Bone Formation on Apatite-Coated Titanium Incorporated with Bone Morphogenetic Protein and Heparin

Takashi Kodama, DDS[1]/Tetsuya Goto, DDS, PhD[2]/Toshiki Miyazaki, PhD[3]/Tetsu Takahashi, DDS, PhD[4]

Purpose: *Bone morphogenetic proteins (BMPs) strongly induce osteogenesis and are enhanced by heparin. In this study, a potent osteoinductive material was developed by coating the surface of titanium with apatite incorporated with BMP and heparin.* ***Materials and Methods:*** *Titanium samples were treated with a 5 N NaOH solution and heated at 600°C for 24 hours. The treated titanium was soaked in simulated body fluid (SBF) for 4 days and additionally soaked in SBF containing recombinant human BMP-2 (rhBMP-2;1,000 ng/mL) with or without heparin (30 μg/mL) for 3 days at 37°C. The surfaces of each sample were examined with scanning electron microscopy. The presence of rhBMP-2 on the surface of the apatite-coated titanium was examined using the immunogold method. MC3T3-E1 osteoblast-like cells were cultured on the surface of each sample. The number and morphology of the adherent cells, alkaline phosphatase activity, and osteocalcin mRNA expression were examined.* ***Results:*** *Apatite was formed on the surface of alkaline heat-treated titanium after soaking in SBF for 7 days. The presence of rhBMP-2 was confirmed by the distribution of BMP-positive immunogold particles. The incorporation of (≥ 3 μg/mL) heparin significantly increased alkaline phosphatase activity and osteocalcin mRNA expression in the cells on apatite-coated titanium containing rhBMP-2.* ***Conclusion:*** *These findings demonstrate that heparin enhanced BMP-2-induced osteogenesis on apatite-coated titanium without the loss of BMP-2 activity. The combination of BMP-2 and heparin was effective even in the thin apatite layer formed on titanium using the alkaline heat treatment method.* Int J Oral Maxillofac Implants 2008;23:1013–1019.

Key words: *BMP-2, heparin, osteogenesis, simulated body fluid, titanium*

Biomaterials such as titanium are designed to induce a specific biological activity. Calcium phosphate is a bioactive material used to coat titanium implants to improve their biological and mechanical performance.[1,2] The plasma-spraying method for coating implants is associated with an increased risk of fatigue-related failure and delamination when the apatite coating is too thick (50 to 200 μm) or when spraying occurs at extremely high temperatures (more than 10,000°C). A more recently developed method of coating implants involves alkaline heat treatment[3] and soaking in simulated body fluid (SBF).[4] This biomimetic method offers several advantages over plasma spraying, such as low temperature reactions (at 37°C) or thin apatite coating (1 to 5 μm), and may be used to incorporate a variety of drugs and growth factors into implant surfaces.[5,6]

Bone morphogenetic proteins (BMPs) play a crucial role in cell growth and differentiation in a variety of cell types, including osteoblasts.[7–9] BMP-2 promotes the development of committed cells into differentiated osteoblasts,[10] and because of their beneficial effects on the development of bone cells, BMPs have been used to accelerate healing after bone implantation.[11–13] However, to effectively deliver BMP-2, a suitable carrier system is required. Apatite is considered a suitable carrier of BMP-2,[14] and the incorporation of BMP-2 into the apatite layer of a titanium implant may enhance its osteoinductive properties. The BMP-2

[1]Assistant Professor, Division of Oral and Maxillofacial Reconstructive Surgery, Kyushu Dental College, Kitakyushu, Japan.
[2]Associate Professor, Division of Anatomy, Kyushu Dental College, Kitakyushu, Japan.
[3]Associate Professor, Graduate School of Life Science and Systems Engineering, Kyushu Institute of Technology, Kitakyushu, Japan.
[4]Professor, Division of Oral and Maxillofacial Reconstructive Surgery, Kyushu Dental College, Kitakyushu, Japan.

Correspondence to: Dr Tetsuya Goto, Division of Anatomy, Kyushu Dental College, Kitakyushu 803-8580, Japan. Fax: +81-93-591-8199. E-mail: tgoto@kyu-dent.ac.jp

incorporation method, which combines an alkaline heat treatment with a soak in SBF with BMP-2, has been shown to preserve the activity of BMP-2.[6] Although the thin apatite layer that forms makes delamination of the apatite from the implant surface difficult, it also limits the amount of BMP-2 that can be taken in. Therefore, a means of enhancing BMP-2 activity is required. Heparin has a binding site on BMP-2 and enhances its activity.[15] Concurrent incorporation of BMP-2 and heparin into apatite-coated titanium may result in potent osteoinduction.

