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Improving osteoblast functions and bone formation upon BMP-2 immobilization on titanium modified with heparin

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Sodium chloride (PubChem CID:5234)
Magnesium chloride (PubChem CID:24584)
Sodium azide (PubChem CID:33557)
4′,6-Diamidino-2-phenylindole (PubChem CID:2954)
Phenylmethylsulfonyl fluoride (PubChem CID:4784)

ABSTRACT

The aim of this study was to develop bone morphogenetic protein-2 (BMP-2) immobilization on titanium (Ti) modified by heparin for improving osteoblast function *in vitro* and bone formation *in vivo*. The Ti surface was first modified with heparin and then immobilized with BMP-2 (BMP-2/Hep-Ti). The Ti and modified Ti were characterized by scanning electron microscopy (SEM), X-ray photoelectron spectroscopy (XPS) and contact angle. *In vitro* studies demonstrated that osteoblasts cultured on BMP-2/Hep-Ti substrate increased ALP activity, calcium deposition, osteocalcin (OCN) and osteopontin (OPN) levels as compared to those cultured on Ti or BMP-2/Ti. In addition, intra-thread bone density and bone to implant contact ratio of BMP-2/Hep-Ti were significantly greater than those of Ti in the *in vivo* study. In conclusion, BMP-2 immobilized Ti modified heparin implants seemed to be a suitable delivery system for BMP-2 to improve osteoblast functions and new bone formation at the defect area around an implant.

1. Introduction

Dental implants are widely used, reliable, and safe; however they have low success rates for patients with poor bone quality and quantity, and for patients whose healing and regeneration capabilities are poor (Retzepi, Lewis, & Donos, 2010). In addition, the most important concern of both patients and dentists is how to reduce

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healing time from placement of the implant fixture to placement of the crown. Studies on the integration of implants and bones have investigated the surface morphology and features of implants and bones, and have attempted to promote their osseointegration *via* more microscopic physical and chemical surface treatment, and even to biomimetically treat the surface (Branemark et al., 1969; Mendonca, Mendonca, Aragao, & Cooper, 2008). Studies on surface treatment techniques are continuously being performed to reduce healing time, improve osseointegration, and enhance augmentation of the surrounding bones (Ponader et al., 2010; von Wilmowsky et al., 2009).

Various growth factor delivery systems have been applied to facilitate bone regeneration by using various polysaccharides such as hyaluronic acid, dextran, chitosan and heparin, *etc.* (Kim et al., 2007, 2011; Lee, Yun, Park, & Kim, 2012; Muzzarelli, 2010; Shi, Neoh, Kang, Poh, & Wang, 2009). Among them, studies on applying growth factors such as bone morphogenetic protein (BMP) to dental implants are underway. BMP enhances bone formation by promoting the differentiation of osteoblasts from the mesenchymal stem cells (MSCs) and by helping in the biosynthesis of the bone matrix through control of essential factors during osteoinduction to regenerate osseous tissue (Bessa, Casal, & Reis, 2008a,b). BMP can be classified into 16 subgroups. Among the subgroups, BMP-2 has been proven by preclinical and clinical studies for use in various medical treatments (Chen, Zhao, & Mundy, 2004). Particularly, one study reported that when the surface of an anodized dental implant is coated with recombinant human BMP-2 (rhBMP-2), which is derived using a genetic recombination technique, the anodized implant can serve as an effective carrier (Hall, Sorensen, Wozney, & Wikesjo, 2007). However, several studies have reported that use of rhBMP-2 does not have a significant bone formation effect (Becker et al., 2006; Wikesjo et al., 2008). Such a result was suggested to be attributable to the early release of a large amount of rhBMP-2, the lack of attaining an optimum concentration, and the fact that only one type of growth factor, rhBMP-2, was used, unlike during the natural regeneration process of the human body wherein multiple growth factors are involved (Schliephake et al., 2005; Stadlinger et al., 2008; Stenport, Johansson, Heo, Aspenberg, & Albrektsson, 2003).

Heparin, a highly sulfated glycosaminoglycan and linear natural polysaccharide, has binding affinities to various growth factors, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and transforming growth factor-ß (Sasisekharan, Ernst, & Venkataraman, 1997). Moreover, biomaterial systems with heparin have advantages for the controlled release of these growth factors (Ishibe et al., 2009; Perets et al., 2003).

