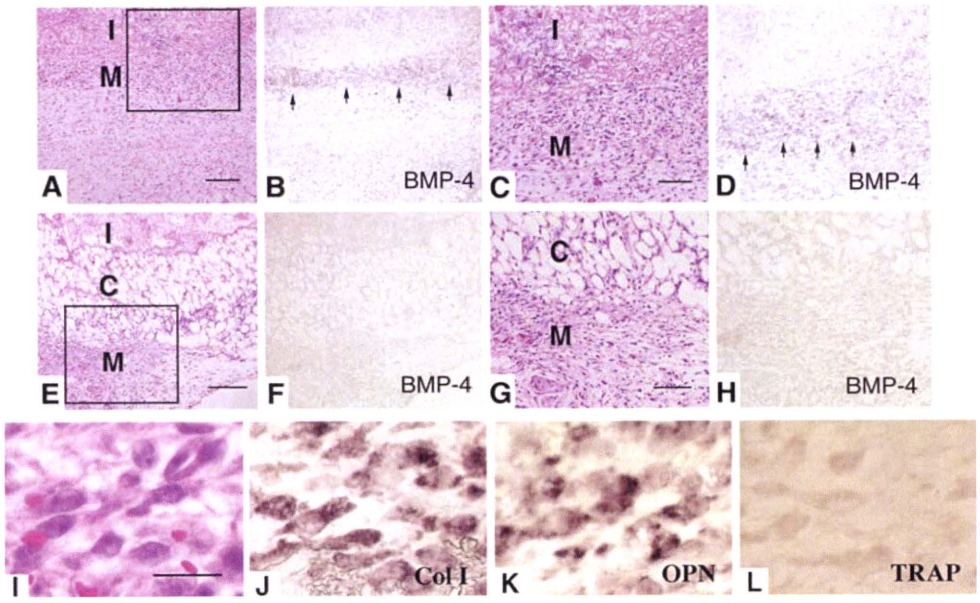

Fig. 2A–L Localization of BMP-4 transcripts in tissue sections on Day 4 after implantation. (**A** and **B**), (**C** and **D**), (**E** and **F**), (**G** and **H**) are adjacent sections. **C** and **G** are higher magnifications of square area of **A** and **B**, respectively. (Scale bar: **A** and **E**; 50 μm. **C** and **G**; 25 μm, I: implant, C: loose connective tissue, M: mesenchymal cell layer) (**A–H**.) Hematoxylin and eosin staining and in situ hybridization for antisense cRNA probe for BMP-4. (**A–D**) Y-27632-treated mice or (**E–H**) PBS-treated mice (original magnifications ×100). (**I–L**) Higher magnification of migrating cells towards rhBMP-2 pellet as shown by: (**I**) hematoxylin and eosin staining; in situ hybridization using antisense cRNA for (**J**) Type I collagen and (**K**) osteopontin; and (**L**) TRAP, stained with tartrate resistant acid phosphatase (TRAP). (Scale bar, 10 μm and original magnifications ×200).

a dose-dependent manner, but also enhanced nodule formation (Fig. 3A–B).

Ten μM of Y-27632 enhanced (two- to threefold compared to the control; $p = 0.0007$) the expression of BMP-4 mRNA level in ST2 cells (Fig. 4A, hatched bars). This effect appeared specific because adding rhBMP-2 did not alter the BMP-4 mRNA level in ST2 cells (Fig. 4B). Expression of the active ROCK mutant (Δ4) in cultured ST2 cells (Fig. 5C) decreased the ALP activity (Fig. 5A, bar 5) in the absence of ROCK inhibitor. The addition of the ROCK inhibitor partly reversed these inhibitory actions (Fig. 5A, bar 6). Conversely the expression of a dominant negative ROCK construct (KDΔ4, Fig. 5C) enhanced ALP activity (Fig. 5A, bar 7) and BMP-4 expression (Fig. 5B, bar 7), and the addition of the ROCK inhibitor stimulated ALP activity and BMP-4 expression (Fig. 5A–B, bar 8). Furthermore, the ST2 cells expressing dominant negative ROCK represented ALP-positive staining (Fig. 5D, lower panel). Active ROCK mutant expressing ST2 cells never showed ALP positive staining (Fig. 5D, upper panels).

Discussion

The small GTPase Rho and Rho-associated protein kinase (Rho kinase, ROCK) signal participates in a variety of biological functions including vascular contraction, tumor invasion, and penile erection. However, whether it is involved in osteoblastic differentiation is unknown. In order to examine the biological role of ROCK in osteogenesis, we asked the following five questions: (1) Does ROCK inhibitor enhance BMP-induced bone formation in vivo? (2) What kinds of cells migrate to the BMP/atelocollagen pellets to induce bone formation in vivo? (3) Does ROCK inhibitor itself enhance osteogenic differentiation of primary cultured calvaria cells in vitro? (4) What is the mechanism for ROCK inhibitor induced osteogenic differentiation? (5) Does ectopic expression of dominant negative ROCK enhance osteoblastic differentiation and does ectopic expression of active ROCK inhibit it?

We acknowledge the following limitations. Since our study has been carried out using a mouse ectopic bone formation system in vivo (mice) and in vitro, the data may not reflect that in human osteogenesis. First, we assume the signaling pathways in vitro are similar to those in vivo, although the in vivo biological environment may have additional or alternate pathways. Indeed, systemic delivery of ROCK inhibitor by osmotic pump only stimulated the ectopic BMP-induced bone formation in mice (Fig. 1 and Table 1), but did not affect the endogenous bone metabolism in tibia (Table 1). Second, given the limited experimental conditions, we consider this a pilot study. Further studies will be required to confirm these observations across a wide range of conditions and models.

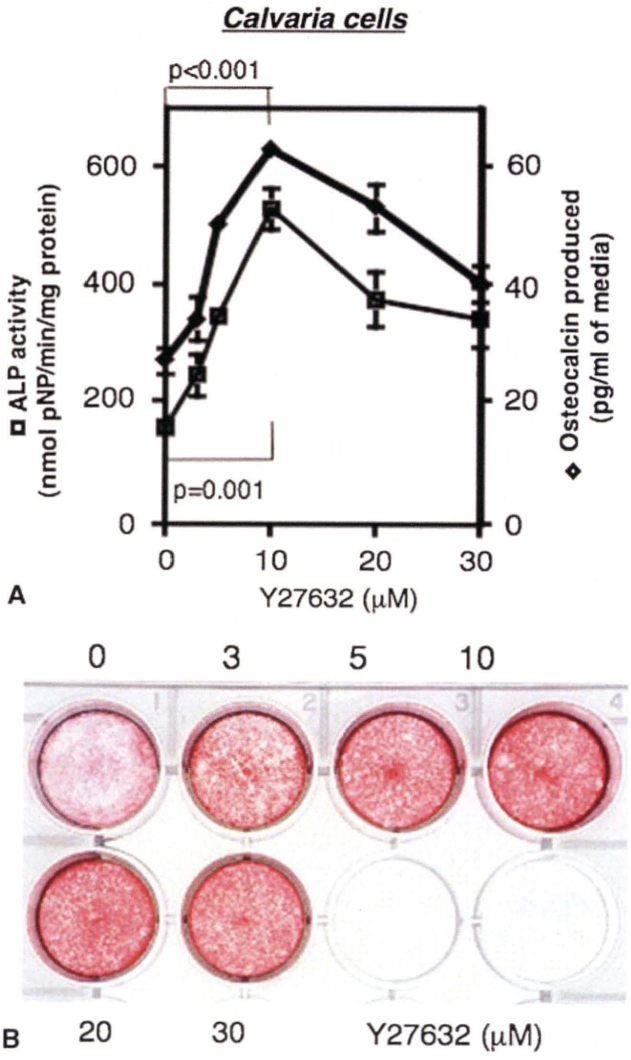

Fig. 3A–B Stimulated ALP activity, osteocalcin production and nodule formation by ROCK inhibitor in primary culture of neonatal murine calvarial cells. (**A**) Stimulated ALP activity (■) and osteocalcin production (◆) by ROCK inhibitor in calvarial cells for 4 days (mean ± SEM, n = 6) (in the absence of Y-27632). (**B**) Nodule formation of calvarial cells for 16 days in the presence of given concentrations of Y-27632.

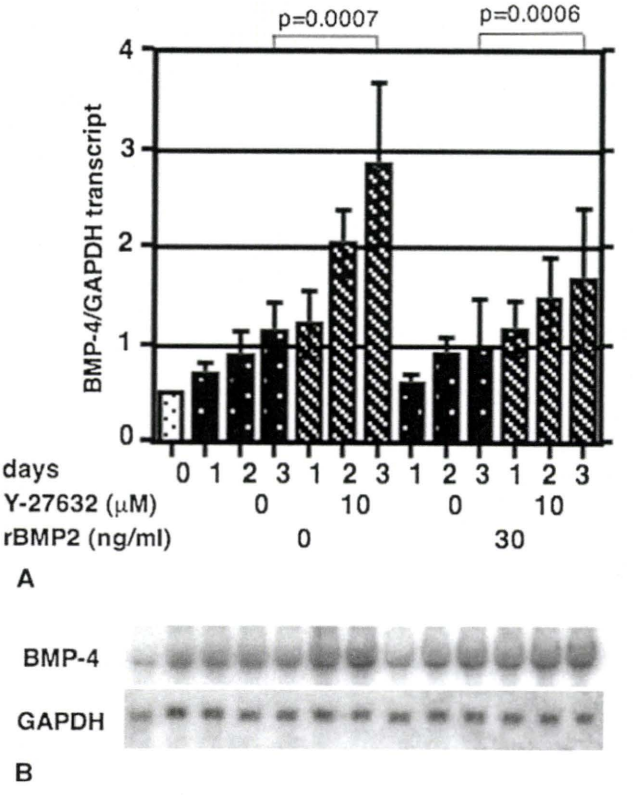

Fig. 4A–B Effects of ROCK inhibitors and BMP-2 on the expression of BMP-4 in vitro. (**A**) The level of transcript was normalized with control GAPDH transcript and shown as mean ± SEM of three determinations. Ten μM of Y-27632 enhanced (two- to threefold compared to the control) the expression of BMP-4 mRNA level in ST2 cells. (**B**) Representative blots of BMP-4 and GAPDH are shown.

We found continuous delivery of a specific ROCK inhibitor (Y-27632) enhanced ectopic bone formation induced by rhBMP-2 impregnated into an atelocollagen carrier in mice without affecting systemic bone metabolism. Treatment with Y-27632 also enhanced the osteoblastic differentiation of cultured murine neonatal calvarial and ST2 cells. These effects were associated with increased expression of BMP-4 gene. Expression of a dominant negative mutant of ROCK in ST2 cells promoted osteoblastic differentiation, while a constitutively active mutant of ROCK attenuated osteoblastic differentiation and the ROCK inhibitor reversed this phenotype. Thus, ROCK apparently plays a negative role in osteogenesis. Mundy et al. [23] reported the stimulation of bone formation in rodents by a statin (inhibitor for HMG-CoA reductase) from the screening of more than 30,000 compounds derived from natural products. The effect of the statin may lower the cellular cholesterol level, which is required for the geranylgeranylation of Rho to show full activity, as the authors postulated. If this is the case, a ROCK inhibitor might be a more promising and specific stimulator for the promoting of osteogenesis, since it exists downstream in the signal transduction cascade. Consistent with the results, Ohnaka et al. [26] reported that pitavastatin, a newly developed statin, enhanced BMP-2 and osteocalcin expression in Rho-associated kinase dependent manner in primary cultured human osteoblasts. They also reported that hydroxyfasudil, a specific inhibitor for ROCK, increased BMP-2 and osteocalcin production. Here, we further confirmed these results by the expression of active and negative ROCK protein in the cells and also presented the enhanced osteogenesis in vivo animal model.