We hypothesized that heparin would enhance the osteoinductive activity of apatite-coated titanium incorporated with BMP-2. We evaluated whether this biomimetic coating would not only improve biointegration, but also promote bone-tissue engineering. We used the MC3T3-E1 cell line as a model system. We investigated the adhesion, morphology, and osteogenic gene expression of osteoblastic cells cultured on titanium surfaces coated with apatite and BMP-2 versus those coated with only apatite.

MATERIALS AND METHODS

Preparation of Apatite-coated Titanium Specimens

Commercially available pure titanium plates (10 × 10 × 1 mm for surface analysis) and disks (30 mm in diameter, 2 mm in thickness for cell culture) (Ti > 99.8%, Tokyo Scientific Instrument Association, Tokyo, Japan) were mechanically polished with 600-grit diamond paper and ultrasonically washed with acetone and distilled water. The polished titanium plates were soaked in 5 mL of a 5 N NaOH solution at 60°C for 24 hours, washed gently with distilled water, and dried at 40°C for 24 hours in an air atmosphere. The treated plates were heated to 600°C at a rate of 5°C per minute in an electric furnace, held for l hour, and allowed to cool to room temperature inside the furnace.

Each titanium plate was soaked in 30 mL of SBF for 4 days. The fluid was prepared by dissolving the reagents of NaCl, $NaHCO_3$, KCl, $K_2HPO_4{\cdot}3H_20$, $MgCl_2{\cdot}6H_20$, $CaCl_2$, and Na_2SO_4 (all from Wako Pure Chemical Industries, Osaka, Japan) into distilled water to adjust the ion concentrations to Na^+ 142.0, K^+ 5.0, Mg^{2+} 1.5, Ca^{2+} 2.5, Cl^- 147.8, HCO^{3-} 4.2, HPO_4^{2-} 1.0, and SO_4^{2-} 0.5 mol/L. The SBF was buffered at pH 7.4 with tris-hydroxymethylaminomethane (4.5 mol/L) and an appropriate amount of hydrochloric acid at 37°C. The samples were sterilized under ultraviolet light for at least 24 hours.

The samples were prepared by soaking the coated plates in SBF (pH 7.4) containing recombinant human BMP-2 (rhBMP-2; Pepro Tech EC, London, UK) (0, 10, 100, 500, or 1,000 ng/mL) and heparin (Wako) (0.3, 3, or 30 µg/mL) for 72 hours at 37°C under sterile conditions.

Surface Analysis

The surface structure of the metals was examined by a thin-film radiographic diffractometer (TF-XRD) (thin-film attachment CN2651A1; Rigaku-Denki, Tokyo, Japan) scanning electron microscopy (SEM) (S-3500CX; Hitachi, Tokyo, Japan). In the TF-XRD analysis, the angle of the incident beam was fixed at 1 degree against the surface of the sample.

Immunogold Electron Microscopy

Immunogold electron microscopy was used to determine the presence of rhBMP-2. The samples were washed in phosphate-buffered saline (PBS) 3 times, dried, and incubated with goat polyclonal BMP-2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100 in PBS for 3 hours at room temperature. Controls were developed by replacing the primary antibody with nonimmune goat serum (Santa Cruz). The samples were washed in PBS 3 times and incubated in rabbit antigoat IgG conjugated with colloidal gold (20 nm particles; EY Laboratories, San Mateo, CA, USA) diluted 1:100 in PBS for 1 hour at room temperature. The samples were coated with carbon. The gold particles were observed under an SEM (S-3500CX, Hitachi) using the secondary electron image mode.

Cell Culture

Mouse osteoblastic MC3T3-E1 cells were seeded on titanium disks (30 mm in diameter) and plates (10 × 10 mm) in concentrations of 5×10^4 and 1×10^4 cells per dish, respectively. The cells were cultured in α-minimum essential medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Wako) and 100 µg/mL penicillin G (Wako), 50 µg/mL gentamicin (Gibco), and 0.3 µmg/mL Fungizone (Gibco). The culture medium was changed twice weekly.

To count the number of cells attached to each substratum, the specimens were washed three times with cytoskeletal-stabilizing (CS) buffer and incubated for 30 min at 37° with tetramethyl rhodamine isothiocyanate (TRITC) phalloidin (Molecular Probes, Eugene, OR, USA) (1:40 dilution). After 3 additional washes in 0.1 M PBS, the cells were enclosed with glycerol-PBS solution containing 1, 4-diazabicyclo [2.2.2] octane (SIGMA, St Louis, MO, USA) (100 mg/mL) to prevent fluorescence decay. The samples were examined using a fluorescence microscope (Olympus Optical, Tokyo, Japan) in 10 randomly selected fields of each sample.