With the popularization of dental implants, the incidence of peri-implantitis is now a growing problem causing local bone destruction and resulting in implant failure. For the above reasons, implant surfaces should have anti-inflammatory activity and should facilitate biomolecular adhesion to enhance osteoblast function. Several studies have suggested that the incorporating BMP-2 and heparin on titanium (Ti) surfaces results in sustained release of the growth factor, well characterized anti-inflammatory activity, and enhanced osteoblastic function (Ishibe et al., 2009; Kodama, Goto, Miyazaki, & Takahashi, 2008; von Walter et al., 2008).

In this study, we investigated whether BMP-2 immobilized heparinized Ti substrates can promote osteoblast function, osseointegration, and bone regeneration in *in vitro* and *in vivo* studies. Ti substrates were modified with heparin to immobilize BMP-2. In addition, we evaluated osteoblast functions such as alkaline phosphatase (ALP) activity, the amount of calcium deposited, and osteocalcin (OCN) and osteopontin (OPN) gene expression *in vitro* as well as the osseointegration and bone regeneration *in vivo*.

2. Materials and methods

2.1. Materials

Titanium (Ti) discs and BMP-2 were purchased from Cowellmedi Co. (Pusan, Korea). Heparin sodium (molecular weight: 12,000–15,000 g/mol) was purchased from Acros Organics (Rahway, NJ, USA). Dopamine hydrochloride (DOPA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), and *N*-hydroxysuccinimide (NHS) were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), and penicillin-streptomycin were purchased from Gibco BRL (Rockville, MD, USA). The BMP-2 enzyme-linked immunosorbent assay (ELISA) kit was purchased from PeproTech Inc. (Rocky Hill, NJ, USA). All other chemicals were of the purest analytical grade available.

2.2. In vitro study

2.2.1. Immobilization of BMP-2 on the heparinized-Ti surface

Prior to immobilizing BMP-2 on the heparinized-Ti surface, DOPA was anchored to the surface of Ti substrates. Briefly, the Ti substrates were immersed in 10 mL of 10 mM Tris–(hydrochloride) HCl buffer solution (pH = 8.0) containing a DOPA concentration of 2 mg/mL and maintained for 24 h under dark conditions. The substrates were washed with distilled water and dried with N_2 gas. Heparin was conjugated onto the surface of the aminated-Ti surfaces by amide bond formation *via* condensation reaction using EDC and NHS. Briefly, heparin (2 mg/mL) was dissolved in 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer solution (pH 5.6) containing EDC (0.59 mg) and NHS (0.18 mg) and then the aminated-Ti substrates were immersed in the aforementioned solution. BMP-2 was immobilized onto the heparinized-Ti surface (BMP-2/Hep-Ti). In brief, the heparinized-Ti substrates were immersed in 0.1 M MES buffer solution (pH 5.6). BMP-2 at a concentration of 50 ng/mL was then added to the 0.1 M MES buffer solution, followed by specific interaction between heparinized-Ti substrates and BMP-2 after 24 h at room temperature (RT). Furthermore, BMP-2/Ti was prepared by the dip and dry method. Briefly, the Ti substrates were immersed in 0.1 M MES buffer solution (pH 5.6). BMP-2 at a concentration of 50 ng/mL was added to the 0.1 M MES buffer solution. The reaction was allowed to proceed for 24 h at RT.

2.2.2. Determining the amount of heparin on the heparinized-Ti surface

The amount of heparin on the heparinized-Ti surface was determined by the toluidine blue method. Briefly, Ti substrates were placed in 1 mL PBS (pH 7.4) solution containing 1 mL of 0.005% toluidine blue solution. After 30 min under gentle shaking, 2 mL of hexane was added. Then, the Ti discs were removed from solution, and the absorbance of the aqueous phase was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at 620 nm. The amount of heparin from the heparinized Ti surface was calculated from a calibration curve that was constructed using various heparin concentrations.