Employing this in vivo animal model using recombinant human BMP-2 containing Type I collagen deposit, we have already screened a number of compounds, including clinically approved antiosteoporotic drugs, such

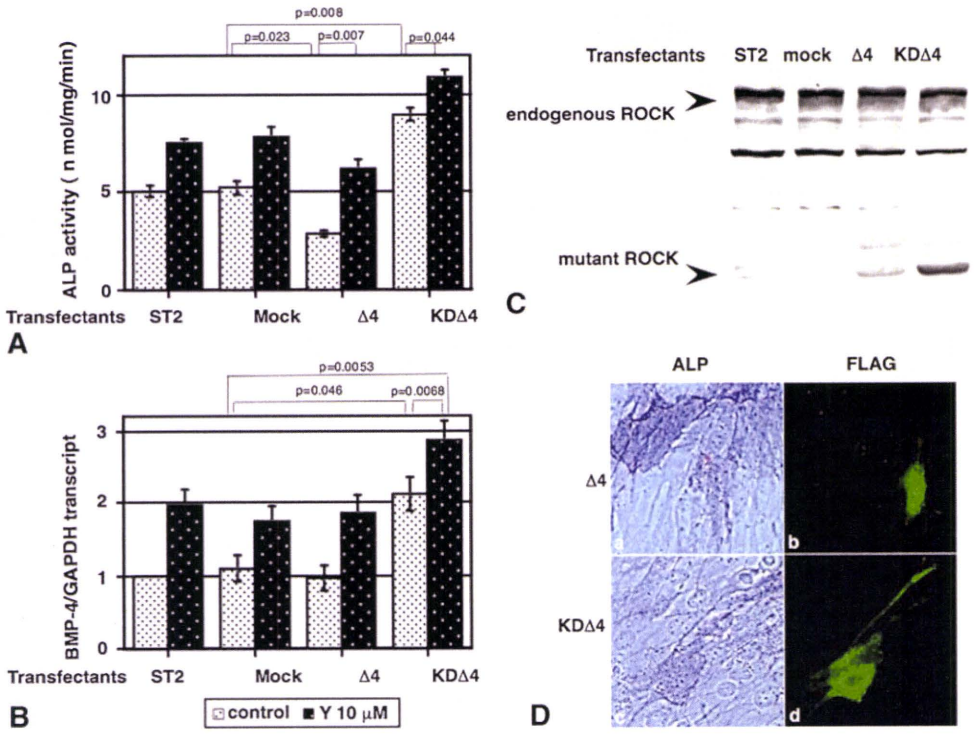

Fig. 5A–D Effects of expression of active (Δ4) or dominant negative ROCK (KDΔ4) mutants on the osteoblastic differentiation in ST2 cells in vitro. (**A** and **B**) ST2 cells were transiently transfected with vectors alone (mock) or with vectors encoding Δ4 or KDΔ4. ALP activity (**A**) and the level of BMP-4 transcript (**B**) were determined in the absence (open bars) or presence (filled bars) of ROCK inhibitor at 72 hours after the transfection. The level of transcript was normalized with control GAPDH transcript. Both ALP activity and the level of BMP-4 were shown as mean ± SEM of six determinations. (**C**) Immunoblot analysis of the same cell lysates with antibodies to ROCK [23] after SDS-7% PAGE. Arrows, positions of endogenous ROCK and recombinant ROCK mutants. (**D**) ALP staining (right panels, original magnification ×200) and anti-FLAG Abs immunostaining (left panels, original magnification ×200) of ST2 cells transfectants.

as ipriflavon, vitamin D analogue, or estrogen analogue. However, none of the compounds demonstrated the positive effect for osteogenesis in vivo. Only estrogen showed high-density bone without changing the ossicle size. We also recently reported the inhibitors for MEK (MAP kinase kinase)-enhanced osteogenesis (ALP activity, osteocalcin secretion, and nodule formation) in the culture cells [6]. However, we could not check the effect of MEK inhibitor on the in vivo BMP-induced osteogenesis because of the limiting availability of the compound. So far the ROCK inhibitor presented here is the only compound in which we found the positive effect for osteogenesis in vivo.

We also found the expression of cbfa-1, a downstream key regulator for osteogenesis [5, 14], was not increased by the ROCK inhibitor and/or rhBMP-2 treatment. Cbfa-1 is a crucial regulator for osteoblastic differentiation; however, the cbfa-1 gene expression during the osteoblastic differentiation is reportedly crucial in some cases and also cell-type specific [5, 14]. Consistent with our results, most recently Mizuno and Kuboki [21] reported that the cbfa-1 gene expression was independent for the osteoblastic differentiation lineage using rat bone marrow stromal cells cultured with Type I collagen. Other posttranslational mechanisms, such as phosphorylation or complex formation of the cbfa-1 protein, should be considered for the activity of this transcriptional factor. In addition, the ROCK inhibitor did not alter the phosphorylation of smad 1/5 [16], a direct downstream target of BMP-2/4 signaling (data not shown).

Enhancement of the healing process for nonunion fractures, reconstruction of local bone defects resulting from surgical resection of bone tumors, and acceleration of an interbody fusion for degenerative spinal disorders are major problems in orthopaedic and craniofacial surgery. Also diseases involving systemic bone loss, such as osteoporosis are major global public health problems. The molecules or genes responsible for this negative control mechanism of osteoblastic differentiation have not yet been identified. The identification of Rho-associated kinase as a negative regulator of bone formation suggests that the ROCK inhibitors such as Y-27632, in conjunction with a local delivery of rhBMPs/collagen, may have therapeutic utility in the clinical setting.

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References

1. Bishop GB, Einhorn TA. Current and future clinical applications of bone morphogenetic proteins in orthopaedic trauma surgery. *Int Orthop.* 2007;6:721–727.
2. Chitaley K, Wingard CJ, Webb RC, Branam H, Stopper VS, Lewis RW, Mills TM. Antagonism of Rho-kinase stimulates rat penile erection via a nitric oxide-independent pathway. *Nature Med.* 2001;7:119–122.
3. Chrzanowska-Wodnicka M, Burridge K. Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J Cell Biol.* 1996;133:1403–1415.
4. Coleman ML, Sahai EA, Yeo M, Bosch M, Dewar A, Olson MF. Membrane blebbing during apoptosis results from caspase-mediated activation of ROCK 1. *Nature Cell Biol.* 2001;3:339–345.
5. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa-1: a transcriptional activator of osteoblast differentiation. *Cell.* 1997;89:747–754.
6. Higuchi C, Myoui A, Hashimoto N, Kuriyama K, Yoshioka K, Yoshikawa H, Itoh K. Continuous inhibition of MAPK signaling promotes the early osteoblastic differentiation and mineralization of extracellular matrix. *J Bone Miner Res.* 2002;17:1785–1794.
7. Hirota S, Takaoka K, Hashimoto J, Nakase T, Takemura T, Morii E, Fukuyama A, Morihana K, Kitamura Y, Nomura S. Expression of mRNA of murine bone-related proteins in ectopic bone induced by murine bone morphogenetic protein-4. *Cell Tissue Res.* 1994;277:27–32.
8. Hoshino M, Namikawa T, Kato M, Terai H, Taguchi S, Takaoka K. Repair of bone defects in revision hip arthroplasty by implantation of a new bone-inducing material comprised of recombinant human BMP-2, Beta-TCP powder, and a biodegradable polymer: an experimental study in dogs. *J Orthop Res.* 2007;25:1042–1051.
9. Ishizaki T, Maekawa M, Fujisawa K, Okawa K, Iwamatsu A, Fujita A, Watanabe N, Saito Y, Kakizuka A, Morii N, Narumiya S. The small GTP-binding protein Rho binds to and activates a 160 kDa Ser/Thr protein kinase homologous to myotonic dystrophy kinase. *EMBO J.* 1996;15:1885–1893.
10. Ishizaki T, Naito M, Fujisawa K, Maekawa M, Watanabe N, Saito Y, Narumiya S. $p160^{ROCK}$, a Rho-associated coiled-coil forming protein kinase, works downstream of Rho and induces focal adhesions. *FEBS Lett.* 1997;404:118–124.
11. Itoh F, Asao H, Sugamura K, Heldin CH, Dijke PT, Itoh S. Promoting bone morphogenetic protein signaling through negative regulation of inhibitory Smads. *EMBO J.* 2001;20:4132–4142.
12. Itoh K, Yoshioka K, Akedo H, Uehata M, Ishizaki T, Narumiya S. An essential part for Rho-associated kinase in the transcellular invasion of tumor cells. *Nature Med.* 1999;5:221–225.
13. Kaito T, Myoui A, Takaoka K, Saito N, Nishikawa M, Tamai N, Ohgushi H, Yoshikawa H. Potentiation of the activity of bone morphogenetic protein-2 in bone regeneration by a PLA-PEG/hydroxyapatite composite. *Biomaterials,* 2005;26:73–79.
14. Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson RT, Gao YH, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S, Kishimoto T. Targeted disruption of *Cbfa1* results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell.* 1997;89:755–764.
15. Krause F, Younger A, Weber M. Recombinant human BMP-2 and allograft compared with autogenous bone graft for reconstruction of diaphyseal tibial fractures with cortical defects. *J Bone Joint Surg Am.* 2008;90:1168–1169.
16. Kretzschmar M, Doody J, Massague J. Opposing BMP and EGF signaling pathways converge on the TGF-β family mediator Smad1. *Nature.* 1997;389:618–622.
17. Kuwabara H, Wada T, Oda T, Yoshikawa H, Sawada N, Kokai Y, Ishii S. Overexpression of the granulocyte colony-stimulating factor gene impairs bone morphogenetic protein responsiveness in mice. *Lab Invest.* 2001;81:1133–1141.
18. Leung T, Manser E, Tan L, Lim L. A novel serine/threonine kinase binding the Ras-related RhoA GTPase which translocates the kinase to peripheral membranes. *J Biol Chem.* 1995;270:29051–29054.
19. Matsui T, Amano M, Yamamoto T, Chihara K, Nakafuku M, Ito M, Nakano T, Okawa K, Iwamatsu A, Kaibuchi K. Rho-associated kinase, a novel serine/threonine kinase, as a putative target for the small GTP binding protein Rho. *EMBO J.* 1996;15:2208–2216.
20. Miyazono K, Kusanagi K, Inoue H. Divergence and convergence of TGF-beta/BMP signaling. *J Cell Physiol.* 2001;187:265–276.
21. Mizuno M, Kuboki Y. Osteoblast-related gene expression of bone marrow cells during the osteoblastic differentiation induced by type I collagen. *J Biochem.* 2001;129:133–138.
22. Mori S, Yoshikawa H, Hashimoto J, Ueda T, Funai H, Kato M, Takaoka K. Antiangiogenic agent (TNP-470) inhibition of ectopic bone formation induced by bone morphogenetic protein-2. *Bone.* 1998;22:99–105.
23. Mundy G, Garrett R, Harris S, Chan J, Chen D, Rossini G, Boyce B, Zhao M, Gutierrez G. Stimulation of bone formation in vitro and in rodents by statins. *Science.* 1999;286:1946–1949.
24. Murakami N, Saito N, Horiuchi H, Okada T, Nozaki K, Takaoka K. Repair of segmental defects in rabbit humeri with titanium fiber mesh cylinders containing recombinant human bone morphogenetic protein-2 (rhBMP-2) and a synthetic polymer. *J Biomed Mater Res.* 2002;62:169–174.
25. Nakase T, Nomura S, Yoshikawa H, Hashimoto J, Hirota S, Kitamura Y, Oikawa S, Ono K, Takaoka K. Transient and localized expression of bone morphogenetic protein 4 messenger RNA during fracture healing. *J Bone Miner Res.* 1994;9:651–659.
26. Ohnaka K, Shimoda S, Nawata H, Shimokawa H, Kaibuchi K, Iwamoto Y, Takayanagi R. Pitavastatin enhanced BMP-2 and osteocalcin expression by inhibition of Rho-associated kinase in human osteoblasts. *Biochem Biophys Res Commun.* 2001;287:337–342.
27. Reddi AH. Role of morphogenetic proteins in skeletal tissue engineering and regeneration. *Nat Biotechnol.* 1998;16:247–252.
28. Sahai E, Alberts AS, Treisman R. RhoA effector mutants reveal distinct effector pathways for cytoskeletal reorganization, SRF activation and transformation. *EMBO J.* 1998;17:1350–1361.
29. Takaoka K, Koezuka M, Nakahara H. Telopeptide-depleted bovine skin collagen as a carrier for bone morphogenetic protein. *J Orthop Res.* 1991;9:902–907.
30. Tamai N, Myoui A, Hirao M, Kaito T, Ochi T, Tanaka J, Takaoka K, Yoshikawa H. A new biotechnology for articular cartilage repair: subchondral implantation of a composite of interconnected porous hydroxyapatite, synthetic polymer (PLA-PEG), and bone morphogenetic protein-2 (rhBMP-2). *Osteoarthritis Cartilage.* 2005;13:405–417.
31. Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M, Narumiya S. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. *Nature.* 1997;389:990–994.
32. Urist MR. Bone: formation by autoinduction. *Science.* 1965;150:893–899.

33. Wozney JM, Rosen V, Celeste AJ, Mitsock LM, Whitters MJ, Kriz RW, Hewick RM, Wang EA. Novel regulators of bone formation: molecular clones and activities. *Science*. 1988;242:1528–1534.
34. Yoshikawa H, Taniguchi S, Yamamura H, Mori S, Sugimoto M, Miyado K, Nakamura K, Nakao K, Katsuki M, Shibata N, Takahashi K. Mice lacking smooth muscle calponin display increased bone formation that is associated with enhancement of bone morphogenetic protein responses. *Genes Cells*. 1998;3:685–695.
35. Yoshioka K, Matsumura F, Akedo H, Itoh K. Small GTP-binding protein Rho stimulates the actomyosin system, leading to invasion of tumor cells. *J Biol Chem*. 1998;273:5146–5154.

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SYMPOSIUM: TRIBUTE TO DR. MARSHALL URIST: MUSCULOSKELETAL GROWTH FACTORS

Synthetic Alginate is a Carrier of OP-1 for Bone Induction

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Abstract Bone morphogenetic proteins (BMPs) can induce bone formation in vivo when combined with appropriate carriers. Several materials, including animal collagens and synthetic polymers, have been evaluated as carriers for BMPs. We examined alginate, an approved biomaterial for human use, as a carrier for BMP-7. In a mouse model of ectopic bone formation, the following four carriers for recombinant human OP-1 (BMP-7) were tested: alginate crosslinked by divalent cations (DC alginate), alginate crosslinked by covalent bonds (CB alginate), Type I atelocollagen, and poly-D,L-lactic acid-polyethyleneglycol block copolymer (PLA-PEG). Discs of carrier materials (5-mm diameter) containing OP-1 (3–30 μg) were implanted beneath the fascia of the back muscles in six mice per group. These discs were recovered 3 weeks after implantation and subjected to radiographic and histologic studies. Ectopic bone formation occurred in a dose-dependent manner after the implantation of DC alginate, atelocollagen, and PLA-PEG, but occurred only at the highest dose implanted with CB alginate. Bone formation with DC alginate/OP-1 composites was equivalent to that with atelocollagen/OP-1 composites. Our data suggest DC alginate, a material free of animal products that is already approved by the FDA and other authorities, is a safe and potent carrier for OP-1. This carrier may also be applicable to various other situations in the orthopaedic field.

