Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 361 (2007) 62–67
BBRC
www.elsevier.com/locate/ybbrc

Platelet-rich plasma stimulates osteoblastic differentiation in the presence of BMPs

Akihiro Tomoyasu [a], Kanji Higashio [a,b], Kazuhiro Kanomata [a], Masaaki Goto [a,c], Kunihiko Kodaira [a,d], Hiroko Serizawa [a], Tatsuo Suda [a], Atsushi Nakamura [a], Junya Nojima [a], Toru Fukuda [a], Takenobu Katagiri [a,*]

[a] *Division of Pathophysiology, Research Center for Genomic Medicine, Saitama Medical University, 1397-1 Yamane, Hidaka-shi, Saitama 350-1241, Japan*
[b] *Metabolome Pharmaceuticals, Inc., 2-20-3 Nakamachi, Meguro-ku, Tokyo 153-0065, Japan*
[c] *Chugai Pharmaceutical Co., Ltd., Fuji Gotemba Research Laboratories, Genome Antibody Product Research Division, 1-135 Komakado, Gotemba, Shizuoka 412-8513, Japan*
[d] *Chugai Pharmaceutical Co., Ltd., Drug Engineering Division, 5-1, Ukima 5, Kita-ku, Tokyo 115-8543, Japan*

Received 25 June 2007
Available online 5 July 2007

Abstract

Platelet-rich plasma (PRP) is clinically used as an autologous blood product to stimulate bone formation in vivo. In the present study, we examined the effects of PRP on proliferation and osteoblast differentiation in vitro in the presence of bone morphogenetic proteins (BMPs). PRP and its soluble fraction stimulated osteoblastic differentiation of myoblasts and osteoblastic cells in the presence of BMP-2, BMP-4, BMP-6 or BMP-7. The soluble PRP fraction stimulated osteoblastic differentiation in 3D cultures using scaffolds made of collagen or hydroxyapatite. Moreover, heparin-binding fractions obtained from serum also stimulated osteoblastic differentiation in the presence of BMP-4. These results suggested that platelets contain not only growth factors for proliferation but also novel potentiator(s) for BMP-dependent osteoblastic differentiation.

Keywords: Bone morphogenetic proteins; Platelet-rich plasma; Osteoblasts; Scaffolds

Bone morphogenetic proteins (BMPs) have a unique and specific activity that is capable of inducing bone formation in vivo [1]. BMPs are responsible for not only artificial ectopic bone formation but also physiological skeletal development [2]. The bone-inducing activity of BMPs should be useful for the development of therapeutic drugs for in vivo bone regeneration [3,4]. However, it has been reported that, compared with rodents, more than 100-fold amounts of BMPs are required to induce bone formation in higher animals such as monkeys and humans [5]. We previously found that sulfated polysaccharides such as heparin and heparan sulfate enhanced BMP-induced osteoblast differentiation in vitro [6,7]. Moreover, heparin enhanced the ectopic bone formation in vivo induced by BMP-2 by protecting BMPs from degradation and inhibition by antagonists [6,7]. This finding suggests that BMP potentiators may be clinically useful for administration of BMPs to stimulate local bone formation in vivo.

Platelets are anuclear cells derived from megakaryocytes, and play important roles as rich sources of growth factors, including platelet-derived growth factor (PDGF), vascular endothelial growth factor, and transforming growth factor-β (TGF-β) [8,9]. These factors stored in α-granules of platelets are released through the activation of platelets by various stimuli [8,9]. Platelet-rich plasma (PRP) is clinically used as an autologous blood product to stimulate tissue regeneration in periodontal defects, extraction sockets, during implant placement, and in

* Corresponding author. Fax: +81 42 985 7214.
E-mail address: katagiri@saitama-med.ac.jp (T. Katagiri).

0006-291X/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2007.06.142

guided bone regeneration around implants [8,9]. The capacity of PRP to stimulate bone regeneration is believed to be due to the stimulatory activities of the growth factors released from activated platelets on proliferation of progenitor cells and vascularization at local sites [8,9].