2.2.3. Characterization of the Ti and surface-modified Ti

The surface morphologies of Ti, BMP-2/Ti and BMP-2/Hep-Ti were investigated by scanning electron microscopy (SEM, S2300, Hitachi, Tokyo, Japan) at the Korea Basic Science Institute Chuncheon Center. The substrates were coated with gold using a sputter-coater (Eiko IB, Tokyo, Japan). The SEM was operated at 3 kV. The surface compositions of Ti, BMP-2/Ti, and BMP-2/Hep-Ti were evaluated by X-ray photoelectron spectroscopy (XPS) on

a *K*-alpha spectrometer (ESCALAB250 XPS System, Theta Probe AR-XPS System, Thermo Fisher Scientific, Cambridge, UK) with an Al *K*α X-ray source (1486, 6 eV photons) at the Korea Basic Science Institute Busan Center. To evaluate the surface modified Ti, we also performed the contact angle method. The Ti and surface-modified Ti were placed on the stage, respectively. Distilled water was then dropped on the substrates from a motorized syringe. The contact angle was calculated by a goniometer (SEO Phoenix 300, Suwon, Korea) with the AuoFAST algorithm. These experiments were repeated three times.

2.2.4. BMP-2 release study from BMP-2/Ti and BMP-2/Hep-Ti

To evaluate the release kinetics of BMP-2 from the BMP-2/Ti and BMP-2/Hep-Ti groups, the substrates were soaked in a 15 mL conical tube (Falcon, Corning, NY, USA) containing 1 mL PBS (pH 7.4) at 100 rpm at 37 °C, respectively. At designated time intervals of 1, 2, 8 h, and 1, 3, 5, 7, 14, 21, and 28 days, the supernatant was collected and replaced with fresh PBS solution. All samples were stored at −20 °C until analysis. The supernatants were determined by ELISA according to the manufacturer's instructions using a microplate reader at a wavelength of 450 nm.

2.2.5. Culture of human osteosarcoma MG-63 cell line

MG-63 cells (1×10^5 cell/mL) were cultured onto pristine and surface-modified Ti with DMEM supplemented with 10% FBS, 50 μg/mL ascorbic acid, 10 nM dexamethasone, 10 mM β-glycerophosphate 100 U/mL penicillin, and 100 μg/mL streptomycin. The medium was exchanged every 3 days. The cultured cells were used to examine cell morphology, adhesion study, cell proliferation, alkaline phosphatase (ALP) activity and calcium concentrations.

2.2.6. Cell morphology and adhesion study

After 24 h, the cell-cultured samples were rinsed with PBS and fixed in 3.7% formaldehyde for 30 min. The samples were stained by *F*-actin staining, in which the samples were immersed in cytoskeleton buffer (5×10^{-3} M sodium chloride (NaCl), 150×10^{-3} M magnesium chloride ($MgCl_2$), 5×10^{-4} M Tris-base and 0.5% Triton X-100) for 5 min at 4 °C, followed by blocking buffer (5% FBS, 0.1% Tween-20 and 0.02% sodium azide (NaN_3) in PBS) for 30 min at 37 °C. The samples were incubated with rhodamine-phalloidin (1:200) and 4′,6-diamidino-2-phenylindole (DAPI) (1:10,000). The samples were mounted on microscope slides using mounting medium and observed by confocal laser scanning microscopy (CLSM700, Zeiss, Oberkochen, Germany).

2.2.7. Cell proliferation

At predesigned time intervals of 1, 3, and 7 days, the cell-cultured samples were rinsed with PBS and cell counting kit-8 (CCK-8) proliferation kit reagents (Dojindo, Japan) were added to the samples. After 1 h incubation, the reagents were carefully transferred to 96-well plates. Optical density was measured using a microplate reader at a wavelength of 450 nm.

2.2.8. Alkaline phosphatase (ALP) activity

At predesigned time intervals of 3, 7, 10, and 14 days, the cell-cultured samples were washed with PBS and then 1× radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% tergitol-type-40 (NP-40), 1 mM ethylenediaminetetraacetic acid (EDTA) including protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin)] was added. The cells in RIPA buffer were sonicated using a Vibra Cell™ instrument (Sonics & Materials Inc., Danbury, CT, USA) for 1 min at 110 W on ice. After sonication, the cell lysates were centrifuged at 13,500 rpm at 4 °C for 3 min to remove cell debris. The supernatants were incubated with *p*-nitrophenyl phosphate solution for 30 min at 37 °C. The reaction was stopped by adding 500 μL of 1 N sodium hydroxide (NaOH). ALP activity was determined by measuring the conversion of *p*-nitrophenyl phosphate to *p*-nitrophenol. Optical density was determined by using a microplate reader at a wavelength of 405 nm.

2.2.9. Calcium concentrations

After 21 days of culture, 0.5 N HCl was added to the cell-cultured samples and the supernatants were used to measure calcium deposition using QuantiChrom Calcium Assay kits (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions. Optical density was determined using a microplate reader at 612 nm.