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Each author certifies that his or her institution has approved the animal protocol for this investigation and that all investigations were conducted in conformity with ethical principles of research.
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Introduction

The repair capacity of human bone appears to depend on different very complex processes, such as vascularization, biomechanics, and topography. When damage is severe, as occurs with comminuted fractures or large bone defects after tumor resection, it is difficult for bone union to be achieved [6]. In such cases, autologous or allogenic bone grafting has been used. Autologous bone grafting is common and is still the gold standard, but has several disadvantages, including a limited supply of suitable bone and the risk of chronic pain, nerve damage, fracture, and cosmetic problems at the donor site. Allografts have no donor site problems, but there is the potential risk of disease or an immunologic reaction [10, 21]. For these reasons, the use of bone substitutes such as calcium phosphate-based porous ceramics has been increasing [18, 33]. These bioceramics are highly biocompatible and demonstrate osteoconduction, which is the ability to bind to bone matrix directly. However, they have no osteoinduction, which is the ability to induce new bone formation at ectopic sites.

Bone morphogenetic proteins (BMPs) belong to the transforming growth factor superfamily, are known to elicit new bone formation in vivo, and may play a leading role in

bone tissue engineering [30, 38]. To date, three types of BMP-based bone tissue engineering have been tried, which are cell therapy, gene therapy, and cytokine therapy [37]. Cell therapy involves the transplantation of autologous bone marrow mesenchymal cells after differentiation has been induced by BMP, but considerable resources and time are needed to culture the necessary cells [23, 34]. Gene therapy involves the transduction of genes encoding BMPs into cells at the site of damage [3, 7]. BMP-transduced cells may work more efficiently, compared with a single dose of recombinant cytokine therapy. However, gene therapy still has unsolved problems such as tumorigenesis and immunogenicity. Cytokine therapy involves the implantation of BMPs together with a carrier material that acts as a drug delivery system. We believe cytokine therapy is the most promising of these three approaches in terms of practical application. Cytokine therapy seems most convenient and safe, but the cost is very high because a large amount of BMP is required to achieve bone growth in humans. To increase the cost effectiveness of BMP, an appropriate carrier material is necessary.

Previous studies have indicated adequate in vivo new bone formation cannot be obtained by simply injecting a solution of BMP into the area where bone is needed [37]. For cytokine therapy, an appropriate carrier material is needed that retains BMP and releases it slowly, while serving as a scaffold for new bone formation [28, 29]. Several materials have already been evaluated as BMP carriers, including collagen obtained from animal sources [8, 11, 13, 31], synthetic polymers [14, 15, 19], tricalcium phosphate [17], and other inorganic materials [16]. Atelocollagen is a well-established BMP carrier, and has already been used clinically. PLA-PEG [20], one of the synthetic polymers, has been reported as a potent carrier for BMPs [28, 29, 36]. Although all of these materials can induce bone formation at ectopic and orthotopic sites, none of them has achieved widespread use because of disadvantages, such as the potential risk of disease transmission, fragility, stickiness, and difficulty in obtaining approval for clinical use [1, 4, 5, 12]. We therefore focused on alginate, which is already approved by the FDA for human use as a wound dressing and food additive [6, 27].

Alginate is a water-soluble linear polysaccharide extracted from brown seaweed that is composed of one to four linked α-L-gluronic and β-D-mannuronic acid monomers [9]. Gelation of alginate occurs as a result of crosslinking by divalent cations or covalent bonds [39]. Therefore, two types of alginate wound dressing products are available on the market and both effectively promote wound healing by maintaining a moist environment. One is an alginate crosslinked by divalent cations (DC alginate) and the other is an alginate crosslinked by covalent bonds (CB alginate).

To determine whether alginate can be a carrier for BMP, we compared four materials as carriers for OP-1(BMP-7) using the bone mineral content (BMC) measurement and alkaline phosphatase (ALP) activity measurement of the bone nodules ectopically induced by carrier materials/OP-1 composites. The four materials were DC alginate, CB alginate, atelocollagen, and PLA-PEG. We hypothesized: (1) BMC of bone nodules ectopically induced by DC alginate/OP-1 composite and/or CB alginate/OP-1 composite are equivalent or superior to those by atelocollagen and PLA-PEG; (2) ALP activity of bone nodules ectopically induced by DC alginate/OP-1 composite and/or CB alginate/OP-1 composite are equivalent or superior to those by atelocollagen and PLA-PEG by radiographic appearance and histology of the ectopic bone nodules; and (3) DC alginate and/or CB alginate have appropriate in vitro release kinetics of OP-1 equivalent to atelocollagen and PLA-PEG.

Materials and Methods

To verify our first hypothesis, we designed the following experiment (Experiment 1; Table 1). For each dose of OP-1 (3, 10, and 30 μg), 24 4-week-old male ICR mice were assigned to four equally sized independent groups after they were housed and acclimatized in cages with free access to food and water for 1 week. The four independent groups were DC alginate group, CB alginate group, atelocollagen group, and PLA-PEG group. The mice were anesthetized by intraperitoneal injection of pentobarbital. As reported previously [14, 15], carrier material/OP-1 composites were implanted beneath the fascia of the back muscles on the left side (one composite per animal). The experiment was designed under the assumption that the justifiable difference (effect size) between the atelocollagen group as a control and the other groups was 6 mg in BMC and the standard deviation within each group was 3 from the result of the previous study [14]. For the experiment to detect the difference at the 5% significance level with 90% power in the one-way analysis of variance, the necessary number of mice per group was six. Three weeks after implantation, these mice were killed and ectopic bone induced at the implantation site was harvested for further evaluation, including BMC measurement, radiography, and histological examination. The experimental protocol was approved by the Animal Experiment Committee of Osaka University, and the experiments were carried out in accordance with the Osaka University guidelines for care and use of laboratory animals.

To verify the second hypothesis, we repeated the Experiment 1 and obtained radiographs and measured ALP activity (Experiment 2; Table 1).

Table 1. Study groups and experimental design

Experiments	Carrier materials	Dose of OP-1	n	Examination
Experiment 1	DC alginate	3, 10, and 30 μg	18*	BMC, radiography, histology
	CB alginate	3, 10, and 30 μg	18*	BMC, radiography, histology
	Atelocollagen	3, 10, and 30 μg	18*	BMC, radiography, histology
	PLA-PEG	3, 10, and 30 μg	18*	BMC, radiography, histology
Experiment 2	DC alginate	3, 10, and 30 μg	18*	Radiography, ALP activity
	CB alginate	3, 10, and 30 μg	18*	Radiography, ALP activity
	Atelocollagen	3, 10, and 30 μg	18*	Radiography, ALP activity
	PLA-PEG	3, 10, and 30 μg	18*	Radiography, ALP activity

*: n = 6, each dose.

The BMC of the harvested discs was determined by dual-energy xray absorptiometry (DXA) using an animal bone densitometer (PIXImus; Lunar Corp, Madison, WI) and was expressed as milligrams per ossicle. Radiographs were obtained with a soft xray apparatus (MX-20 Faxitron®; Torrex and Micro Focus Systems, Wheeling, IL).

To measure ALP activity, the harvested discs were crushed, homogenized in 0.2% Nonidet® P-40 containing 1 mmol/L $MgCl_2$, and centrifuged at 10,000 rpm for 1 minute at 4°C. The supernatants thus obtained were assayed for ALP activity with an Alkaline Phospha B-Test Wako kit (Wako Pure Chemical Industries, Ltd, Osaka, Japan) using p-nitrophenyl phosphate (p-NP) as a substrate. The protein content was measured with a Pierce® BCA protein assay kit (Thermo Fisher Scientific Inc, Rockford, IL), and ALP activity was standardized by the protein content and expressed as nmol p-NP/minute/mg protein.

After radiography and BMC measurement, the samples were fixed in 10% neutral formalin, decalcified with ethylenediaminetetraacetic acid (pH 7.4), dehydrated in a graded ethanol series, and embedded in paraffin. One section per group with the largest tissue area (5-μm thick) were cut and stained with hematoxylin and eosin for observation under a light microscope. The formation of new bone, new bone marrow, degradation of the materials, and inflammatory change were evaluated by a pathologist (AM) and an orthopaedic surgeon (KN).

To verify the third hypothesis that DC alginate and/or CB alginate have appropriate in vitro release kinetics of OP-1, we incubated carrier materials/OP-1 composites in centrifuge tubes containing 1000 μL phosphate-buffered saline (PBS; Invitrogen, Carlsbad, CA) and kept for 21 days at 37°C. For each composite group, three samples were examined. The PBS in the tubes was replaced every 2 days, and then 100 μL was collected for assay after 24 hours. The amount of OP-1 was determined by measurement with a commercial BMP-7 ELISA kit (R&D Systems Inc, Minneapolis, MN) on days 1, 3, 7, 13, and 21 according to the manufacturer's instruction.

OP-1 (BMP-7 in a lyophilized 5% lactose formulation) was provided by Stryker Biotech (Hopkinton, MA). OP-1 was dissolved in distilled water at a concentration of 2 μg/μL. DC alginate (ARGODERM®; crosslinked by Ca^{2+}), CB alginate (KURABIO®), and atelocollagen (INSTAT®) were purchased from Smith & Nephew (London, UK), Koyo Sangyo Co, Ltd (Tokyo, Japan), and Johnson & Johnson (New Brunswick, NJ), respectively. PLA-PEG with a total molecular weight of 11,400 Da and a PLA:PEG molar ratio of 51:49 was synthesized and provided by Taki Chemicals Co, Ltd (Hyogo, Japan).

To prepare carrier material/OP-1 composites, sheets of DC alginate, CB alginate, and atelocollagen were cut into discs (5-mm diameter). Then 25 μL of a solution containing 3, 10, or 30 μg OP-1 was added dropwise to each disc, after which the discs were freeze-dried and stored at −20°C until implantation into mice. All procedures were carried out under sterile conditions.

PLA-PEG/OP-1 composites were prepared as described previously [25]. Briefly, 10 mg of the polymer was liquefied in 50 μL acetone and mixed with 3, 10, or 30 μg OP-1. Each mixture was evaporated to dryness to remove acetone in a safety cabinet, fabricated into a disc-shaped implant, and stored at −20°C until implantation into mice.

To verify the first and second hypotheses, we used one-way analysis of variance (ANOVA), followed by a post hoc Scheffe's test. For each of these statistical analyses, the data sets met the assumptions of normality ($p > 0.15$ by the Jarque-Bera test [12]) justifying the use of parametric models. All analyses were performed using the R software program (Version 2.8.1;R Foundation for Statistical Computing).

Results

With 3 μg OP-1, BMC of the new bone in the DC alginate group was greater than that in atelocollagen group ($p = 0.0234$) and PLA-PEG group ($p = 0.0009$). With

30 μg OP-1, however, we observed no differences among the DC alginate, atelocollagen, and PLA-PEG. On the other hand, BMC of CB alginate group was very low compared with the other groups (Fig.). The results suggest that the BMC of DC alginate group was superior to those of atelocollagen and PLA-PEG groups, especially with a low dose of OP-1.

In the DC alginate group, ALP activity was high independent of the OP-1 dose. With 3 μg OP-1, DC alginate/OP-1 composites exhibited higher ALP activity than atelocollagen group ($p = 0.0071$) and PLA-PEG group ($p = 0.0001$) (by Scheffe's test). ALP activity of the CB alginate group was very low compared with the other groups (Fig.). The results suggest that ALP activity of the DC alginate group was superior to those of atelocollagen and PLA-PEG groups, especially with a low dose of OP-1.

In the release study of OP-1, the maximum concentration of OP-1 in the supernatant was detected on Day 1, followed by a steady decline. The decline of OP-1 levels in the atelocollagen group was faster than that in the other groups. In the DC alginate group, the decrease of OP-1 levels was the slowest and the concentration of OP-1 was still higher than 200 ng/mL on Day 21 (Fig.). These data suggested that DC alginate retains OP-1 and releases it most slowly compared to atelocollagen and PLA-PEG.