In the present study, we examined the effects of PRP on proliferation and osteoblastic differentiation in vitro in the presence of small amounts of BMPs, and found that PRP stimulated the osteoblastic differentiation. Moreover, a soluble fraction prepared from activated PRP also exhibited stimulatory effects on the BMP activity in 3D cultures on scaffolds. These findings suggest that simultaneous administration of BMPs and PRP with scaffolds is a simple and useful method of stimulating bone formation in vivo. They also suggest that platelets contain novel potentiator(s) of BMPs that stimulate osteoblastic differentiation of progenitor cells.

Materials and methods

Materials. Bovine plasma was purchased from Funakoshi, Tokyo, Japan. Dulbecco's modified Eagle's medium (DMEM) α-minimal essential medium (α-MEM) were obtained from Sigma–Aldrich (St. Louis, MO). Fetal bovine serum (FBS) and calf serum (CS) were obtained from Moregate Biotech (Bulimba, Qld, Australia). Bovine thrombin was obtained from Wako Pure Chemical Industries (Osaka, Japan). Recombinant BMP-2 produced in *Escherichia coli* was obtained from Acris Antibodies GmbH (Nordhein-Westfalen, Germany). Recombinant human BMP-4, BMP-6, and BMP-7 were purchased from R&D Systems (Minneapolis, MN). SuperScript III and Platinum Pfx DNA polymerase were from Invitrogen (Carlsbad, CA). Collagen sponge and porous hydroxyapatite (Celllyard, HA) were obtained from Wako Pure Chemical Industries and Pentax (Tokyo, Japan), respectively. A Cell Count Reagent SF kit was from Nacalai Tesque, Tokyo, Japan. Heparin sepharose CL-6B column was obtained from GE Healthcare (Fairfield, CT).

Cell cultures. A mouse cell line, C2C12, was maintained in DMEM containing 15% FBS [10], and ST-2, MC3T3-E1 and 3T3-L1 cells were maintained in α-MEM containing 10% FBS. C2C12 cells were inoculated at 1.0×10^4 cells/well in type I collagen-coated 96-well plates (IWAKI, Tokyo, Japan) and cultured overnight before treatment with stimuli. ST-2, MC3T3-E1, and 3T3-L1 cells were inoculated at 500 cells/well in type I collagen-coated plates and cultured for 2 days. BMPs at various concentrations were added to the cultures in fresh medium containing 1% FBS with or without PRP or a soluble fraction prepared from PRP or platelet-poor plasma (PPP). Osteoblastic differentiation was determined by measuring alkaline phosphatase (ALP) activity and mRNA levels as described below.

Preparations of PRP and PPP, and soluble fractions of PRP and PPP. Human or bovine plasma was centrifuged at 2100*g* for 20 min to precipitate platelets. PRP was prepared by re-suspending the precipitates in 1/100 volume of PPP, which was the supernatant fraction derived from centrifugation of plasma. The PRP and PPP were diluted 1/10 in DMEM containing 1% FBS and then activated with 0.01 U/μl of bovine thrombin in the presence of 0.025% $CaCl_2$. The activated PRP and PPP were centrifuged at 3400*g* for 10 min to remove fibrin clots, and the supernatants were prepared as soluble fractions of PRP and PPP, respectively.

