported that the amounts of PTN varied with degree of maturation in cartilage, and the response of mature chondrocytes was dependent on the dose of PTN (*29*).

A third possibility is that osteoblasts might be a direct target of PTN. We have previously studied the expression of PTN in osteoblasts (*16, 35*). Moreover osteoblasts express PTN receptors, N-syndecan and RPTPβ/ζ, that change the balance of phosphorylation/dephosphorylation of F-actin binding protein contactin and β-catenin, respectively. Their transmembrane signaling systems regulate cell motility and migration response of osteoblast/osteoblastic precursor cells that provided the recent reports (*14, 26*). Therefore, it is possible that PTN activates the migration of osteoblast/osteoblastic precursor cells in our experiments. Our finding that bone formation is inhibited by high ratios of concentration of PTN, motility of local osteoblast/osteoblastic precursor cells may be reduced due to down-regulation of transmembrane signaling cascade. Using PTN coated microbeads and measurement of localization of cell motility–associated mRNAs, Rauvala *et al.* also found that an excess of PTN is able to block the effect of matrix-bound PTN (*26*). In contrast, osteoblast/osteoblastic precursor cells have well-known BMP-2 receptors and intracellular signaling pathways such as Smad. It is also possible that PTN and BMP-2 bind both receptors on the same cell surface, and then the phosphorylation/dephosphorylation balance of a certain signaling molecule of the BMP-2 pathway is modulated by PTN signaling.

Many other cytokines may affect the efficacy of BMP functions (*34–37*). Bentz *et al.* first reported a factor that enhanced BMP-induced osteogenesis (*36*). They showed that the addition of TGF-β1 to the implants of a BMP-carrier composite for ectopic bone induction increased bone formation about 1.8-fold. Later, the addition of activin was shown to increase BMP-induced bone production (*37*). Takita *et al.* (*38*) also showed that basic fibroblast growth factor acted synergistically with rhBMP-2 to promote ectopic osteogenesis in rat skin. On the other hand, noggin (*39*) and follistatin (*40*) were reported to inhibit some functions of BMP. These cytokines may have synergistic effects when administered in combination with BMP, and therefore be useful for clinical application of BMP. However, from the standpoint of tissue engineering, PTN may have advantages over other cytokines, since it is abundantly present in bone tissues.

Bone metabolism is under the concerted control of both systemic calcitrophic hormones and various local regulators. Our experiments demonstrate that PTN has diverse effects on BMP-2–induced endochondral ossification at different concentrations *in vivo*. These results suggest a possible physiological role of PTN in bone tissue, but its molecular mechanisms remain to determined.

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