In the additionally performed radiographic examination of the bone nodules, obvious bone formation was only detected in the DC alginate and atelocollagen groups with 3 μg OP-1 (Fig. A–D). In the CB alginate group, new bone formation was observed only with 30 μg OP-1. The results of the additionally performed histological examination were consistent with the radiographic findings. In the DC alginate and atelocollagen groups, abundant new bone formation that contained normal hematopoietic bone marrow was observed even at low dose of OP-1. In the CB alginate group, however, new bone formation was very poor at low dose of OP-1. With 30 μg OP-1, irrespective of the carrier materials, newly formed bone had a thin cortex

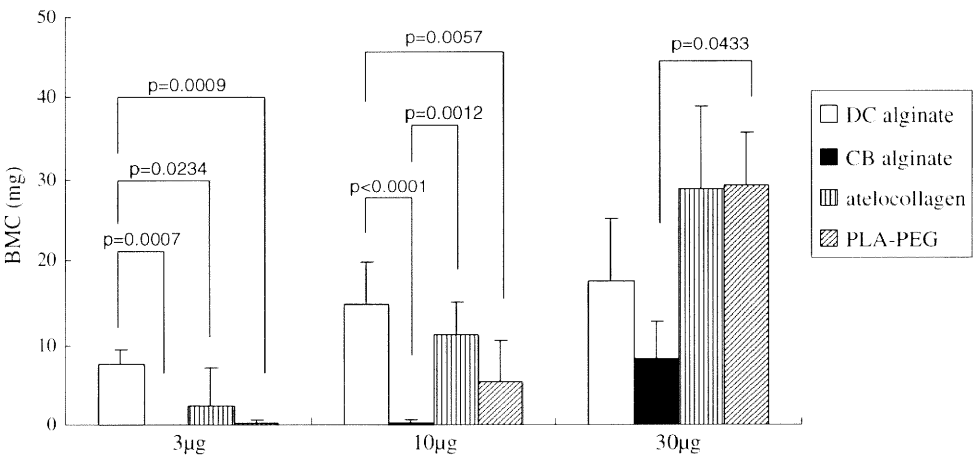

Fig. 1 In each carrier material group, BMC was measured by DXA using a PIXImus animal densitometer. BMC increased in an OP-1-dose dependent manner with every carrier material. With 3 μg and 10 μg OP-1, the BMC of the new bone in the DC alginate group was greater than that in the other groups.

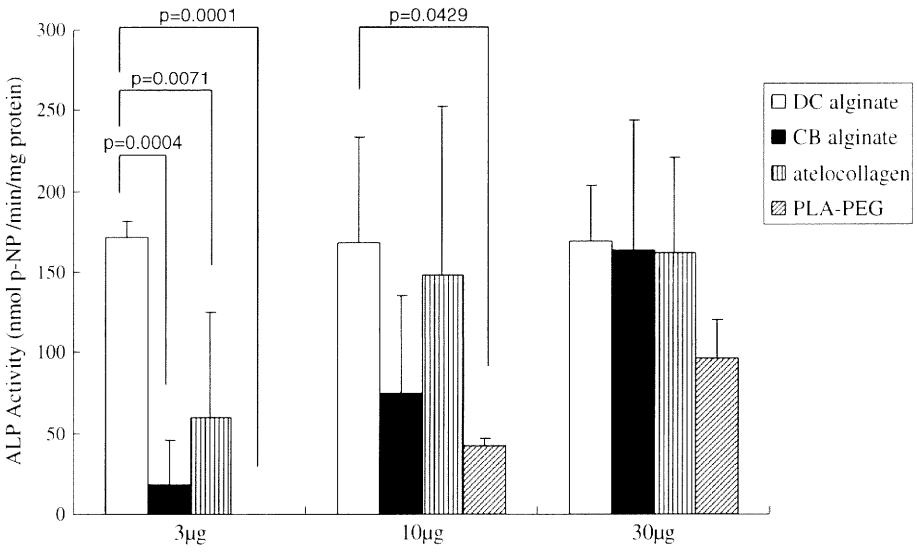

Fig. 2 ALP activity of ectopic bone was measured by using p-NP as a substrate. In the CB alginate, atelocollagen, and PLA-PEG groups, ALP activity increased in a dose-dependent manner. In the DC alginate group, ALP activity was relatively high independent of the dose of OP-1.

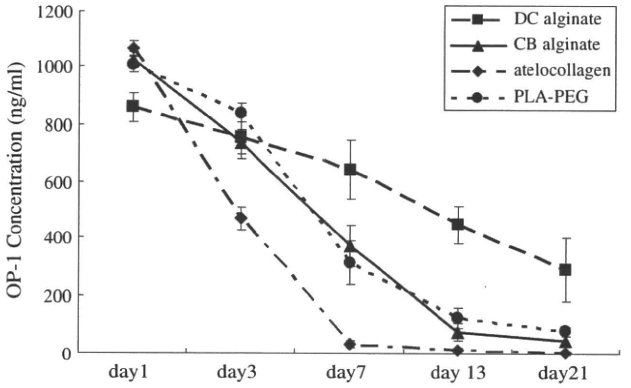

Fig. 3 OP-1 release from each carrier material/OP-1 (30 μg) composite was measured over time by a commercial BMP-7 ELISA kit. With each carrier material, the maximum concentration of OP-1 was detected on Day 1 and it decreased afterward. The decline was slowest in the DC alginate group.

surrounding cancellous bone that contained hematopoietic bone marrow, and no inflammatory change was observed (Fig. 5A–D). These additional results were compatible with the results of BMC and ALP activity, suggesting that DC alginate can be an equivalent or superior carrier for a low dose of OP-1 compared with atelocollagen and PLA-PEG.

Discussion

Various materials have already been evaluated as carriers for BMPs, but they all have some disadvantages as mentioned previously. This study was designed to examine whether alginate, a material with no animal product content, is an equivalent or superior carrier for OP-1(BMP-7) compared with atelocollagen and PLA-PEG. Specifically we hypothesized: (1) BMC of bone nodules ectopically induced by DC alginate/OP-1 composite and/or CB alginate/OP-1 composite are equivalent or superior to those by atelocollagen and PLA-PEG; (2) ALP activity of bone nodules ectopically induced by DC alginate/OP-1 composite and/or CB alginate/OP-1 composite are equivalent or superior to those by atelocollagen and PLA-PEG; and (3) DC alginate and/or CB alginate have appropriate in vitro release kinetics of OP-1 equivalent to atelocollagen and PLA-PEG.

This study has several limitations. First, DC alginate was originally approved for clinical use as a cutaneous wound dressing [8, 37]. Therefore, its biodegradability and immunogenicity are unclear during use at a deeper site. Second, in the histological examination, DC alginate remained at the center of the new ectopic bone, indicating it had not degraded within 3 weeks. Although no inflammatory reaction was found, longer observation will be necessary before this material can be used with confidence

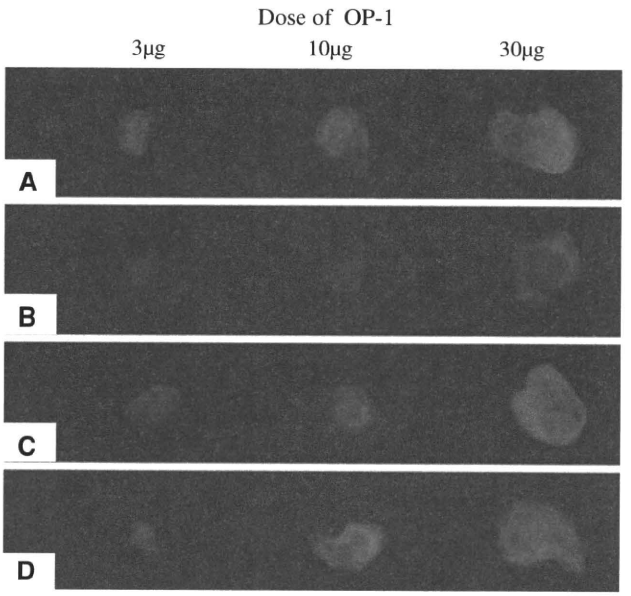

Fig. 4A–D The carriers for OP-1 tested were (**A**) DC alginate, (**B**) CB alginate, (**C**) atelocollagen, and (**D**) PLA-PEG. Soft xray photographs of ectopic bone induced by OP-1 (3, 10, or 30 μg) show bone formation with the DC alginate/OP-1 composite is equivalent or superior to that induced by the other carrier/OP-1 composites.

at deeper sites. Third, ALP activity is a marker for osteoblastic differentiation, and is high in the early stage of osteoblast lineage. ALP activity is not necessarily parallel to the activity of bone formation. Fourth, in the release study, a commercial BMP-7 ELISA kit can only detect the amount of BMP-7(OP-1) protein, but cannot evaluate the activity of OP-1. The result of a release test may not reflect the actual activity of OP-1 released from carriers in vivo.

To determine whether DC alginate and/or CB alginate are equivalent or superior carriers for OP-1 compared with atelocollagen and PLA-PEG, we measured BMC of ectopic bone nodules as a primary research question. A previous study [24] reported that BMC of the ectopic bone induced by PLA-PEG/BMP-2 composite is about 6 mg higher than that by atelocollagen/BMP-2 composite. In our study, the BMC of DC alginate/OP-1 (3 μg) composite was about 6 mg higher than that of the atelocollagen/OP-1 (3 μg) composite and even much higher than that of CB alginate and PLA-PEG. The result of BMC measurement suggested that DC alginate is a highly effective carrier that enhances the bone-inducing effect of OP-1 even when OP-1 content is low.

The result of ALP activity measurement was compatible with the result of BMC, which reinforced the hypothesis that DC alginate is an equivalent or superior carrier compared with the other materials. Upon histological examination, not only trabecular bone but also normal hematopoietic bone marrow was observed, and we found

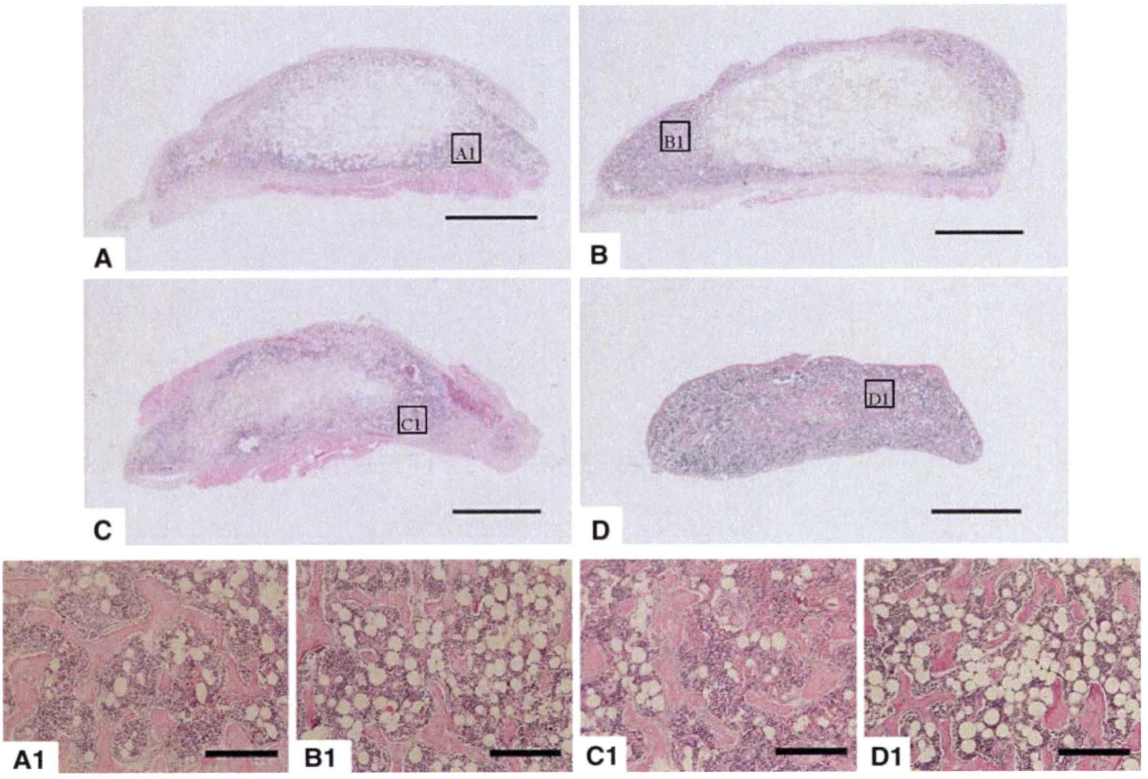

Fig. 5A–D Representative photomicrographs of the ectopic bone formation induced by OP-1 (30 μg) are shown (**A**, A1: DC alginate; **B**, B1: CB alginate; **C**, C1: atelocollagen; **D**, D1: PLA-PEG) (**A–D**: stain, hematoxylin and eosin; original magnification, ×10; scale bar = 2 mm; A1–D1: stain, hematoxylin and eosin; original magnification, ×100; scale bar = 200 μm). Irrespective of the carrier material, the newly formed bone had a thin cortex surrounding cancellous bone that contained highly cellular bone marrow.

no accumulation of inflammatory cells, such as monocyte/macrophages. The histological appearance of the ectopic bone induced by DC alginate/OP-1 composite seemed similar to that by atelocollagen/OP-1 composite, which is considered a safe biomaterial in terms of immunological response. These data suggested that DC alginate appears likely a safe material with no inflammatory response even when used in a deep site.

In contrast, CB alginate achieved relatively poor bone formation, especially with a low dose of OP-1. DC alginate and CB alginate only differ in the mode of crosslinking, but the release of OP-1 from these two alginates was quite different. It is known crosslinking by divalent cations forms a characteristic egg box structure that is suitable for trapping proteins in alginate [9]. Thus, the difference of bone formation between these two types of alginate may be partly due to a difference in their ability to retain OP-1 and release it slowly. It is also known the number of carboxyl residues in DC alginate is larger than that in CB alginate. The carboxyl residues induce apatite nucleation followed by the deposition of hydroxyapatite crystals on the alginate [35]. Furthermore, the Ca^{2+} contained in DC alginate can be utilized for new calcified bone, which is an advantage compared with CB alginate.