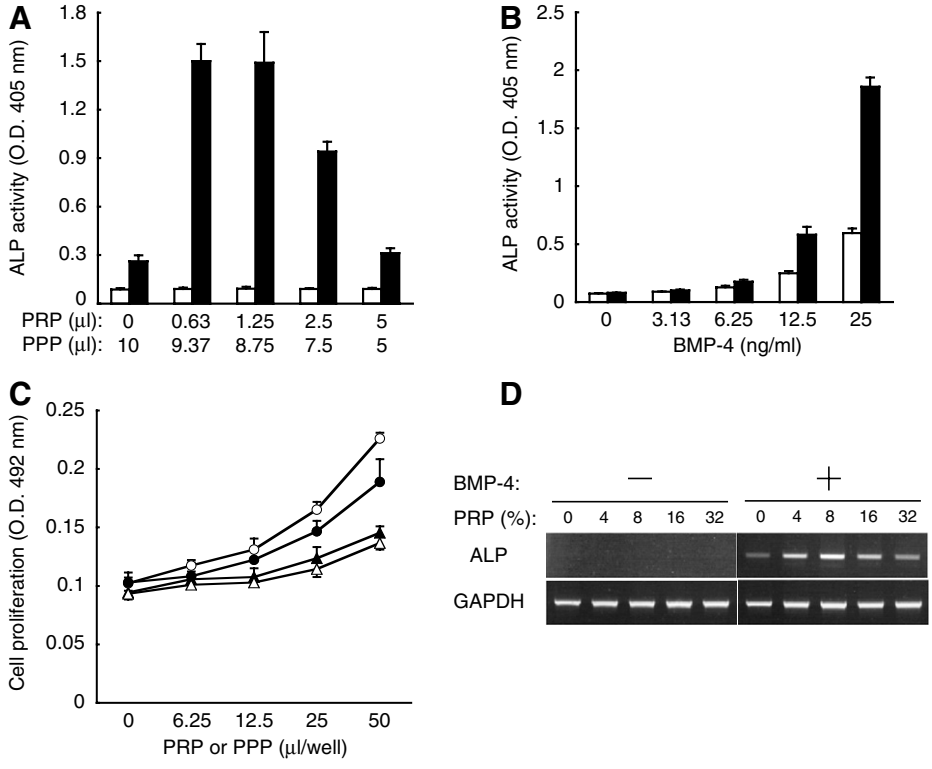

Fig. 1. Human and bovine PRP stimulate proliferation and osteoblastic differentiation of C2C12 myoblasts in the presence of BMP-4. (A) Human PRP diluted with PPP was added to cultures of C2C12 cells in the presence (solid bars) and absence (open bars) of 20 ng/ml of BMP-4. Total amount of the mixture was fixed at 10 μl in 150 μl of total culture volume. On day 6, ALP activity was determined as a marker of osteoblastic differentiation. (B) Soluble fraction prepared from bovine PRP (solid bars) or PPP (open bars) was added to C2C12 cultures with increasing concentrations of BMP-4. ALP activity was determined on day 4. (C) Effects of soluble PRP (circles) and PPP (triangles) fractions on proliferation of C2C12 cells in the presence (solid symbols) or absence (open symbols) of 25 ng/ml of BMP-4. Cell proliferation capacity was determined on day 4. (D) RT-PCR analysis of levels of expression of ALP mRNAs. C2C12 cells were treated for 4 days with increasing amounts of soluble PRP fraction in the presence or absence of 25 ng/ml of BMP-4.

Measurements of ALP activity and cell proliferation. ALP activity was measured as described previously [11]. In brief, the cells were rinsed with PBS and enzyme activity was determined by directly adding ALP buffer containing a substrate (0.6 M diethanolamine, 0.6 mM $MgCl_2$, 0.1% Triton X-100, and 30 mM *p*-nitrophenylphosphate) to each well. After incubation for 15 min at room temperature, the reaction was terminated by adding 3 M NaOH, and absorbance at 405 nm was measured using a microplate reader (ASYS Hitech Gmbh, Eugendorf, Austria). Cell proliferation was measured by absorbance at 492 nm using a Cell Count Reagent SF kit according to the manufacture's instructions.

Reverse transcription-polymerase chain reaction (RT-PCR). Levels of expression of ALP mRNA were analyzed by a RT-PCR technique as described [12]. In brief, cDNA was reverse transcribed from 5 μg of total RNA, and then each sequence was amplified by PCR (94 °C, 45 s; 60 °C, 1 min; 68 °C, 1 min; 30 cycles). Primers for ALP and glyceraldehyde-3-dehydrogenase were used [12].