Alkaline Phosphate Activity Assay

Eight days after cell seeding, the culture medium was removed. The cells were rinsed twice with PBS and harvested in 1 mL of demineralized water using a disposable cell scraper. The cells were then homogenized for 2 minutes and centrifuged at 2,000 rpm for 10 minutes. The supernatant was used to measure the alkaline phosphate activity according to the protocol of the ALP activity assay kit (Rab Assay, Wako) using p-nitrophenyl phosphate as the substrate. The assay was performed in 96-well microtiter plates. Alkaline phosphate activity was monitored spectrophotometrically (at an adsorption wavelength of 415 nm) by the release of p-nitrophenol from p-nitrophenyl phosphate (at pH 9.8) using a microplate reader (Model 550; Bio-Rad, Tokyo, Japan), as previously described.[16] Alkaline phosphatase activity was expressed as the concentration of p-nitrophenyl phosphate (in micromoles) transformed per microgram of protein. The enzyme activity was expressed as micromoles of p-nitrophenol per microgram of protein. The protein concentration was measured with a commercially available kit (Protein Assay Rapid Kit; Wako). The assay was performed in 96-well microtiter plates. The absorbance was measured at 477 nm with a microplate reader (Bio-Rad). Bovine serum albumin was used as the standard.

Semiquantitative RT-PCR

Fourteen days after cell seeding, cellular mRNA was extracted using a Total RNA Extraction Miniprep System (Viogene, Sunnyvale, CA, USA) in accordance with the manufacturer's protocol. Total RNA was extracted from the cells after a culture duration of 2 weeks. To reduce DNA contamination, RNA samples were treated with RNase-free DNase1 (Takara Bio, Shiga, Japan) for 10 hours at 37°C. cDNA was synthesized from total RNA (2 μg) in reaction buffer (30 μL) composed of dNTPs (500 μM), ribonuclease inhibitor (20 U) (Promega, Madison, WI, USA), and Superscript II reverse transcriptase (200 U) (Invitrogen Life Technology, Carlsbad, CA, USA). The reaction was carried out for 7 minutes at 70°C, 60 minutes at 45°C, and 10 minutes at 70°C and then cooled to 4°C.

The oligonucleotide reverse transcriptase-polymerase chain reaction (RT-PCR) primer sequences were designed and their specificities were confirmed using a BLAST-assisted Internet search of a nonredundant nucleotide sequence database (National Library of Medicine, Bethesda, MD, USA). The primers used to amplify osteocalcin (OCN) were 5′-CCT CAG TCC CCA GCC CAG ATC C-3′ and 5′-CAG GGC AGA GAG AGA GGA CAG G-3′. The primers for the β-actin internal control were 5′-TGG ACT TCG AGC AAG AGA TGG -3′ and 5′-ATC TCC TTC TGC ATC CTG TCG -3′. Each cycle consisted of a denaturation step (OCN: 94°C for 30 seconds; β-actin: 94°C for 1 minute), an annealing step (OCN: 58°C for 30 seconds; β-actin 56°C for 1 minute), and an extension step (OCN: 72°C for 1 minute; β-actin: 72°C for 1 minute). The reproducibility of the RT-PCR analysis was confirmed with 3 or more replicate experiments. Each PCR mixture consisted of cDNA (reverse-transcribed RNA), Taq polymerase buffer, and 1 μL each of sense and antisense primers, for a total volume of 20 μL. The PCR products were electrophoresed in 2% agarose gels and visualized with ethidium bromide. The data were analyzed using NIH Image (NIH, Bethesda, MD, USA).

Statistical Analysis

The data were analyzed using StatView (Abacus Concepts, Berkeley, CA, USA). One-way analysis of variance (ANOVA) was used to evaluate the effect of BMP-2 and heparin on cell number and alkaline phosphatase activity. Significant differences were examined by post hoc Scheffé test.

RESULTS

Surface Characterization

SEM photographs of the pure titanium samples demonstrated rough, scratched surfaces (Fig 1a). After alkaline heat treatment, the surfaces of the samples were porous and exhibited a honeycomb-like structure (Fig 1b). After alkaline heat treatments and soaking in SBF for 10 days, the surfaces of the titanium samples exhibited an apatite-like structure (Fig 1c). The TF-XRD pattern of nontreated samples showed broad peaks, which were assigned to titanium (Fig 1d). Samples with an alkaline heat treatment demonstrated broad peaks, which were assigned to sodium titanate (Fig 1e). After the alkaline heat treatment and soaking in SBF for 10 days, the titanium surface was coated with apatite. Broad peaks were assigned to apatite, with a low level of crystallinity observed after the soaking (Fig 1f). The TF-XRD pattern indicated that the domed structure observed under SEM was low crystalline apatite.

rhBMP-2 Detection on Apatite-Coated Titanium

The rhBMP-2 on apatite-coated titanium was visualized by immunogold electron microscopy (Figs 2a and 2b). We tested concentrations of rhBMP-2 ranging from 0 to 1,000 ng/mL. Because a large number of gold particles were observed with 1,000 ng/mL rhBMP-2, we used this concentration in all experiments.