In conclusion, our data suggest DC alginate, a material with no animal product content that is approved by the FDA and other authorities, is a safe and potent carrier for OP-1. It is of note that DC alginate strongly potentiates osteoinduction of OP-1 even at a low dose. Thus, its use may reduce the cost of OP-1-based bone regeneration therapy.

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References

1. Bach FH, Fishman JA, Daniels N, Proimos J, Anderson B, Carpenter CB, Forrow L, Robson SC, Fineberg HV. Uncertainty in xenotransplantation: individual benefit versus collective risk. *Nature Med.* 1998;4:141–144.
2. Bonadio J, Smiley E, Patil P, Goldstein S. Localized, direct plasmid gene delivery in vivo: prolonged therapy results in reproducible tissue regeneration. *Nature Med.* 1999;5:753–759.
3. Burkus JK, Dorchak JD, Sanders DL. Radiographic assessment of interbody fusion using recombinant human bone morphogenetic protein type 2. *Spine.* 2003;28:372–377.
4. Butler D, Wadman M, Lehrman S, Schiermeier Q. Last chance to stop and think on risks of xenotransplants. *Nature.* 1998;391:320–324.

5. Delustro F, Dasch J, Keefe J, Ellingsworth L. Immune responses to allogeneic and xenogeneic implants of collagen and collagen derivatives. *Clin Orthop Relat Res.* 1990;260: 263–279.
6. Einhorn TA. Enhancement of Fracture-Healing. *J Bone Joint Surg Am.* 1995;77:940–956.
7. Fang J, Zhu YY, Smiley E, Bonadio J, Rouleau JP, Goldstein SA, McCauley LK, Davidson BL, Roessler BJ. Stimulation of new bone formation by direct transfer of osteogenic plasmid gene. *Proc Natl Acad Sci USA.* 1996;93:5753–5758.
8. Gensheimer D. A review of calcium alginate. *Ostomy Wound Manage.* 1993;39:34–38, 42–43.
9. George M, Abraham TE. Polyionic hydrocolloids for the intestinal delivery of protein drugs: alginate and chitosan—a review. *J Controlled Release.* 2006;114:1–14.
10. Goldberg VM, Stevenson S. Natural history of Autografts and Allografts. *Clin Orthop Relat Res.* 1987;225:7–16.
11. Greesink RG, Hoefnagels NH, Bulstra SK. Osteogenic activity of OP-1 bone morphogenetic protein (BMP-7) in a human fibular defect. *J Bone Joint Surg Br.* 1999;81:710–718.
12. Jarque CM, Bera AK. Efficient tests for normality, homoscedasticity and serial independence of regression residuals. *Economics Letters.* 1980;6:255–259.
13. Johnsson R, Stromqvist B, Aspenberg P. Randomized radiostereometric study comparing osteogenic protein-1 (BMP-7) and autograft bone in human noninstrumented posterolateral lumbar fusion. *Spine.* 2002;27:2654–2661.
14. Kato M, Namikawa T, Terai H, Hoshino M, Miyamoto S, Takaoka K. Ectopic bone formation in mice associated with a lactic acid/dioxanone/ethylene glycol copolymer-tricalcium phosphate composite with added recombinant human bone morphogenetic protein-2. *Biomaterials.* 2006;27:3927–3933.
15. Kato M, Toyoda H, Namikawa T, Hoshino M, Terai H, Miyamoto S, Takaoka K. Optimized use of biodegradable polymer as a carrier material for the local delivery of recombinant human bone morphogenetic protein-2 (rhBMP-2). *Biomaterials.* 2006;27:2035–2041.
16. Kirker-Head CA. Potential applications and delivery strategies for bone morphogenetic proteins. *Adv Drug Deliv Rev.* 2000;43: 65–92.
17. Lee DD, Tofighi A, Aiolova M, Chakravarthy P, Catalano A, Majahad A, Knaack D. Alpha-BSM: a biomimetic bone substitute and drug delivery vehicle. *Clin Orthop Relat Res.* 1999;367: S396–S405.
18. Matsumine A, Myoui A, Kusuzaki K, Araki N, Seto M, Yoshikawa H, Uchida A. Calcium hydroxyapatite ceramic implants in bone tumor surgery: a long-term follow-up study. *J Bone Joint Surg Br.* 2004;86:719–725.
19. Miyamoto S, Takaoka K, Okada T, Yoshikawa H, Hashimoto J, Suzuki S, Ono K. Evaluation of polylactic acid homopolymers as carriers for bone morphogenetic protein. *Clin Orthop Relat Res.* 1992;278:274–285.
20. Miyamoto S, Takaoka K, Okada T, Yoshikawa H, Hashimoto J, Suzuki S, Ono K. Polylactic acid-polyethylene glycol block copolymer: a new biodegradable synthetic carrier for bone morphogenetic protein. *Clin Orthop Relat Res.* 1993;294:333–343.
21. Nemzek JA, Arnoczky SP, Swenson CL. Retroviral transmission by the transplantation of connective-tissue allografts. *J Bone Joint Surg Am.* 1994;76:1036–1041.
22. Ohgushi H, Dohi Y, Toshikawa T, Tamai S, Tabata S, Suwa Y. In vitro bone formation by rat marrow cell culture. *J Biomed Mater Res.* 1996;32:341–348.
23. Saito N, Murakami N, Takahashi J, Horiuchi H, Ota H, Kato H, Okada T, Nozaki K, Takaoka K. Synthetic biodegradable polymers as drug delivery systems for bone morphogenetic proteins. *Adv Drug Deliv Rev.* 2005;57:1037–1048.
24. Saito N, Okada T, Horiuchi H, Murakami N, Takahashi J, Nawata M, Ota H, Miyamoto S, Nozaki K, Takaoka K. Biodegradable poly-D,L-lactic acid-polyethylene glycol block copolymers as a BMP delivery system for inducing bone. *J Bone Joint Surg Am.* 2001;83 Suppl 1(Pt 2):S92–S98.
25. Saito N, Okada T, Horiuchi H, Ota H, Takahashi J, Murakami N, Nawata M, Kojima S, Nozaki K, Takaoka K. Local bone formation by injection of recombinant human bone morphogenetic protein-2 contained in polymer carriers. *Bone.* 2003;32:381–386.
26. Saito N, Okada T, Toba S, Miyamoto S, Takaoka K. New synthetic absorbable polymers as BMP carriers: plastic properties of poly-D,L-lactic acid-polyethylene glycol block copolymers. *J Biomed Mater Res.* 1999;47:104–110.
27. Saito N, Takaoka K. New synthetic biodegradable polymers as BMP carriers for bone tissue engineering. *Biomaterial.* 2003;24: 2287–2293.
28. Schmidmaier G, Schwabe P, Strobel C, Wiidemann B. Carrier systems and application of growth factors in orthopaedics. *Injury.* 2008; 39 Suppl 2:S37–43.
29. Seeherman H, Wozney J M. Delivery of bone morphogenetic proteins for orthopedic tissue regeneration. *Cytokine Growth Factor Rev.* 2005;16:329–345.
30. Suzuki Y, Tanihara M, Nishimura Y, Suzuki K, Yamawaki Y, Kudo H, Kakimaru Y, Shimizu Y. In vivo evaluation of a novel alginate dressing. *J Biomed Mater Res.* 1999;48:522–527.
31. Takaoka K, Koezuka M, Nakamura H. Telopeptide-depleted bovine skin collagen as a carrier for bone morphogenetic protein. *J Orthop Res.* 1991;9:902–907.
32. Takaoka K, Nakahara H, Yoshikawa H, Masuhara K, Tsuda T, Ono K. Ectopic bone induction on and in porous hydroxyapatite combined with collagen and bone morphogenetic protein. *Clin Orthop Relat Res.* 1988;234:250–254.
33. Tamai N, Myoui A, Tomita T, Nakase T, Tanaka J, Ochi T, Yoshikawa H. Novel hydroxyapatite ceramics with an interconnective porous structure exhibit superior osteoconduction in vivo. *J Biomed Mater Res.* 2002;59:110–117.
34. Tamura S, Kataoka H, Matsui Y, Shionoya Y, Ohno K, Michi KI, Takahashi K, Yamaguchi A. The effects of transplantation of osteoblastic cells with bone morphogenetic protein (BMP)/carrier complex on bone repair. *Bone.* 2001;29:169–175.
35. Tanahashi M, Matsuda T. Surface functional group dependence on apatite formation on self-assembled monolayers in a simulated body fluid. *J Biomed Mater Res.* 1997;34:305–315.
36. Urist MR. Bone formation by autoinduction. *Science.* 1965;150: 893–899.
37. Vanstraelen P. Comparison of calcium sodium alginate (KALTOSTAT) and porcine xenograft (E-Z DERM) in the healing of split-thickness skin graft donor sites. *Burns.* 1992;18:145–148.
38. Wang EA, Rosen V, D'Alessandro JS, Baudny M, Cordes P, Harada T, Israel DI, Hewick RM, Kerns KM, LaPan P, Luxenberg DP, McQuaid D, Moutsatsos IK, Nove J, Wozeny EA. Recombinant human bone morphogenetic protein induces bone formation. *Proc Natl Acad Sci USA.* 1990;87:2220–2224.

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Isolation and characterization of a novel peptide, osteoblast activating peptide (OBAP), associated with osteoblast differentiation and bone formation

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ABSTRACT

A long-standing goal in bone loss treatment has been to develop bone-rebuilding anabolic agents that can potentially be used to treat bone-related disorders. To purify and isolate a novel anabolic that acts to osteoblasts, we monitored changes in intracellular calcium concentrations ($[Ca^{2+}]i$). We identified a novel, 24 amino-acid peptide from the rat stomach and termed this peptide osteoblast activating peptide (OBAP). Furthermore, we examined the effects of OBAP in osteoblasts. First, osteoblast differentiation markers (alkaline phosphatase [ALP], osteocalcin [OCN]) were analyzed using quantitative RT-PCR. We also examined the ALP activity in osteoblasts induced by OBAP. OBAP significantly increased the expression of osteoblast differentiation markers and the activity of ALP *in vitro*. Next, to address the *in vivo* effects of OBAP on bone metabolism, we examined the bone mineral density (BMD) of gastrectomized (Gx) rats and found that OBAP significantly increased BMD *in vivo*. Finally, to confirm the *in vivo* effects of OBAP on bone, we measured serum ALP and OCN in Gx rats and found that OBAP significantly increased serum ALP and OCN. Taken together, these results indicate that the novel peptide, OBAP, positively regulates bone formation by augmenting osteoblast differentiation. Furthermore, these results may provide a new therapeutic approach to anabolically treat bone-related disorders.

1. Introduction

Osteoporosis is characterized by low bone mass and structural bone deterioration, both of which are associated with reduced bone strength and an increased risk of fracture. In the United States alone, 10 million people are estimated to have osteoporosis, and osteoporosis contributes to 1.5 million fractures each year [1]. Bone undergoes a normal remodeling process, mediated by the coordinated actions of osteoclasts and osteoblasts. Bone loss and skeletal fragility in osteoporosis are caused by an imbalance in bone remodeling, in which the rate of osteoclast-mediated bone resorption is higher than the rate of osteoblast-mediated bone formation. A variety of therapeutics are currently used to treat osteoporosis, with the vast majority being antiresorptive agents that exert their clinical effects by decreasing the rate of bone resorption, thereby preventing further bone loss and reducing fractures [2]. Along with ongoing efforts to develop improved antiresorptive agents, a long-standing goal has been to develop therapeutics that can stimulate bone formation to increase bone mass and bone strength. It is thought that such bone-rebuilding anabolics could provide important treatment options, not only for low bone mass conditions but also for fracture healing, orthopaedic procedures, etc. Currently, the only approved bone anabolic agents for osteoporosis are full-length and truncated PTH, both of which are administered by daily subcutaneous injections [2]. Therefore, it is important to identify new bone anabolic agents.

Here, we report the purification and isolation of a novel peptide that acts to osteoblasts. In addition, we demonstrate that this novel peptide regulates osteoblast activation and bone formation.

2. Materials and methods

2.1. Animals

Sprague–Dawley (SD) rats (Charles River Co., Yokohama, Japan) were used to purify the peptide and to conduct both osteoblast-like cell culture experiments and *in vivo* studies. The rats were housed in a regulated environment (22 ± 2 °C, 55 ± 10% humidity, 12-h light, 12-h dark cycle with lights on at 0700 h) with free access to food and water. All experiments were conducted in accordance with the Japanese Physiological Society's guidelines for animal care.

2.2. Cell culture

Primary osteoblast-like cells were isolated by digesting a 21-day-old fetal rat calvaria with collagenase (Sigma Chemical

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Co., St. Louis, MO, USA) as previously described [3,4]. Digests 3–5 were pooled and grown in 10-cm cell culture plates in primary culture media consisting of α-Minimal Essential Medium (α-MEM; containing L-glutamine and nucleosides) (Life Technologies-GIBCO, Cergy Pontoise, France) supplemented with 10% fetal bovine serum (Thermo Trace, Melbourne, Australia) and antibiotics, including 100 μg/ml penicillin G (Life Technologies-GIBCO), 50 μg/ml streptomycin sulfate, and 0.3 μg/ml Fungizone (Life Technologies-GIBCO). Cells were grown to confluence before experimentation.