Heparin sepharose CL-6B column chromatography. One liter of CS was pre-fractionated using a heparin sepharose CL-6B column equilibrated with 50 mM Tris–HCl, pH 7.5, containing 0.1% Chaps by a linear gradient of NaCl. This procedure was repeated five times. Fractions eluted with 1.0–1.5 M NaCl were pooled and applied to a new heparin sepharose CL-6B column. The bound proteins were eluted with a linear gradient of NaCl from 0 to 2.0 M. Each fraction was examined for ALP-inducing activity in C2C12 cells in the presence or absence of 20 ng/ml of BMP-4.

Results

PRP and soluble fraction of PRP stimulate BMP-4-induced osteoblastic differentiation and proliferation of C2C12 myoblasts

BMP-4 at 20 ng/ml slightly induced ALP activity in C2C12 cells (Fig. 1A). Addition of human PRP to the cultures stimulated ALP activity at lower concentrations, although the degree of stimulation gradually decreased at higher concentrations (Fig. 1A). In contrast, human PPP failed to stimulate ALP activity at all concentrations examined (Fig. 1A). We further examined the stimulatory activity in the soluble fractions prepared from bovine activated PRP and PPP. The ALP activity induced by BMP-4 at 12.5

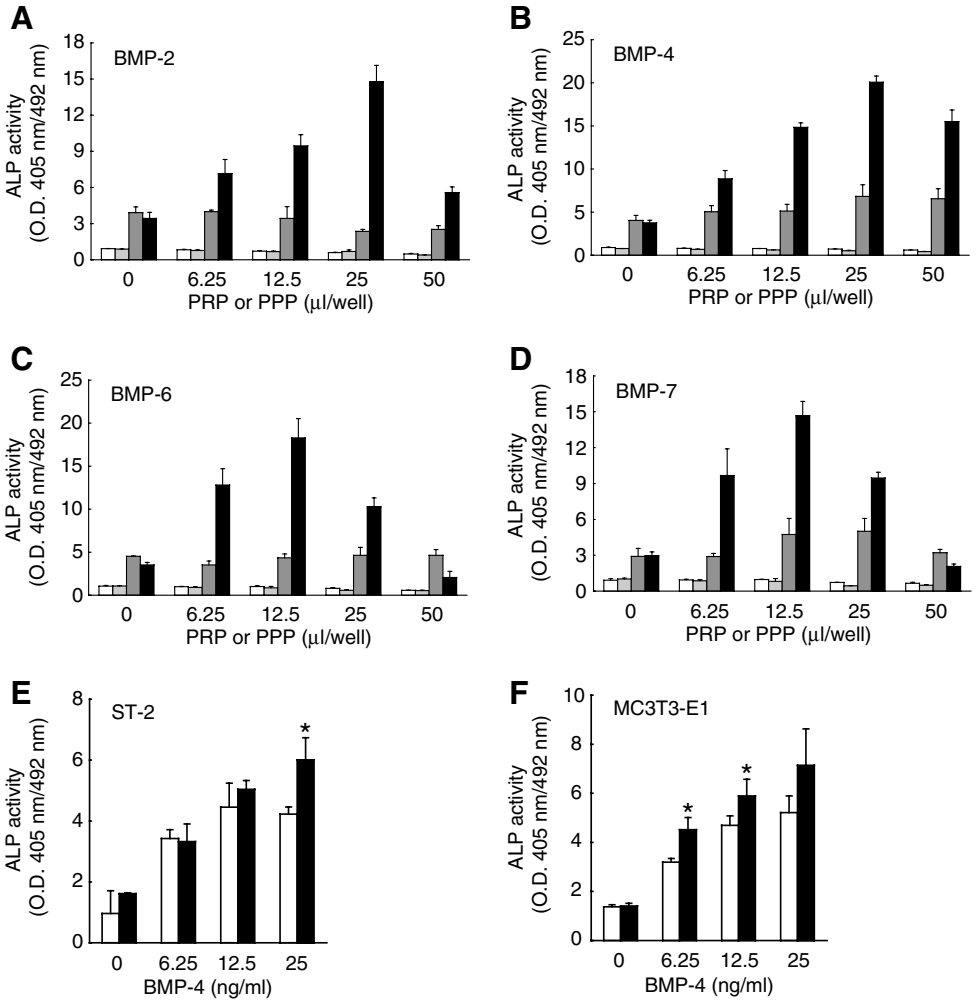