Although the apatite-coated titanium disks incorporated with rhBMP-2 were thoroughly washed with PBS 3 times, the presence of rhBMP-2 was still

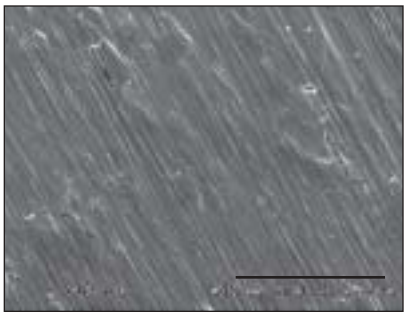


Fig 1a Scanning micrographic images of the pure titanium surface. Bar = 50 μm.

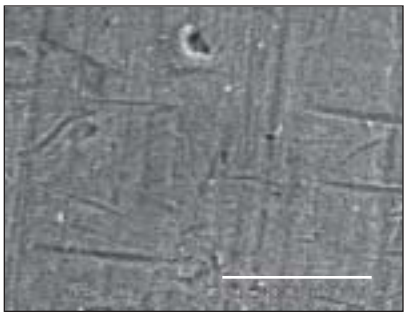


Fig 1b Scanning micrographic images of the titanium surface treated with 5 N NaOH for 24 hours and heated to 600°C for I h. Bar = 50 μm.

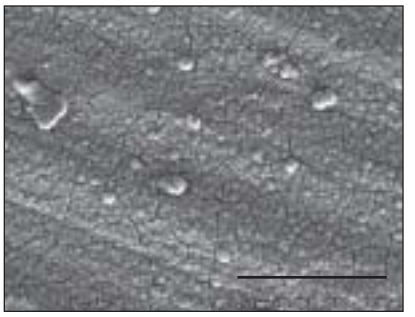


Fig 1c Scanning micrographic images of the titanium surface subjected to an alkaline heat treatment and soaked in SBF for 10 days. Bar = 100 μm.

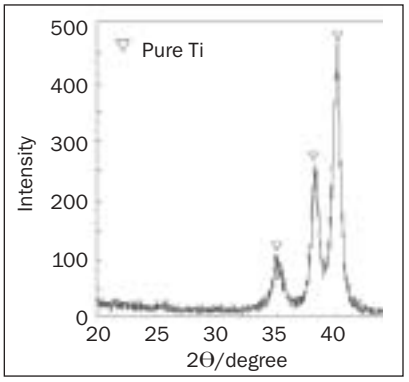


Fig 1d A TF-XRD pattern on the surface of sandblasted pure titanium.

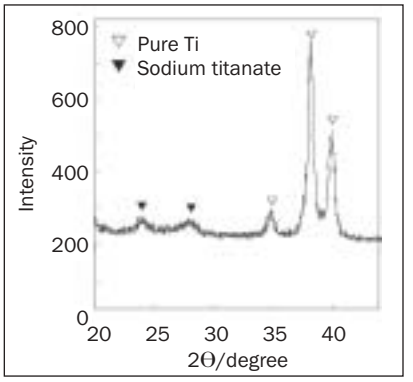


Fig 1e A TF-XRD pattern on the surface of titanium treated with 5 N NaOH and subjected to an alkaline heat treatment.

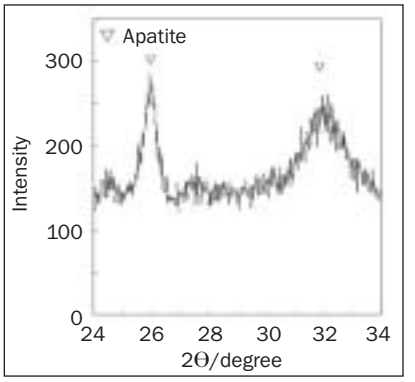
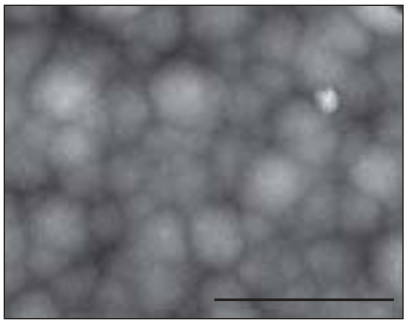


Fig 1f A TF-XRD spectrum pattern of apatite-coated titanium. The alkaline heat-treated titanium was soaked in SBF for 10 days, with apatite subsequently forming on the surface.