UMR-106 cells, a rat osteoblastic cell line, were obtained from Dainippon Pharmaceutical Co. (Osaka, Japan). Cells were plated in 10-cm plates at a density of 2×10^5 cells/plate and maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Life Technologies-GIBCO) supplemented with 10% fetal bovine serum and antibiotics.

All cultures were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2.

2.3. Purification of the novel peptide

The novel peptide was isolated and purified by the same method as previously described [5]. A fresh rat stomach (50 g) was diced and boiled for 5 min in five volumes of water to inactivate the intrinsic proteases. The solution was adjusted to 1 M AcOH, 20 mM HCl. Peptides were extracted by homogenizing with a Polytron mixer. The extracts were centrifuged for 30 min at 11,000 rpm, and the supernatants were concentrated to approximately 40 ml with an evaporator. The residual concentrate was precipitated with 66% acetone. After the precipitates were removed, the acetone-containing supernatant was evaporated. The solution was loaded onto a 10-g cartridge of Sep-Pak C18 (Waters), which was pre-equilibrated with 0.1% trifluoroacetic acid (TFA). The Sep-Pak cartridge was washed with 10% CH_3CN/0.1% TFA, and then eluted with 60% CH_3CN/0.1% TFA. The eluate was evaporated and lyophilized. The residual materials were redissolved in 1 M AcOH and then adsorbed on a SP-Sephadex C-25 column (II + form) that was pre-equilibrated with 1 M AcOH. Successive elution with 1 M AcOH, 2 M pyridine and 2 M pyridine AcOH (pH 5.0) yielded three fractions of SP-I, SP-II, and SP-III. The lyophilized SP-III fraction was applied on a Sephadex G-50 gel-filtration column. A portion of each fraction was subjected to the intracellular calcium concentration ($[Ca^{2+}]i$)-change assay using UMR-106 cells. Active fractions (43–48) were then separated by reverse-phase high-performance liquid chromatography (HPLC) using a symmetry300 C18 column (3.9 × 150 mm; Waters). The active fractions were further fractionated manually.

2.4. Structural analysis

The amino-acid sequence of the peptide was analysed with a protein sequencer (494; Applied Biosystems, Foster City, USA). The molecular weight was determined using MS. The fully protected peptide was synthesized by the Fmoc solid-phase method on a peptide synthesizer (433A; Applied Biosystems). Purified peptides were compared to synthetic peptides by reverse-phase HPLC. The activities of the novel peptide, HPLC-fractionated peptides, and the synthetic peptides were examined by monitoring the effects of these samples in the $[Ca^{2+}]i$-change assay.

2.5. Changes in intracellular Ca^{2+} concentrations

Changes in $[Ca^{2+}]i$ were measured using the FLEXstation Calcium Assay Kit (Molecular Devices), fluorescent dye Fluo-4 AM (Molecular Probes), and black-walled, clear-bottomed 96-well microplates (Costar, cat# 3603 and Greiner, E&K cat# 655090). Compound plates used were 96-well, V-bottomed, clear polypropylene plates (E&K, cat# 651201). A 1X Reagent Buffer containing 1× Hank's Balanced Salt Solution and 20 mM HEPES pH 7.4 was used to wash cells, dilute compound, and dissolve dye solutions. All buffers contained a final concentration of 2.5 mM probenecid in order to inhibit endogenous efflux pumps. Cells were seeded the night before the experiment at 5×10^4 cells/well in a volume of 100 μl per well of a 96-well microplate. Cells were incubated at 37 °C in 5% CO_2 overnight. The next day, the cells were incubated with Fluo-4 dye loading buffer at room temperature for 1 h. Then, the Fluo-4 dye-loaded cells were manually washed three times with 1× Reagent Buffer, and 100 μl of 1× Reagent Buffer was added to the wells. The compound was added to the plates of cells via FLEXstation and subsequent changes in fluorescent signals were monitored.

2.6. Cell proliferation assays

Primary osteoblast-like cells were seeded in 96-well plates at a density of 6000 cells/well. After 24 h, the media was changed to serum-free medium with 1% bovine serum albumin, and the cells were incubated for an additional 24 h before the experimental compounds were added.

The relative number of viable cells in each well was determined after a 48-h incubation with the compounds using the cell count reagent SF (Nacalai Tesque, Kyoto, Japan), 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8). Briefly, 10 μl of the WST-8 solution was added to each well, including three wells containing medium alone that were used to subtract the background fluorescence. Then, the cells were incubated at 37 °C for 1 h. The absorbance at 450 nm in each well was determined using a microplate reader, model 550 (Bio-Rad Laboratories, Hercules, CA, USA). This technique produces a linear relationship between the number of viable cells and the absorbance at 450 nm.

2.7. Assessment of the expression of osteoblast differentiation markers

Cells in 6-well plates were treated with or without peptide (10^{-4} or 10^{-5} M) and then analyzed after 3 and 14 days of *in vitro* culture. Cells were plated at a density of 4×10^4 cells/well and grown until they reached confluency, which was designated day 0. Cells were grown in primary culture media with 50 μg/ml ascorbic acid (Sigma Chemical Co.) and 10 mmol/L β-glycerophosphate (Sigma Chemical Co.). Total RNA was extracted from the cell pellets using an RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA using the Super Script Preamplification System for First-Strand cDNA Synthesis Kit (Life Technologies-GIBCO, Cergy Pontoise, France). cDNA was analyzed by quantitative real-time PCR using the ABI PRISM 7700 sequence detection system (Applied Biosystems). Specific primers that produce short PCR products suitable for SYBR-Green detection were designed using Primer Express software (version 1.0, PE Applied Biosystems). The primer sequences were as follows: Runx2 (Runx2; 67-bp product; GenBank accession no. AF053953), 5′-GCTTCATTCGCCTCACAAACA-3′ (sense) and 5′-TGCTGTCCTCCTGGAGAAAGTT-3′ (antisense); alkaline phosphatase (ALP; 101-bp product; GenBank accession no. J03572), 5′-CGTCTCCATGGTGGATTATGC-3′ (sense) and 5′-TGGCAAAGACCGCCACAT (antisense); osteocalcin (OCN; 63-bp product; GenBank accession no. X04141), 5′-GAGCTAGCGGACCACATTGG-3′ (sense) and 5′-CCTAAACGGTGGTGCCATAGA-3′ (antisense); and beta-actin (β-actin; 67-bp product; GenBank accession no. NM031144), 5′-TTCAACACCCCAGCCATGT-3′ (sense) and 5′-GTGGTACGACCAGAGGCATACA-3′ (antisense). Samples were examined in triplicate. The reaction volume was 50 μl and included 3 μl diluted cDNA (1:30), 10 μl SYBR green buffer, and 10 pmol of each primer. The samples were subjected to 45 cycles of amplification at 95 °C for 15 s, followed

by 52 °C for 60 s. The concentration of amplified cDNA in each sample was calculated relative to that of β-actin cDNA. After RT-PCR amplification, a dissociation analysis was performed to ensure that only one product was amplified in each PCR reaction. Products were also separated on a 2% agarose gel to confirm that reaction produced a single, correctly sized product.

2.8. Alkaline phosphatase (ALP) activity

The dose-dependent effects of the novel peptide were analyzed after a 5-day incubation with or without the peptide (10^{-4} or 10^{-5} M). ALP activity was analyzed histochemically using an alkaline phosphatase kit (WAKO Pure Chemical Industry, Osaka, Japan). Briefly, assay mixtures containing 0.1 M 2-amino-2-methyl-1-propanol, 1 mM $MgCl_2$, 8 mM p-nitrophenyl phosphate disodium, and cell homogenates were incubated for 5 min at 37 °C, after which the reaction was stopped with 0.1 N NaOH and the absorbance at 405 nm was measured. A standard curve was prepared with p-nitrophenol. Each value was normalized to the total protein concentration in the cell layer, which was measured by the Lowry method using a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and BSA as a standard.

2.9. Bone densitometry and μCT

We used gastrectomized (Gx) rats as an osteoporosis model. Gastrectomies were performed by a total resection of the stomach of 5-week-old male SD rats. Two weeks after recovering from the surgery, the gastrectomized (Gx) rats were randomly divided into two groups: Gx with vehicle and Gx with peptide.

Gx rats were infused intraperitoneally with the peptide ($n = 4$) (0.2 mg/kg per hour) or saline ($n = 5$) using osmotic minipumps (Alzet; Alza Corp., Palo Alto, CA, USA). We measured the body weight and food intake of the rats daily. After 4 weeks of treatment, the BMD of the femur was measured by dual-energy X-ray absorptiometry (DXA; model DCS-600; Aloka, Tokyo, Japan). In addition, we obtained two-dimensional images of the distal femurs by microcomputed tomography (μCT, Scan Xmate-L090, Comscan).

2.10. Assay for serum chemistry

Just before death, blood samples were taken, and serum was prepared. The osteocalcin (OC) levels in the serum were determined by ELISA with a rat OCN immunoassay kit (R&D Systems). Serum ALP was measured with an alkaline phosphatase kit. Assays were performed according to the manufacturer's recommendations.

2.11. Statistical analysis

Data are presented as the mean ± SD. A two-factor analysis of variance (ANOVA) was used to determine if differences between mean values were statistically significant, and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Isolation of a novel peptide

Osteoblasts were monitored for changes in $[Ca^{2+}]i$ that were induced by tissue extracts from a rat stomach. Because the highest activity was detected in stomach extracts, these extracts were further purified by successive chromatography, including Sephadex G-50 gel filtration and reverse-phase HPLC (Fig. 1A and B). Gel filtration showed that fractions with a relative molecular mass of ~3000 potently increased $[Ca^{2+}]i$. Active fractions (fractions 43–48) were further purified by RP-HPLC to yield 20 μg of pure peptide from 50 g of rat stomach tissue (Fig. 1C).

3.2. Structural analysis of the novel peptide

Protein sequence analysis revealed that the amino-acid sequence of the purified peptide was LDLNLDLSKFRLPQPSSGRESPRH (Fig. 1D). The calculated mass of the novel peptide was 2763.4. To confirm that the native and synthetic peptides had similar structures, the peptides were analyzed by HPLC. The elution profiles of both the native and synthetic peptide were identical by C-18 reverse-phase. Moreover, mixtures of the native and synthetic

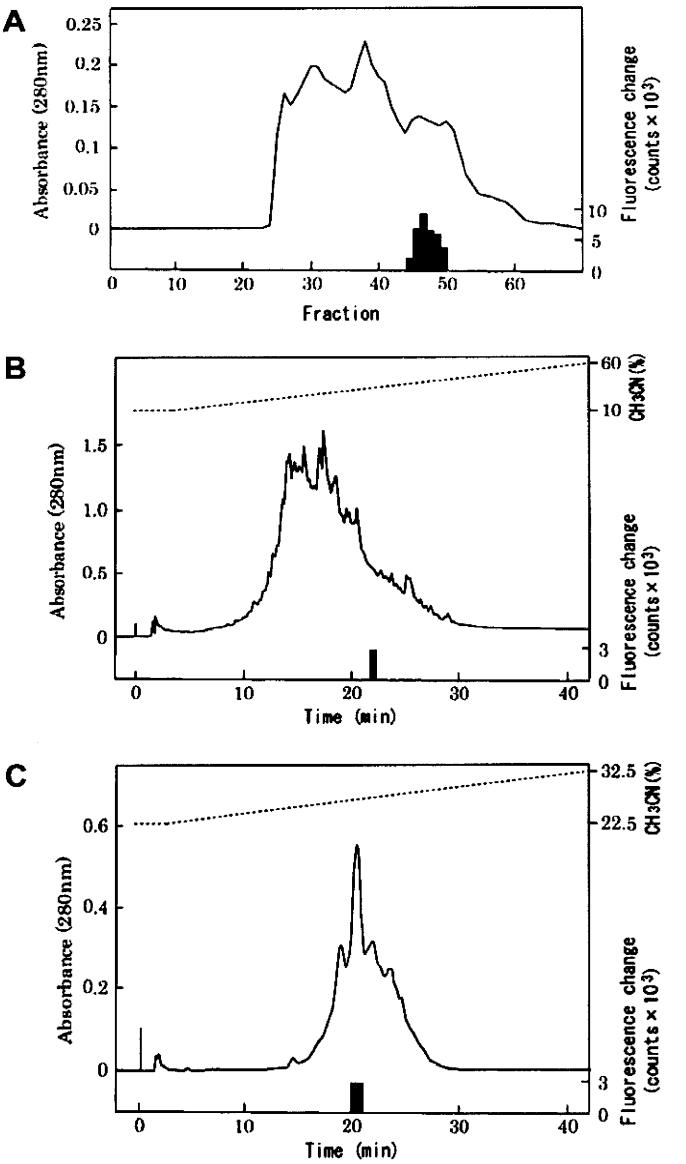

Fig. 1. Purification of the novel peptide. (A) Sephadex G-50 gel filtration of the SP-III fraction from 50 g of rat stomach. The black bars indicate the changes in fluorescence due to the increase in $[Ca^{2+}]i$ in UMR-106 cells. The active fractions eluted at approximately M_r 3000. (B) Reverse-phase HPLC of the active fractions in A. The broken line indicates the concentration of CAN in the eluting solvent. Bioactive fractions are represented by black bars. (C) Final purification by reverse-phase HPLC of the active fractions in B. (D) Structure of the novel peptide.

peptides eluted as a single peak (Fig. 2A). Next, to confirm that the native and synthetic peptides have similar effects, the peptides were analyzed in the $[Ca^{2+}]i$-change assay. The native and synthetic peptides produced similar $[Ca^{2+}]i$ changes (Fig. 2B). These results confirmed the sequence of the peptide. As this peptide acts on osteoblasts, we termed this peptide osteoblast activating peptide (OBAP).