Fig. 2. Soluble PRP fraction stimulates osteoblastic differentiation of C2C12, ST-2, and MC3T3-E1 cells induced by various BMPs. Soluble fraction prepared from bovine PPP (open and gray bars) or PRP (light gray and solid bars) was added to cultures of C2C12 cells in the absence (open and light gray bars) or presence (gray and solid bars) of 300 ng/ml of BMP-2 (A), 25 ng/ml of BMP-4 (B), 150 ng/ml of BMP-6 (C), or 200 ng/ml of BMP-7 (D). ST-2 bone marrow stromal cells (E) and MC3T3-E1 osteoblasts (F) were cultured with graded concentrations of BMP-4 in the presence of soluble fraction prepared from bovine PPP (open bars) or PRP (solid bars). ALP activity was determined on day 4. $^*P < 0.05$ vs. PPP.

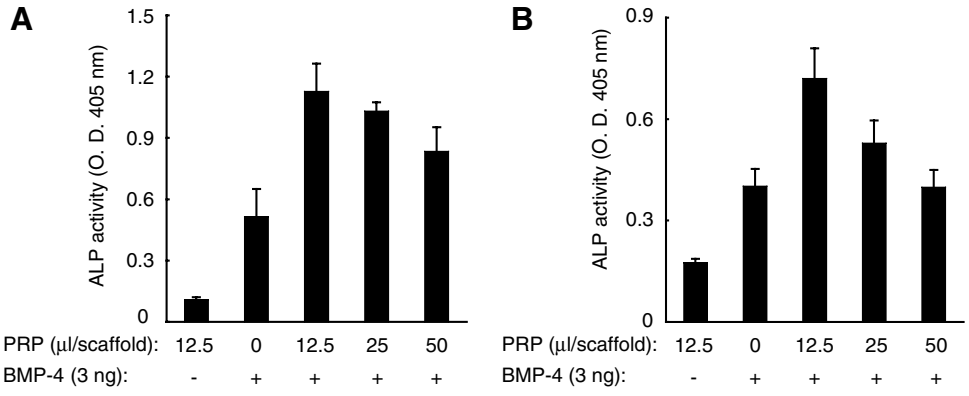

Fig. 3. Soluble PRP fraction and BMP-4 are retained in scaffolds and stimulate osteoblastic differentiation in vitro. Scaffolds made of collagen (A) and hydroxyapatite (B) were pre-incubated for 1 h with graded concentrations of soluble PRP fraction in the presence or absence of 3 ng of BMP-4 at room temperature, and then rinsed with PBS. C2C12 cells were inoculated on these scaffolds without additional PRP or BMP-4. ALP activity was determined on day 7.

and 25 ng/ml was much higher in the presence of the soluble fraction of PRP than that of PPP (Fig. 1B). Both soluble fractions of PRP and PPP stimulated proliferation at higher concentrations regardless of the presence of BMP-4, and higher stimulatory capacity was observed with the PRP fraction (Fig. 1C). The soluble PRP fraction increased levels of expression of ALP mRNA at lower concentrations in the presence of BMP-4 (Fig. 1D).