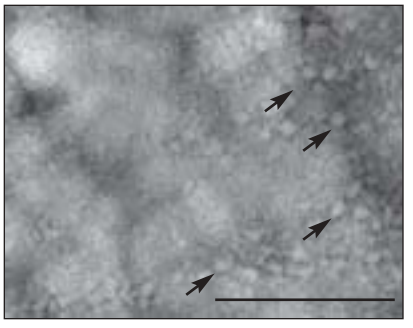


Fig 2a *(Left)* Scanning micrographic images of the surface of apatite-coated titanium (original magnification, × 5,000). Bar = 1 μm.

Fig 2b *(Right)* Scanning micrographic images of colloidal gold particles, which represent the distribution of BMP-2 on the apatite-coated titanium. Arrows indicate colloidal gold particles (original magnification, × 5000). Bar = 1 μm.

observed under immunogold electron microscopy. In this experiment, we tested concentrations ranging from 0 to 1,000 ng/mL rhBMP-2.

Cell Number and Morphology

The morphology of the osteoblastic cells 24 hours after cell seeding showed differences in cell shape depending on the substrate. The cells on the pure titanium were spindle-shaped, whereas the cells on the apatite and the apatite with rhBMP-2 were round (Figs 3a to 3c). The cells on the pure titanium were dispersed to a greater degree than those on the apatite alone or apatite with rhBMP-2.

The number of attached cells was determined with the micrographs of the actin-stained cells at 24 hours. The number of attached cells was not significantly different among the pure titanium, apatite alone, and apatite with rhBMP-2 samples (Fig 3d).

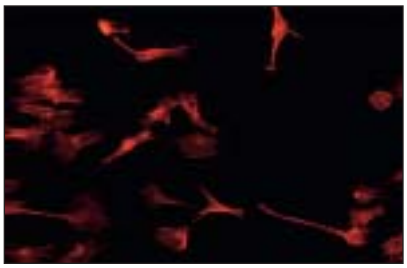


Fig 3a Fluorescent images of actin fibers in the osteoblastic cells on pure titanium. Bar = 30 μm.

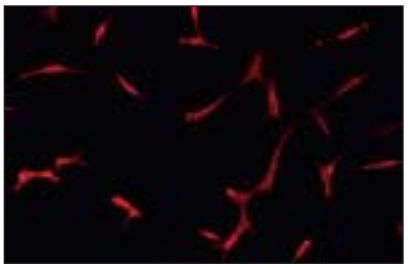


Fig 3b Fluorescent images of actin fibers in the osteoblastic cells on HA-coated titanium incorporated with BMP-2. Bar = 30 μm.

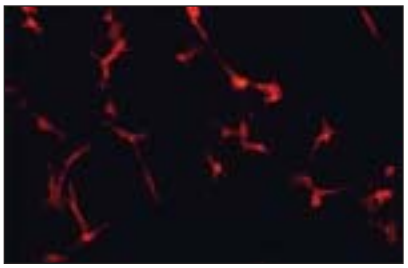


Fig 3c Fluorescent images of actin fibers in the osteoblastic cells on HA-coated titanium. Bar = 30 μm.

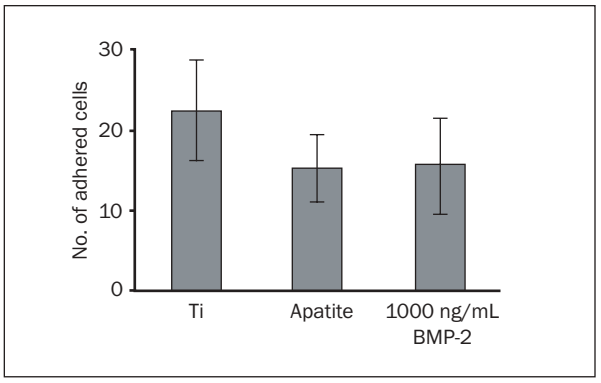


Fig 3d Number of cells attached to the pure titanium, apatite-coated titanium, and apatite-coated titanium incorporated with 500 ng/mL BMP-2. Data represent means ± SD.