2.2.10. Quantitative real-time polymerase chain reaction (PCR)

After 21-day incubation, complementary DNA (cDNA) was synthesized on the cell-cultured samples using the Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's directions, using 1 μg total ribonucleic acid (RNA) with oligo (dT). The cDNA was amplified *via* PCR, using an RNA PCR kit (Bioneer Inc., Daejeon, South Korea), according to the manufacturer's instructions. The following oligonucleotide primers were used: osteocalcin (OCN) [(F) 5′-TGA GAG CCC TCA CAC TCC TC-3′, (R) 5′-ACC TTT GCT GGA CTC TGC AC-3′, osteopontin (OPN) [(F) 5′-GAG GGC TTG GTT GTC AGC-3′, (R) 5′-CAA TTC TCA TGG TAG TGA GTT TTC C-3′, GAPDH [(F) 5′-ACT TTG TCA AGC TCA TTT CC-3′, (R) 5′-TGC AGC GAA CTT TAT TGA TG-3′]. The real-time PCR reaction was performed with the above-mentioned specific primers. DyNAmo™ SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland) and PCR amplification and detection were carried out on an ABI7300 Real-Time Thermal Cycler (Applied Biosystems, Foster, CA, USA). The relative levels of OCN and OPN were normalized to those of glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

2.3. In vivo study

2.3.1. Fabrication of implants and experimental animals

Twelve-four implants (7.0 mm in length, 3.5 mm in diameter; Cowell Medi Co., Busan, Korea) were fabricated. All implants were made of pure titanium. The animal study was performed with three experimental groups such as Ti (control group, $n = 8$), BMP-2 (0.75 mg/mL)/Ti (Ex-1 group, $n = 8$), and BMP-2 (0.75 mg/mL)/Hep-Ti (Ex-2 group, $n = 8$). All groups were fabricated with the same methods introduced for the *in vitro* study. To apply BMP-2 coating, each implant was immersed during 12 h in the protein solution (0.75 mg/mL for BMP-2) and freeze dried under sterile conditions (Freeze drying at −40 °C, followed by vacuum drying at maximum 20 °C). This study was carried out with approval from the Ethics Committee on Animal Experimentation of Chun Nam National University (CNU IACUC-TB-2012-10). Three dogs, approximately 13–15 kg in weight, were used for this study and given 2-weeks to acclimatize. The animals were fed a soft-dog food diet and had free access to water.

2.3.2. Teeth extraction

The premolars were extracted and first molars of upper and lower jaws of the animals during the first surgery. They were pre-anaesthetized with atropine sulfate (0.05 mg/kg intramuscular (IM); Dai Han Pharm Co., Seoul, Korea) and maintained with gas anesthesia using isoflurane (Choongwae Co., Seoul, Korea). 1 mL of lidocaine (Yu-Han Co., Gunpo, Korea) with 1:100,000 epinephrine infiltrated into the mucosa at the surgical site. The upper and lower premolars and first molars were separated into the mesial and distal

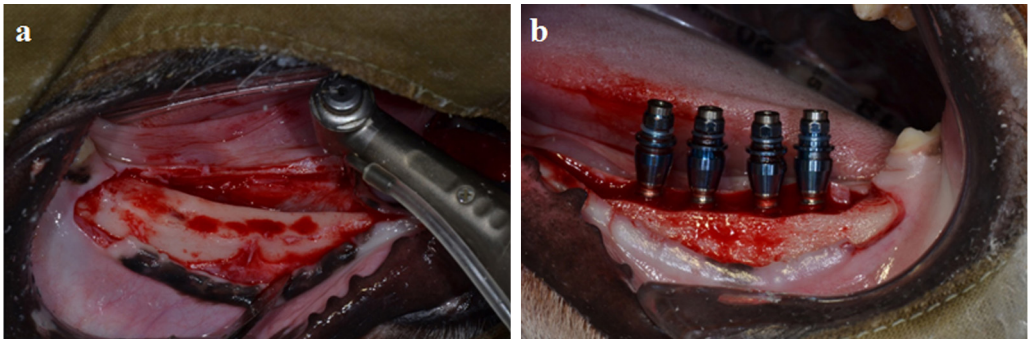

Fig. 1. Implant surgery procedure for the *in vivo* study; (a) the alveolar ridge was trimmed about 1.5 mm to make a flat ridge, (b) and a vertical defect model with a 2.5 mm depth was formed, This model was not bone in 2.5 mm upper portion of implant after implant insertion.

roots. Care was taken to preserve the buccal, lingual, and lateral walls of the alveolar sockets. They were carefully extracted without any damage to the extraction site. The extraction sites were sutured with 4–0 nylon (Mersilk, Ethicon Co., Livingston, UK) to enhance healing. The extraction sites were allowed to heal for 2 months.