Ligand- and cell type-specificities of stimulatory activity of PRP

We examined the stimulatory effect of the soluble PRP fraction on osteoblastic differentiation in the presence of other BMPs. The soluble fraction of PRP, but not that of PPP, stimulated ALP activity in C2C12 cells in the presence of BMP-2, BMP-4, BMP-6, and BMP-7 (Fig. 2A–D). Higher concentrations of the soluble PRP fraction suppressed the ALP activity induced by each BMP examined, suggesting some inhibitors were also present in it. We next examined the stimulation of the BMP activity by the soluble fraction of PRP in two types of osteoblastic cells, ST-2 bone marrow stromal cells and MC3T3-E1 osteoblasts. In both types of cells, BMP-4 induced ALP activity at lower concentrations than in C2C12 myoblasts (Fig. 2E and F). As in C2C12 cells, ALP activity was much higher in the presence of the soluble fraction of PRP than that of PPP in both osteoblastic cell lines (Fig. 2E and F). 3T3-L1 preadipocytes also exhibited ALP activity on stimulation by the PRP fraction with more than 2 μg/ml of BMP-4 (data not shown).

Soluble PRP fraction stimulates osteoblastic differentiation of C2C12 cells cultured on scaffolds

BMPs are usually used in vivo with scaffolds made of collagen or hydroxyapatite to maintain local concentrations at sites of implantation [13,14]. We therefore pre-treated scaffolds of collagen sponge or hydroxyapatite with a mixture of BMP-4 and increasing amounts of the soluble PRP fraction. C2C12 cells were cultured on these scaffolds without additional BMPs or PRP. ALP activity was

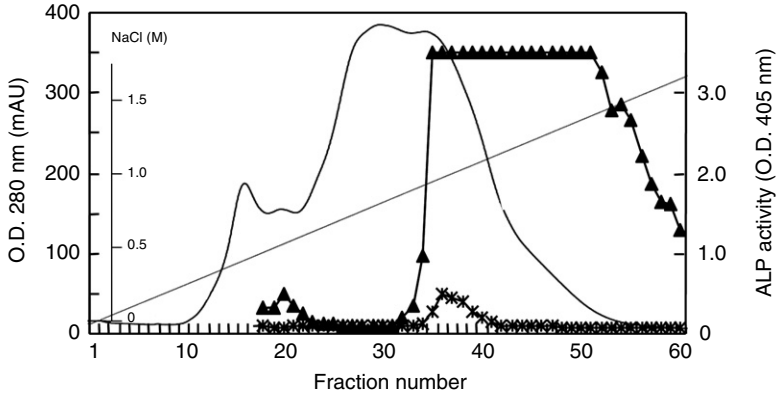

Fig. 4. BMP potentiator in CS binds to a heparin sepharose CL-6B column. Pre-fractionated CS was applied to a heparin sepharose CL-6B column as described in Materials and methods. The bound proteins were eluted by a linear gradient of NaCl from 0 to 2.0 M. Absorbance at 280 nm was monitored. The BMP potentiator activity in each fraction was determined by measuring ALP activity in C2C12 cells cultured with (triangles) or without (asterisks) 20 ng/ml of BMP-4.

induced on scaffolds pre-treated with the soluble PRP fraction and BMP-4, although enzyme activity gradually decreased with increasing amounts of the PRP fraction (Fig. 3A and B).

BMP potentiator in serum binds to a heparin sepharose CL-6B column

We have reported purification and identification of BMP-4 from bovine serum [11]. In our preliminary experiments, BMP potentiator was also detected in CS, which contains various factors released from platelets. To characterize the BMP potentiators, CS was applied to a heparin sepharose CL-6B column, and the bound proteins were eluted with a linear gradient of NaCl (Fig. 4). Fractions 35–53 induced high ALP activity in the presence of BMP-4 (Fig. 4). The potentiator activity on BMP-4 in a pool of the fractions 35–53 was lost by digestion with trypsin (data not shown).

Discussion

In the present study, we found that the soluble fraction prepared from activated PRP stimulates osteoblastic differentiation in the presence of BMPs in mesenchymal progenitor cells. Pretreatment of scaffolds with BMP-4 and the soluble PRP fraction stimulated osteoblastic differentiation in vitro. Because PRP is an autologous blood product, simultaneous application of PRP and BMPs with scaffolds may be a simple and useful method for enhancement of bone formation in vivo.