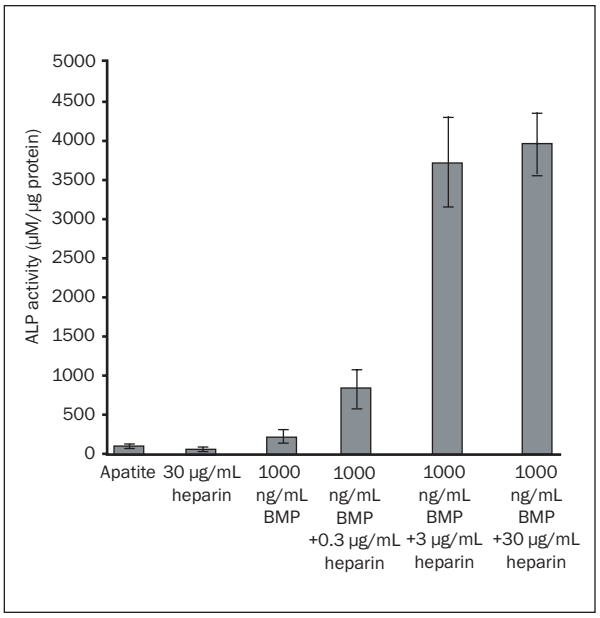


Fig 4 Alkaline phosphatase activity in the osteoblastic cells cultured on apatite-coated titanium for 8 days. In total, 1,000 ng/mL BMP-2 or 0.3 to 30 μg/mL heparin were added to the SBF. Data represent means ± SD. **P* < .01, ***P* < .001 versus 1,000 ng/mL BMP-2.

Alkaline Phosphatase Activity

Alkaline phosphatase is an early-stage marker of osteoblast differentiation. Alkaline phosphatase activity 8 days after cell seeding varied according to the substrate (Fig 4). The activity of alkaline phosphatase increased significantly in the cells on the samples incorporated with apatite and rhBMP-2 (1,000 ng/mL) compared to those with apatite alone. Alkaline phosphatase activity in the cell layers on the apatite-coated titanium increased significantly in the cells incorporated with heparin (0.3 to 30 μg/mL) in a dose-dependent manner. The alkaline phosphatase activity in the cell layers on apatite-coated titanium containing heparin (3 μg/mL) and rhBMP-2 (1,000 ng/mL) was similar to that of the cell layers incorporated with heparin (30 μg/mL) and rhBMP-2 (1,000 ng/mL). The activity of alkaline phosphatase did not significantly increase with the incorporation of 30 μg/mL heparin alone.

Osteogenesis Differentiation

OCN is a late-stage marker of osteoblast differentiation. Expression of OCN mRNA at 14 days after cell seeding differed according to the substrate (Figs 5a and 5b). Expression levels of OCN mRNA increased on the titanium coated with apatite and rhBMP-2 (1,000 ng/mL) compared to the titanium coated with apatite alone. Furthermore, rhBMP-2 expression of OCN mRNA increased on the apatite-coated titanium with rhBMP-2 (1,000 ng/mL) and heparin (30 μg/mL) compared to the apatite-coated titanium with rhBMP-2 alone (1;000 ng/mL).

DISCUSSION

In this study, heparin enhanced rhBMP-2–induced osteogenesis on alkaline heat-treated titanium soaked in SBF. Such potent osteoinduction could be advantageous in a clinical setting.

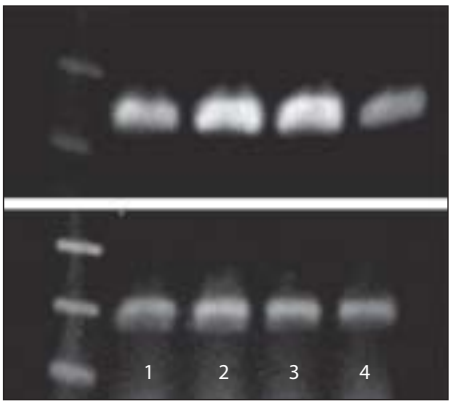


Fig 5a Expression of OCN mRNA on apatite-coated titanium (1) and apatite-coated titanium with 1,000 ng/mL BMP-2 (2), 1,000 ng/mL BMP-2 plus 30 μg/mL heparin (3), or 30 μg/mL heparin (4). β-actin mRNA expression corresponds with OCN expression. MW indicates molecular weight markers.

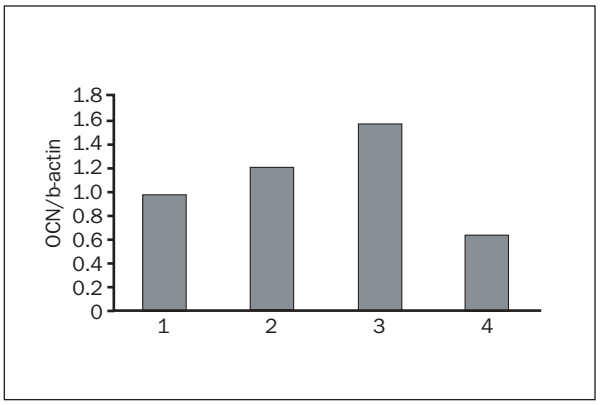


Fig 5b Relative density of OPG expression per β-actin expression. One through 4 represent the same groups described in Fig 5a.