3.3. Effects of the novel peptide on $[Ca^{2+}]i$ in cells

A novel peptide potently induced an increase in $[Ca^{2+}]i$ in UMR-102 cells, whereas other peptides such as neuromedin that acts through GPCRs had no effect on cells under the same conditions

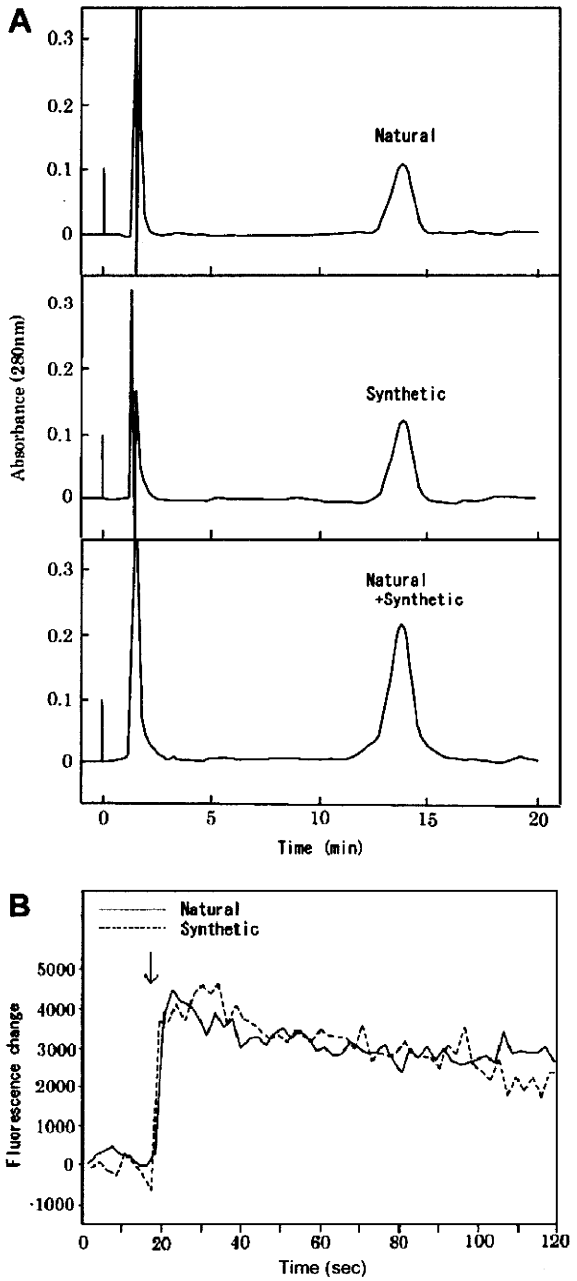

Fig. 2. Comparison of the natural and synthetic peptides. (A) Comparison of the elution profiles of natural, synthetic, and mixed peptides applied to reverse-phase HPLC. (B) Time courses of $[Ca^{2+}]i$ changes in UMR-106 cells that were treated with the natural and synthetic peptides. Each peptide (2×10^{-4} M) was added at the time indicated by the arrow.

(data not shown). OBAP induced $[Ca^{2+}]i$ in primary osteoblasts and osteoblast-like cells but not RAW264.5 or CHO cells (Fig. 3A). This peptide also induced a dose-dependent increase in $[Ca^{2+}]i$ (Fig. 3B).

3.4. Effects of the novel peptide on osteoblast proliferation

Next, to assess the effects of the novel peptide on primary osteoblast-like cells, we measured cell viability in response to peptide treatment. In these assays, OBAP did not affect cell proliferation (data not shown).

3.5. Effects of the novel peptide on osteoblast differentiation

To further examine the effects of OBAP on osteoblast differentiation, we analyzed the expression of several markers of osteoblast differentiation, including Runx2, ALP and OCN, by quantitative RT-PCR. The expression of Runx2, a transcriptional factor necessary for osteoblast differentiation, was not significantly changed (data not shown). However, compared to vehicle-treated cells, 10^{-5} and 10^{-4} M OBAP significantly increased the expression of the early osteoblast differentiation marker, ALP, by up to 2.26- and 3.07-fold, respectively, at day 3 ($P < 0.01$; Fig. 3C). Furthermore, the expression of OCN, a marker of late-stage differentiation, began to increase at day 7 by up to 2.75- and 3.45-fold ($P < 0.01$; Fig. 3D).

Next, we evaluated the ALP activity of osteoblast-like cells. As shown in Fig. 3E, OBAP significantly increased the ALP activity of osteoblast-like cells after 5 days of culture by up to 1.58- and 2.24-fold compared to vehicle-treated cells. These results indicate that OBAP stimulates osteoblast differentiation.

3.6. Effects of the novel peptide on BMD in vivo

To explore the *in vivo* effects of OBAP in the bone, we measured BMD by DXA in Gx rats. Seven-week-old male Gx rats were infused intraperitoneally with the novel peptide for 4 weeks. Compared with the vehicle control, OBAP significantly increased BMD in Gx rats (Fig. 4A–C).

3.7. Effects of the novel peptide on serum ALP and osteocalcin in Gx rats

To confirm the *in vivo* effects of OBAP on bone, we measured serum ALP and OCN in Gx rats. Compared with the vehicle control, OBAP significantly increased ALP and OCN in Gx rats by up to 1.51- and 3.25-fold ($P < 0.01$), respectively (Fig. 4D and E).

4. Discussion

In this study, we isolated a novel peptide from the rat stomach and termed this peptide osteoblast activating peptide (OBAP). Furthermore, we demonstrated that OBAP directly promotes osteoblast differentiation *in vitro* and increases BMD *in vivo*.

First, we searched for novel peptides that are produced in various organs, such as the brain, liver, kidney and small intestine, and act on osteoblasts, but were unable to isolate these putative peptides. Next we examined peptides in the stomach because the stomach produces physiologically active substances (e.g., ghrelin). Furthermore, the stomach is thought to play a role in bone metabolism, and it is well documented that surgically removing the whole stomach (gastrectomy) or the acid-producing part of the stomach (fundectomy) results in osteopenia [6–15]. In fact, gastrectomy-evoked bone loss was observed in young dogs in 1938 [7]. Although the phenomenon of gastrectomy-evoked osteopathy has been known for over 70 years, the mechanism remains poorly

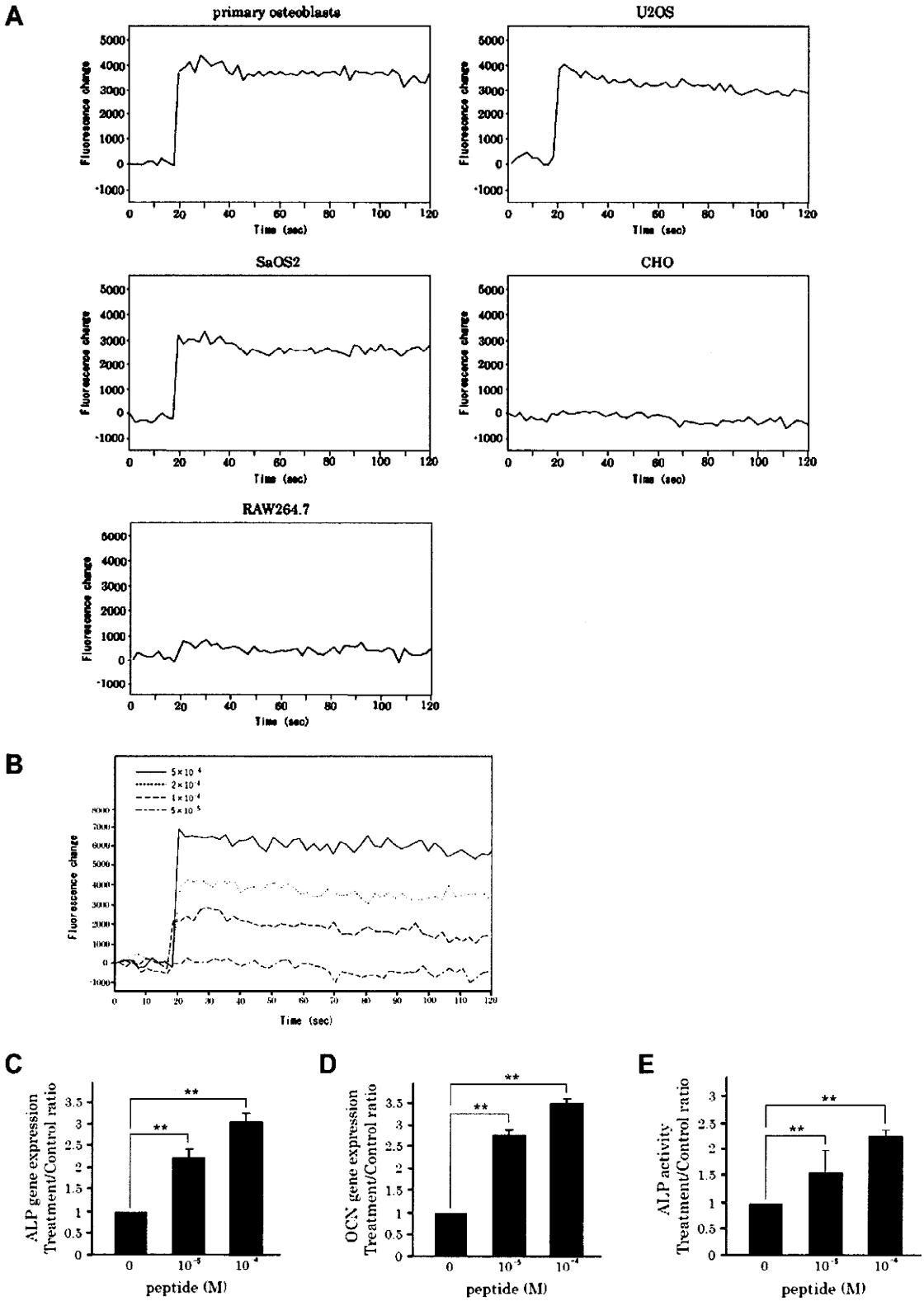

Fig. 3. Effects of the novel peptide *in vitro*. (A) Effects of the peptide on $[Ca^{2+}]i$ changes in osteoblastic cells (primary osteoblasts, U_2OS cells, and $SaOS_2$ cells), RAW264.7 cells, and CHO cells. (B) Dose-dependent response of $[Ca^{2+}]i$ changes in primary osteoblasts. $^{**}P < 0.01$ Quantitative RT-PCR analysis of the osteoblast differentiation marker genes (C) ALP and (D) OCN in cells treated with or without 10^{-5} and 10^{-4} M peptide. The novel peptide increased the expression of ALP and OCN. $^{**}P < 0.01$ (E) Dose-dependent effects of the novel peptide on ALP activity at day 5. Stimulating cells with 10^{-5} and 10^{-4} M peptide significantly increased ALP activity. $^{**}P < 0.01$.

understood. Previously, it was thought that bone loss was the result of an impaired capacity to utilize dietary calcium or a vitamin D deficiency. However, dietary supplementation with $CaCl_2$ [8,9] and continuous subcutaneous infusions of $CaCl_2$ [10] do not prevent bone loss, and $1,25\text{-}(OH)_2D_3$ concentrations are reportedly increased rather than decreased after gastrectomies [11–14]. Based