The stimulatory activity of PRP on bone healing in vivo is believed to be due to stimulation of proliferation of progenitor cells by growth factors released from activated platelets [8,9]. Interestingly, we found dual effects of the soluble fraction prepared from PRP on proliferation and differentiation. At lower concentrations, the PRP fraction markedly stimulated the osteoblastic differentiation in the presence of BMPs, while at higher concentrations the same fraction stimulated proliferation and suppressed osteoblastic differentiation. PDGF and TGF-β, which are abundantly stored in platelets, may be involved in stimulation of proliferation and suppression of differentiation, since such activities of PDGF and TGF-β have been reported in osteoblast progenitor cells [10,15,16]. These findings suggest that optimal dosages of PRP and BMPs must be determined to stimulate bone formation in vivo.

BMP activities are regulated positively and negatively by various types of molecules in the extracellular environment [17–19]. Although many inhibitors of BMPs have been identified as antagonists, such as noggin, chordin, follistatin, and the DAN family members, potentiators of BMPs have yet to be clearly identified [17–19]. We previously reported that some native and synthetic sulfated polysaccharides act as BMP potentiators in vitro and in vivo [6,7]. This activity is strongly dependent on both their size and the number of sulfated residues [6]. Recently, KCP, a Kielin/chordin-like protein, was identified as a novel BMP potentiator in the kidney [20]. KCP plays an important role in attenuating the pathology of renal fibrotic disease [21]. Our findings here suggest that platelets contain not only growth factors for proliferation but also novel potentiator(s) for BMP-dependent differentiation. Synergism between novel potentiator(s) in platelets and BMPs may also be involved in the stimulation of bone healing with use of PRP alone, since BMPs are present in bone matrix [22]. It is possible that a similar stimulation of the BMP activity by novel potentiator(s) plays an important role in native fracture healing in vivo. Identification of this novel potentiator and establishment of conditions of application of it with BMPs should enable clinically use of it for stimulation of local bone formation in vivo. Our findings suggested that this potentiator is a heparin-binding protein rather than a polysaccharide. This novel BMP potentiator produced by platelets is presently being studied in greater detail in our laboratory.

In conclusion, we found that PRP stimulates osteoblastic differentiation of mesenchymal progenitor cells in vitro in the presence of BMPs. Simultaneous administration of PRP and BMPs with scaffolds should be useful for stimulation of bone formation in vivo.

Acknowledgments

This work was supported in part by the Ministry of Education, Culture, Sports, Science and Technology of Japan, and in particular by a Ministry Grant to the Saitama Medical University Research Center for Genomic Medicine.

References

[1] T. Katagiri, T. Suda, K. Miyazono, The bone morphogenetic proteins, in: K. Miyazono, R. Derynck (Eds.), The TGF-β Superfamily Monograph, Cold Spring Harbor, New York, in press..
[2] J.T. Thomas, M.W. Kilpatrick, K. Lin, L. Erlacher, P. Lembessis, T. Costa, P. Tsipouras, F.P. Luyten, Disruption of human limb morphogenesis by a dominant negative mutation in CDMP1, Nat. Genet. 17 (1997) 58–64.
[3] T.D. Alden, P. Varady, D.F. Kallmes, J.A. Jane Jr., G.A. Helm, Bone morphogenetic protein gene therapy, Spine 27 (2002) S87–S93.
[4] P.S. Issack, P.E. DiCesare, Recent advances toward the clinical application of bone morphogenetic proteins in bone and cartilage repair, Am. J. Orthop. 32 (2003) 429–436.
[5] K. Kusumoto, K. Bessho, K. Fujimura, J. Akioka, Y. Okubo, Y. Wang, T. Iizuka, Y. Ogawa, Osteoinduction by recombinant human bone morphogenetic protein-2 in muscles of non-human primates, J. Int. Med. Res. 30 (2002) 251–259.
[6] T. Takada, T. Katagiri, M. Ifuku, N. Morimura, M. Kobayashi, K. Hasegawa, A. Ogamo, R. Kamijo, Sulfated polysaccharides enhance the biological activities of bone morphogenetic proteins, J. Biol. Chem. 278 (2003) 43229–43235.
[7] B. Zhao, T. Katagiri, H. Toyoda, T. Takada, T. Yanai, T. Fukuda, U.I. Chung, T. Koike, K. Takaoka, R. Kamijo, Heparin potentiates the in vivo ectopic bone formation induced by bone morphogenetic protein-2, J. Biol. Chem. 281 (2006) 23246–23253.
[8] L. Boyapati, H.L. Wang, The role of platelet-rich plasma in sinus augmentation: a critical review, Implant Dent. 15 (2006) 160–170.