2.3.3. Implant surgery

Implant surgery were performed 2 months after complete healing of the extraction sockets. Local and general anesthesia was performed in the same way as for tooth extraction. The experimental implants were installed into the edentulated mandibular alveolar ridge 2 months following the surgical extractions. The alveolar ridge was trimmed about 1.5 mm to make a flat ridge before inserting the implant, and a vertical defect model that had 2.5 mm exposure of implant upper portion was formed. This model was not bone in the 2.5 mm upper portion of implant. (Fig. 1). Each of the four experimental implants was installed on the right and left edentulated mandibular alveolar ridge area using a split-mouth design. Treatments were randomized between left and right jaw quadrants in consecutive animals. Four and a half mm of the implant was placed within the reduced alveolar ridge to the level of the reference line, creating 2.5 mm peri-implant vertical defects. (Fig. 1) The exposed bone was marked at the implant placement sites using a ruler to place the implants at the same location on both sides.

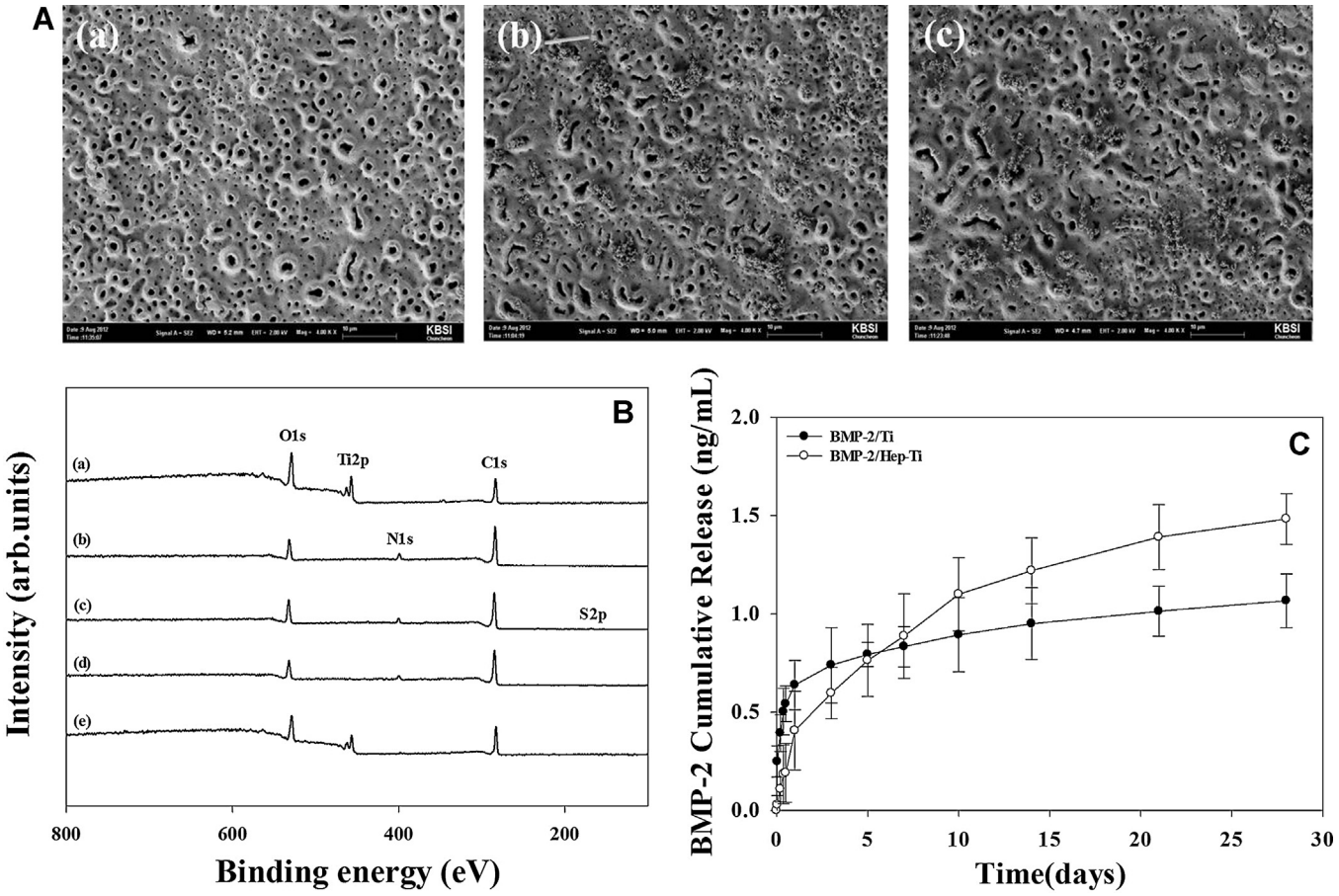

Fig. 2. (A) Scanning electron microscopy (SEM) for the surface morphologies; the control and surface-modified groups had similar morphologies. (a) Ti, (b) BMP-2/Ti, (c) BMP-2/Hep-Ti. (Magnification: ×4000). (B) X-ray photoelectron spectroscopy (XPS) wide-scan spectra of various Ti surfaces: (a) Ti, (b) aminated-Ti, (c) Hep-Ti, (d) BMP-2/Ti, and (e) BMP-2/Hep-Ti. (C) Release kinetics of BMP-2 from BMP-2/Ti and BMP-2/Hep-Ti, respectively.

Table 1
Percentages of C1s, O1s, N1s, Ti2p and S2p expressed at the surfaces of Ti and functionalized Ti with heparin and/or BMP-2 as determined by XPS.

Substrate	Ti2p (%)	C1s (%)	N1s (%)	O1s (%)	S2p (%)
Ti	9.3 ± 0.3	52.3 ± 0.5	1.9 ± 0.1	36.5 ± 0.4	–
Aminated-Ti	0.7 ± 0.1	76.4 ± 1.0	6.2 ± 0.2	16.8 ± 0.6	–
Hep-Ti	0.5 ± 0.1	76.1 ± 0.9	4.7 ± 0.2	18.0 ± 0.4	0.6 ± 0.1
BMP-2/Ti	8.5 ± 0.4	62.5 ± 0.9	3.0 ± 0.1	26.1 ± 0.5	–
BMP-2/Hep-Ti	0.5 ± 0.1	76.4 ± 0.7	5.4 ± 0.4	17.3 ± 0.3	0.4 ± 0.1