Apatite formation by alkaline heat treatment and soaking in SBF was originally introduced by Li et al,[17] who suggested that a material possessing and/or developing both a negatively charged surface and abundant OH groups in a physiologically related fluid would likely be an efficient apatite inducer. This method was subsequently employed to create an apatite coating on titanium and titanium alloy using SBF.[3,18] Alkaline heat treatment results in the formation of a thin apatite layer (1 to 5 μm) and a graded structure between the titanium and apatite layer, which allows for tight integration of the titanium with living bone.[19,20] We confirmed the presence of a thin apatite layer using TF-XRD analysis and observed that the domed structure of the apatite exhibited a low level of crystallinity. It has been previously reported that after soaking in SBF, amorphous calcium phosphate may convert into bonelike crystalline apatite.[21]

The osteoblastic cells on the apatite surface with the incorporated rhBMP-2 shrank compared to the cells on the untreated titanium. The substrates on which cell layers were attached differed: One was mechanically polished with 600-grit diamond paper, and the other was covered with a round crystalline matrix. Although substrates with a rough surface have been shown to promote better osteointegration,[22–24] rhBMP-2 may affect cell attachment and result in changes in cell morphology. We previously observed similar morphological changes in osteoblastic cells that exhibited a more round and compact shape on apatite than on titanium.[25] After seeding osteoblastic cells on the substrate, the cells quickly exhibited compact shape over the apatite layer, which may have induced earlier osteogenesis. These findings suggest that shrunken-shaped cells on apatite-coated titanium incorporated with rhBMP-2 may comprise a suitable environment for osteogenesis.

Calcium phosphate is a suitable carrier for growth factors such as BMP, and biomimetic calcium phosphate coating has been shown to release BMP slowly and at a rate sufficient enough to support local osteogenic activity.[26] In this study, the presence of rhBMP-2 on the apatite-coated titanium was confirmed by the distribution of BMP-positive immunogold particles after soaking in SBF. The activity of rhBMP-2 was confirmed by the enhanced alkaline phosphatase activity and OCN mRNA expression. Although the apatite coating formed by the alkaline heat treatment and soaking in SBF with BMP-2 is suitable for osteoinductive material, its thinness (1 to 5 μm) limits the amount of possible BMP-2 incorporation. Therefore, we applied heparin, which protects BMPs from degradation and inhibition by BMP antagonists such as noggin,[27] to enhance the activity of BMP-2. The activity of alkaline phosphate in osteoblastic cells on HA-coated titanium was more than 15-fold greater in samples incorporated with rhBMP-2 and 3 or 30 μg/mL of heparin compared to those containing BMP-2 alone. Furthermore, heparin enhanced OCN mRNA expression. A previous study showed that the biological activity of rhBMP-2 was prolonged in the presence of heparin, as demonstrated by the prolonged degradation of BMP-2 in culture media.[27] In the osteoblasts, noggin mRNA was rapidly induced by BMP-2; noggin failed to inhibit BMP-2 activity in the presence of heparin. Our findings suggest that the rhBMP-2 retained in the apatite layer had not lost its biological activity and

was capable of binding to the signaling receptors. Furthermore, heparin retained in the apatite layer remained active while enhancing rhBMP-2 activity. These findings suggest that the combination of BMP-2 and heparin in the thin apatite layer formed on titanium using the alkaline heat treatment may shorten a peri-implant bone healing.

CONCLUSION

The results of the present study demonstrated that heparin (≥ 3 μg/mL) retained in the apatite layer enhanced rhBMP-2–induced osteogenesis on apatite titanium. The combination of rhBMP-2 and heparin in a thin apatite surface on titanium formed by the alkaline heat treatment and soaking in SBF stimulated alkaline phosphatase activity and OCN mRNA expression in osteoblastic cells.

ACKNOWLEDGMENTS

This work was supported by the Ministry of Education, Science, Sports, and Culture of Japan (Grant-in-Aid for Scientific Research 18592194 to Dr T. Takahashi).