[9] T.F. Tozum, B. Demiralp, Platelet-rich plasma: a promising innovation in dentistry, J. Can. Dent. Assoc. 69 (2003) 664.
[10] T. Katagiri, A. Yamaguchi, M. Komaki, E. Abe, N. Takahashi, T. Ikeda, V. Rosen, J.M. Wozney, A. Fujisawa-Sehara, T. Suda, Bone morphogenetic protein-2 converts the differentiation pathway of C2C12 myoblasts into the osteoblast lineage, J. Cell Biol. 127 (1994) 1755–1766.
[11] K. Kodaira, M. Imada, M. Goto, A. Tomoyasu, T. Fukuda, R. Kamijo, T. Suda, K. Higashio, T. Katagiri, Purification and identification of a BMP-like factor from bovine serum, Biochem. Biophys. Res. Commun. 345 (2006) 1224–1231.
[12] H. Hattori, M. Ishihara, T. Fukuda, T. Suda, T. Katagiri, Establishment of a novel method for enriching osteoblast progenitors from adipose tissues using a difference in cell adhesive properties, Biochem. Biophys. Res. Commun. 343 (2006) 1118–1123.
[13] Y. Kuboki, H. Takita, D. Kobayashi, E. Tsuruga, M. Inoue, M. Murata, N. Nagai, Y. Dohi, H. Ohgushi, BMP-induced osteogenesis on the surface of hydroxyapatite with geometrically feasible and nonfeasible structures: topology of osteogenesis, J. Biomed. Mater. Res. 39 (1998) 190–199.
[14] H. Toyoda, H. Terai, R. Sasaoka, K. Oda, K. Takaoka, Augmentation of bone morphogenetic protein-induced bone mass by local delivery of a prostaglandin E EP4 receptor agonist, Bone 37 (2005) 555–562.
[15] A.F. Kells, S.R. Coats, H.S. Schwartz, R.L. Hoover, TGF-beta and PDGF act synergistically in affecting the growth of human osteoblast-enriched cultures, Connect. Tissue Res. 31 (1995) 117–124.
[16] T. Katagiri, T. Lee, H. Takeshima, T. Suda, H. Tanaka, S. Omura, Transforming growth factor-beta modulates proliferation and differentiation of mouse clonal osteoblastic MC3T3-E1 cells depending on their maturation stages, Bone Miner. 11 (1990) 285–293.
[17] E. Abe, Function of BMPs and BMP antagonists in adult bone, Ann. N. Y. Acad. Sci. 1068 (2006) 41–53.
[18] V. Rosen, BMP and BMP inhibitors in bone, Ann. N. Y. Acad. Sci. 1068 (2006) 19–25.
[19] E. Gazzerro, E. Canalis, Bone morphogenetic proteins and their antagonists, Rev. Endocr. Metab. Disord. 7 (2006) 51–65.
[20] J. Lin, S.R. Patel, X. Cheng, E.A. Cho, I. Levitan, M. Ullenbruch, S.H. Phan, J.M. Park, G.R. Dressler, Kielin/chordin-like protein, a novel enhancer of BMP signaling, attenuates renal fibrotic disease, Nat. Med. 11 (2005) 387–393.
[21] M. Yanagita, Modulator of bone morphogenetic protein activity in the progression of kidney diseases, Kidney Int. 70 (2006) 989–993.
[22] M.R. Urist, Bone: formation by autoinduction, Science 150 (1965) 893–899.