2.3.4. Postoperative care and sacrifice

Broad spectrum antibiotics (penicillin G procaine and penicillin G benzathine) were administered immediately after surgery and again after 48 h by intramuscular injection (1 mL/5 kg). Plaque control was maintained by daily flushing of the oral cavity with 2% chlorhexidine gluconate until completion of the study. Observations of the experimental sites with regards to mucosal health, maintenance of suture line closure, edema, and evidence of tissue necrosis or infection were made daily until the sutures were removed. Suture materials were removed 1 week after placement of the implants. The animals were given a soft diet for 2 weeks, followed by a conventional regular diet. The animals were anesthetized and euthanized at 8 weeks post-surgery by intravenous injection of concentrated sodium pentobarbital (Euthasol, Delmarva Laboratories Inc., Midlothian, VA, USA). Following euthanasia, block sections including implants, alveolar bone, and the surrounding mucosa were collected.

2.3.5. Histological analysis

Specimens were fixed in neutral buffered formalin (Sigma Aldrich, St Louis, MO, USA) for 2 weeks and dehydrated in ascending concentrations of 70%, 80%, 90% and 100% ethanol. The dehydrated specimens were embedded in Technovit 7200 resin (Heraeus KULZER, South Bend, IN, USA). The blocks of polymerized specimens were sectioned longitudinally from the center of each implant using an EXAKT diamond cutter (KULZER EXAKT 300, EXAKT, Norderstedt, Germany). Thirty μm final slides were prepared from the initial 400 μm slides by grinding the sections with an EXAKT grinding machine (KULZER EXAKT 400CS, EXAKT). The specimens were stained with hematoxylin and eosin. Images were captured using a computer connected to a light microscope (Olympus BX, Tokyo, Japan) attached to a CCD camera (Polaroid DMC2 digital Microscope camera; Polaroid Corp., Cambridge, MA, USA). All measurements were made using SPOT Software V4.0 (Diagnostic Instrument, Inc., Sterling Height, MI, USA).