REFERENCES

1. Osborn JF, Newesley H. Dynamic aspects of implant/bone interface. In: Heimke G (ed). Dental Implant. Munich: Carl Hansen Verlag, 1980:111–123.
2. de Groot K, Wolke JG, Jansen JA. Calcium phosphate coatings for medical implants. Proc Inst Mech Eng 1998;212: 137–147.
3. Kokubo T, Miyaji F, Kim HM, Nakamura T. Spontaneous formation of bone-like apatite layer on chemically treated titanium metals. J Am Ceram Soc 1996;79:1127–1129.
4. Kim HM, Miyaji F, Kokubo T, Nakamura T. Effect of heat treatment on apatite-forming ability of Ti metal induced by alkali treatment. J Mater Sci Mater Med 1997;8:341–347.
5. Stigter M, de Groot K, Layrolle P. Incorporation of tobramycin into biomimetic hydroxyapatite coating on titanium. Biomaterials 2002;23:4143–4153.
6. Liu Y, Hunziker EB, Layrolle P, De Bruijn JD, de Groot K. Bone morphogenetic protein 2 incorporated into biomimetic coatings retains its biological activity. Tissue Eng 2004;10:101–108.
7. Hanada K, Dennis JE, Caplan AI. Stimulatory effects of basic fibroblast growth factor and bone morphogenetic protein-2 on osteogenic differentiation of rat bone marrow-derived mesenchymal stem cells. J Bone Miner Res 1997;12:1606–1614.
8. Tate Y, Kawasaki K, Ishibashi S, Ikeda U, Shimada K. Effects of N-acetylcysteine on nitroglycerin-induced relaxation and protein phosphorylation of porcine coronary arteries. Heart Vessels 1998;13:263–268.
9. Puleo DA. Dependence of mesenchymal cell responses on duration of exposure to bone morphogenetic protein-2 in vitro. J Cell Physiol 1997;173:93–101.
10. Takuwa Y, Ohse C, Wang EA, Wozney JM, Yamashita K. Bone morphogenetic protein-2 stimulates alkaline phosphatase activity and collagen synthesis in cultured osteoblastic cells, MC3T3-E1. Biochem Biophys Res Commun 1991;174:96–101.
11. Kirker-Head CA. Potential applications and delivery strategies for bone morphogenetic proteins. Adv Drug Deliv Rev 2000; 43:65–92.
12. Herr G, Hartwig CH, Boll C, Kusswetter W. Ectopic bone formation by composites of BMP and metal implants in rats. Acta Orthop Scand 1996; 67: 606-610.
13. Asahina I, Watanabe M, Sakurai N, Mori M, Enomoto S. Repair of bone defect in primate mandible using a bone morphogenetic protein (BMP)-hydroxyapatite-collagen composite. J Med Dent Sci 1997;44:63–70.
14. Seeherman H, Wozney JM. Delivery of bone morphogenetic proteins for orthopedic tissue regeneration. Cytokine Growth Factor Rev 2005;16:329–345.
15. Ruppert R, Hoffmann E, Sebald W. Human bone morphogenetic protein 2 contains a heparin-binding site which modifies its biological activity. Eur J Biochem 1996;237:295–302.
16. Boyan BD, Schwartz Z, Bonewald LF, Swain LD. Localization of 1,25-(OH)2D3-responsive alkaline phosphatase in osteoblast-like cells (ROS 17/2.8, MG 63, and MC 3T3) and growth cartilage cells in culture. J Biol Chem 1989;264:1187911886.
17. Li P, Ohtsuki C, Kokubo T, Nakanishi K, Soga N, de Groot K. The role of hydrated silica, titania, and alumina in inducing apatite on implants. J Biomed Mater Res 1994;28:715.
18. Kim HM, Miyaji F, Kokubo T, Nakamura T. Preparation of bioactive Ti and its alloys via simple chemical surface treatment. J Biomed Mater Res 1996;32:409-417.
19. Nishiguchi S, Kato H, Fujita H, et al. Enhancement of bone-bonding strengths of titanium alloy implants by alkali and heat treatments. J Biomed Mater Res 1999;48:689-696.
20. Kim HM, Takadama H, Kokubo T, Nishiguchi S, Nakamura T. Formation of a bioactive graded surface structure on Ti-15Mo-5Zr-3Al alloy by chemical treatment. Biomaterials 2000;21: 353-358.
21. Takadama H, Kim HM, Kokubo T, Nakamura T. TEM-EDX study of mechanism of bonelike apatite formation on bioactive titanium metal in simulated body fluid. J Biomed Mater Res 2001; 57:441-448.
22. Groessner-Schreiber B, Tuan RS. Enhanced extracellular matrix production and mineralization by osteoblasts cultured on titanium surfaces in vitro. J Cell Sci 1992;101:209-217.
23. Perizzolo D, Lacefield WR, Brunette DM. Interaction between topography and coating in the formation of bone nodules in culture for hydroxyapatite- and titanium-coated micromachined surfaces. J Biomed Mater Res 2001;56:494-503.
24. Schneider GB, Perinpanayagam H, Clegg M, et al.Implant surface roughness affects osteoblast gene expression. J Dent Res. 2003;82:372-376.
25. Okumura A, Goto M, Goto T, et al. Substrate affects the initial attachment and subsequent behavior of human osteoblastic cells (Saos-2). Biomaterials 2001;22:2263-2271.
26. Liu Y, Hunziker EB, Van de Vaal C, de Groot K. Biomimetic coating vs. collagen sponges as a carrier for BMP-2: A comparison of the osteogenic responses triggered in vitro using an ectopic rat model. Bioceramics 16 2004;254-2:619-622.
27. Zhao B, Katagiri T, Toyoda H, et al. Heparin potentiates the in vivo ectopic bone formation induced by bone morphogenetic protein-2. J Biol Chem 2006;281:23246-23253.