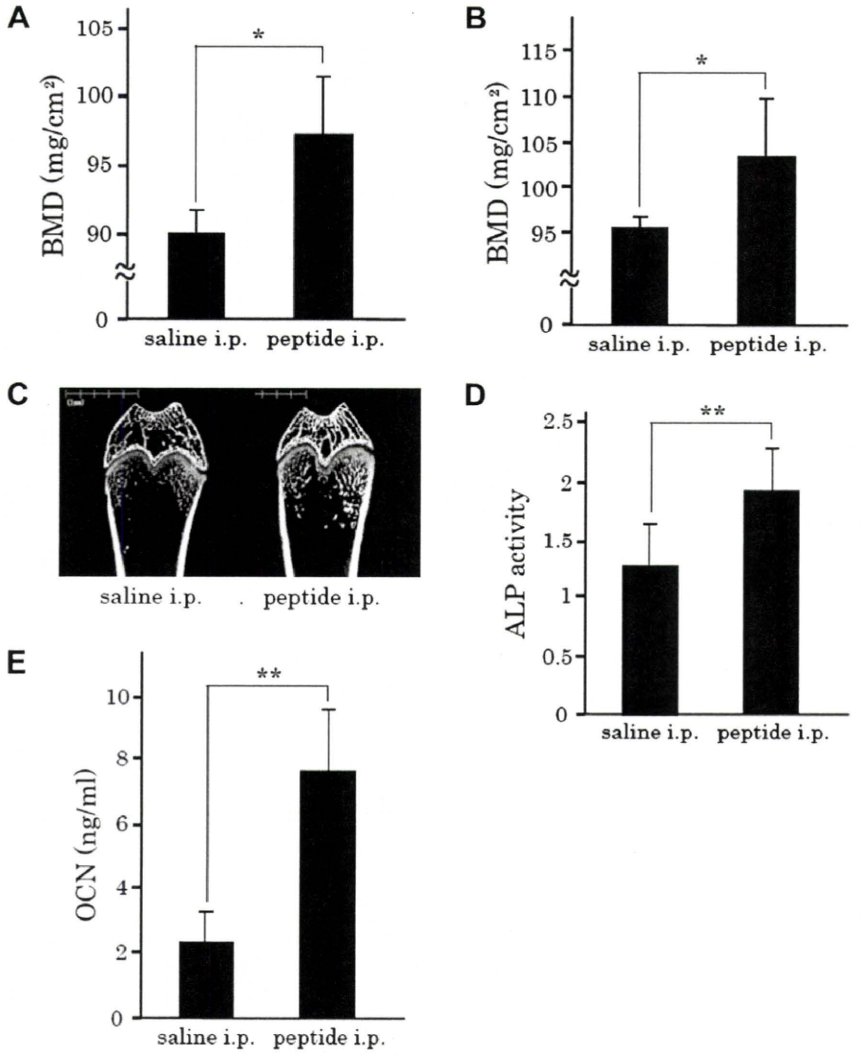

Fig. 4. Effects of novel peptide in gastrectomized rats. Rats were infused intraperitoneally with the peptide (0.2 mg/kg per hour, $n = 4$) or saline ($n = 5$) for 4 weeks. BMD was measured by DXA. The peptide treatment significantly increased both the (A) total and (B) trabecular BMD compared with saline treatment. $^{*}P < 0.05$ (C) Two-dimensional images of distal femurs by μCT. $^{*}P < 0.05$, $^{**}P < 0.01$ Effects of the novel peptide on serum alkaline phosphatase and osteocalcin in Gx rats. Rats were infused intraperitoneally with peptide (0.2 mg/kg per hour, $n = 4$) or saline ($n = 5$) for 4 weeks. Compared with the vehicle control, the peptide significantly increased (D) ALP and (E) OC. $^{**}P < 0.01$.

on these points, Larsson et al. suggested that ECL-cells harbor a novel peptide hormone that is involved in bone metabolism. In addition, they found that extracts of ECL-cell granule/vesicle enriched preparations of the oxyntic mucosa as well as extracts of isolated ECL cells evoked a typical Ca^{2+}-mediated second messenger response in different osteoblast-like cell lines [16,17]. We isolated OBAP, which is composed of 24 amino acids that are identical to the C-terminus of NADH dehydrogenase (ubiquinone) flavoprotein 3 (Ndufv3; MIPP65), which is a mitochondrial phosphoprotein [18] with an unknown function.

OBAP induced $[Ca^{2+}]i$ in primary osteoblasts and osteoblast-like cells but not RAW264.5 or CHO cells. These findings suggest that OBAP binds to a specific receptor in osteoblasts. To analyze the pharmacological activity of OBAP, we first measured osteoblast differentiation markers to confirm that OBAP potently affects osteoblasts. Furthermore, we demonstrated that OBAP increases ALP activity. These data confirm that OBAP stimulates osteoblast differentiation.

Next, we explored the *in vivo* effects of OBAP in the bone. It has been reported that PTH acts on osteoblasts, similar to the OBAP-induced increase in BMD [19]. These findings suggest that OBAP upregulates bone formation *in vivo*. In support of this hypothesis, we found that treating Gx rats with OBAP significantly increased BMD compared with saline treatment. We used Gx rats as an osteoporosis model because native peptide may be released by the stomach and enter the bloodstream, which could confound the effects of the native peptide.

OBAP resulted in a significant response *in vitro* and *in vivo*. However, if OBAP is used to treat osteoporosis, the effects should be stronger. Therefore, we need to further investigate and identify the OBAP receptor and improve the structure of OBAP to enhance its pharmacological effects.

The stomach is thought to play a role not only in bone metabolism but also in calcium metabolism. It is interesting that gastrin releases a hypocalcemic agent from the oxyntic mucosa and that subcutaneous injections of gastrin reduce blood calcium and stimulate Ca^{2+} uptake into the bone in rats [20–22]. Gastrin has been shown to induce hypocalcemia in thyroparathyroidectomized rats, indicating that calcitonin is not involved. However, gastrin does not affect bone calcium uptake in gastrectomized rats, whereas extracts of the oxyntic mucosa stimulate this process equally well in unmanipulated and gastrectomized rats [20]. These findings are thought to indicate that gastrin releases a hypocalcemic agent, currently referred to as gastrocalcin, from the oxyntic mucosa.

Because OBAP was purified and isolated from stomach and affects bone formation, it is possible that OBAP is gastrocalcin. Additional studies need to investigate this possibility.

In conclusion, we discovered a novel peptide, OBAP, which is a positive regulator that acts directly on osteoblasts. The discovery of OBAP may provide a new treatment option for osteoporosis and fracture healing.

Conflict of interest statement

All authors have no conflicts of interest.

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References

[1] L.S. Stepnick, The frequency of bone disease, in: J.A. McGowan, L.G. Raisz, A.S. Noonan, A.L. Elderkin (Eds.), Bone Health and Osteoporosis: A Report of the Surgeon General, Office of the US Surgeon General, Washington, DC, 2004, pp. 68–87.
[2] D. Shoback, Update in osteoporosis and metabolic bone disorders, J. Clin. Endocrinol. Metab. 92 (2007) 747–753.
[3] C.G. Bellows, J.E. Aubin, J.N. Heersche, Physiological concentrations of glucocorticoids stimulate formation of bone nodules from isolated rat calvaria cells in vitro, Endocrinology 121 (1987) 1985–1992.
[4] N. Fukushima, R. Hanada, H. Teranishi, Y. Fukue, T. Tachibana, H. Ishikawa, S. Takeda, Y. Takeuchi, S. Fukumoto, K. Kangawa, K. Nagata, M. Kojima, Ghrelin directly regulates bone formation, J. Bone Miner. Res. 20 (2005) 790–798.
[5] M. Kojima, H. Hosoda, Y. Date, M. Nakazato, H. Matsuo, K. Kangawa, Ghrelin is a growth-hormone-releasing acylated peptide from stomach, Nature 402 (1999) 656–660.
[6] Editorial, Osteomalacia after gastrectomy, Lancet 1 (1986) 77–78.
[7] R.A. Bussabarger, S. Freeman, A.C. Ivy, The experimental production of severe homogeneous osteoporosis by gastrectomy in puppies, Am. J. Physiol. 121 (1938) 137–148.
[8] F.I. Tovey, M.L. Hall, P.J. Ell, M. Hobsley, Postgastrectomy osteoporosis, Br. J. Surg. 78 (1991) 1335–1337.
[9] B. Klinge, D. Lehto-Axtelius, M. Åkerman, R. Håkanson, Structure of calvaria after gastrectomy. An experimental study in the rat, Scand. J. Gastroenterol. 30 (1995) 952–957.
[10] P. Persson, R. Gagnemo-Persson, D. Chen, J. Axelson, A.G. Nylander, O. Johnell, R. Håkanson, Gastrectomy causes bone loss in the rat: is lack of gastric acid responsible? Scand. J. Gastroenterol. 28 (1993) 301–306.
[11] L. Nilas, C. Christiansen, J. Christiansen, Regulation of vitamin D and calcium metabolism after gastrectomy, Gut 26 (1985) 252–257.
[12] J. Axelson, P. Persson, G. Gagnemo-Persson, R. Håkansson, Importance of the stomach in maintaining calcium homeostasis in the rat, Gut 32 (1991) 1298–1302.
[13] M. Davies, S.E. Heys, P.L. Selby, J.L. Berry, E.B. Mawer, Increased catabolism of 25-hydroxyvitamin D in patients with partial gastrectomy and elevated 1,25-dihydroxyvitamin D levels. Implications for metabolic bone disease, J. Clin. Endocrinol. Metab. 82 (1997) 209–212.
[14] G.W. Maier, M.E. Kreis, T.T. Zittel, H.D. Becker, Calcium regulation and bone mass loss after total gastrectomy in pig, Ann. Surg. 225 (1997) 181–192.
[15] D. Lehto-Axtelius, M. Stenström, O. Johnell, Osteopenia after gastrectomy, fundectomy, or antrectomy: an experimental study in the rat, Regul. Pept. 78 (1998) 41–50.
[16] B. Larsson, A. Gritli-Linde, P. Norlén, E. Lindström, R. Håkanson, A. Linde, Extracts of ECL-cell granules/vesicles and of isolated ECL cells from rat oxyntic mucosa evoke a Ca^{2+} second messenger response in osteoblastic cells, Regul. Pept. 97 (2–3) (2001) 153–161.
[17] B. Larsson, P. Norlén, E. Lindström, D. Zhao, R. Håkanson, A. Linde, Effects of ECL cell extracts and granule/vesicle-enriched fractions from rat oxyntic mucosa on cAMP and IP(3) in rat osteoblast-like cells, Regul. Pept. 106 (1–3) (2002) 13–18.
[18] M. Kitagawa, H. Mukai, Y. Ono, Molecular cloning and characterization of a novel mitochondrial phosphoprotein, MIPP65, from rat liver, Exp. Cell Res. 235 (1) (1997) 71–78.
[19] M.J. Mahon, M. Donowitz, C.C. Yun, G.V. Segre, Na(+)/H(+) exchanger regulatory factor 2 directs parathyroid hormone 1 receptor signalling, Nature 417 (6891) (2002) 858–861.
[20] P. Persson, R. Håkanson, J. Axelson, F. Sundler, Gastrin releases a blood calcium-lowering peptide from the acid-producing part of the rat stomach, Proc. Natl Acad. Sci. USA 86 (1989) 2834–2838.
[21] R. Håkanson, P. Persson, J. Axelson, O. Johnell, F. Sundler, Evidence that gastrin enhances ^{45}Ca uptake into bone through release of gastric hormone, Regul. Pept. 28 (1990) 107–118.
[22] R. Håkanson, P. Persson, J. Axelson, Elevated serum gastrin after food intake or acid blockade evokes hypocalcemia, Regul. Pept. 28 (1990) 131–136.

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MCP-1 expressed by osteoclasts stimulates osteoclastogenesis in an autocrine/paracrine manner

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ABSTRACT

Monocyte chemoattractant protein-1 (MCP-1) is a chemokine that plays a critical role in the recruitment and activation of leukocytes. Here, we describe that multinuclear osteoclast formation was significantly inhibited in cells derived from MCP-1-deficient mice. MCP-1 has been implicated in the regulation of osteoclast cell-cell fusion; however defects of multinuclear osteoclast formation in the cells from mice deficient in DC-STAMP, a seven transmembrane receptor essential for osteoclast cell-cell fusion, was not rescued by recombinant MCP-1. The lack of MCP-1 in osteoclasts resulted in a down-regulation of DC-STAMP, NFATc1, and cathepsin K, all of which were highly expressed in normal osteoclasts, suggesting that osteoclast differentiation was inhibited in MCP-1-deficient cells. MCP-1 alone did not induce osteoclastogenesis, however, the inhibition of osteoclastogenesis in MCP-1-deficient cells was restored by addition of recombinant MCP-1, indicating that osteoclastogenesis was regulated in an autocrine/paracrine manner by MCP-1 under the stimulation of RANKL in osteoclasts.

Osteoclasts are multinuclear cells derived from hematopoietic stem cells or monocyte/macrophage lineage cells [1]. Since osteoclasts are unique cells responsible for bone resorption, the control of osteoclast function or differentiation is critical to protect bones from bone diseases. Osteoclast formation is highly stimulated in destructive bone diseases such as rheumatoid arthritis (RA), multiple myeloma and bone metastasis [2–4]. Macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor kappa B ligand (RANKL) have been reported to be expressed in osteoblast/stroma cells [5,6], and described to play a pivotal role in regulating osteoclastogenesis through their receptors, c-Fms and RANK, respectively, which are expressed in osteoclast progenitor cells [7–9]. Various factors such as vitamin D, PTHrP and PGE2 were reported to indirectly stimulate osteoclast differentiation through the upregulation of RANKL in osteoblast/stroma cells [6,10,11], however, the existence of an autocrine/paracrine system that stimulates osteoclastogenesis remains largely unclear.

Monocyte chemoattractant protein-1 (MCP-1)/Chemokine (C–C motif) ligand 2 (Ccl2) is a chemokine that belongs to the CC chemokine family and plays a critical role in the recruitment and activation of leukocytes during acute inflammation [12]. MCP-1 has been shown to play a critical role in the pathogenesis of arteriosclerosis and other vascular diseases by recruiting monocytes into the arterial wall [13]. MCP-1 has also been reported to be involved in osteoclast differentiation [14–17], however, studies of osteoclast differentiation using MCP-1-deficient mice have not been reported. Since MCP-1 has been implicated in the cell–cell fusion of osteoclasts [14–17], and MCP-1 family ligands share multiple receptors, it is possible that MCP-1 binds to an unknown receptor to stimulate osteoclast cell–cell fusion. Dendritic cell specific transmembrane receptor (DC-STAMP), an orphan seven transmembrane receptor, is essential for cell–cell fusion of osteoclasts, and DC-STAMP-deficient osteoclasts show a complete lack of cell–cell

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