The following parameters were assessed:

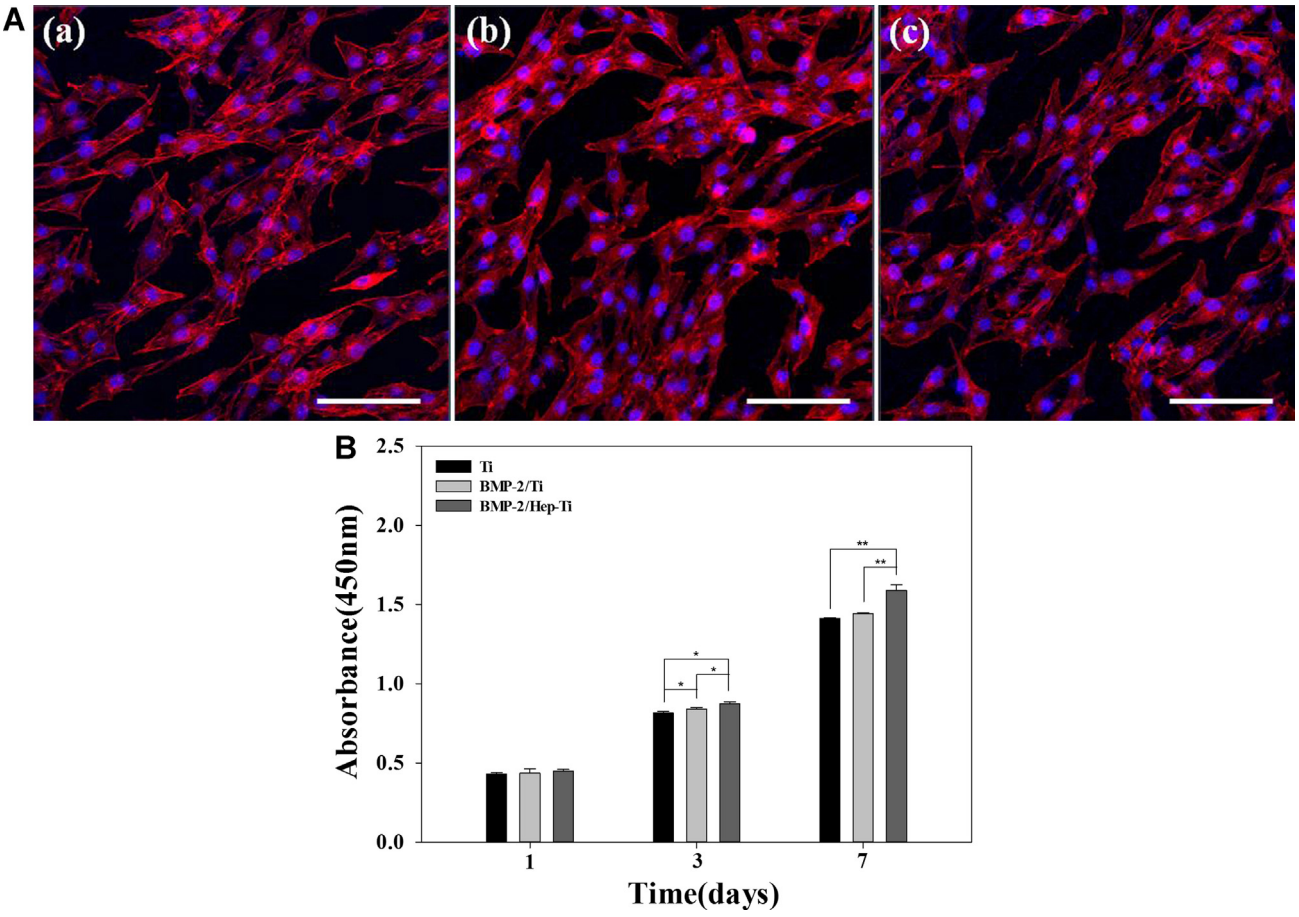

Fig. 3. (A) *F*-actin staining images of MG-63 cells grown on (a) Ti, (b) BMP-2/Ti, and BMP-2/Hep-Ti after 24 h incubation. (B) Cell proliferation of MG-63 cells grown on Ti, BMP-2/Ti, and BMP-2/Hep-Ti after 1, 3, and 7 days of incubation. (* $P < 0.05$ and ** $P < 0.001$).

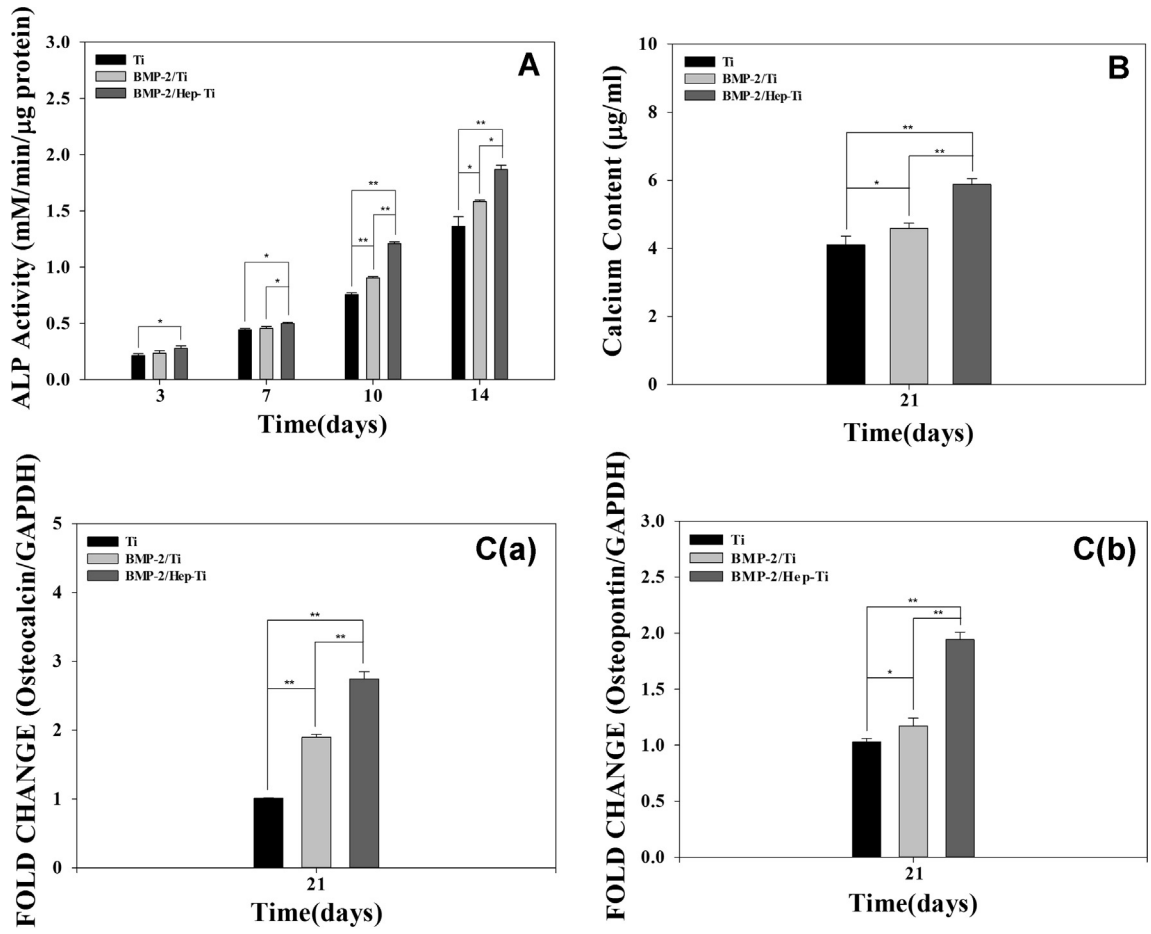

Fig. 4. (A) Alkaline phosphatase (ALP) activity of MG-63 cells grown on Ti, BMP-2/Ti, and BMP-2/Hep-Ti after 3, 7, 10, and 14 days of incubation. ($^{*}P<0.05$ and $^{**}P<0.001$). (B) The amount of calcium deposition by MG-63 cells grown on Ti, BMP-2/Ti, and BMP-2/Hep-Ti after 21 day of incubation. ($^{*}P<0.05$ and $^{**}P<0.001$). (C) Real-time PCR analysis for (a) osteocalcin and (b) osteopontin expression of MG-63 cells grown on Ti, BMP-2/Ti, and BMP-2/Hep-Ti after 21 day of incubation. ($^{*}P<0.05$ and $^{**}P<0.001$).

(A) Bone growth height in defect area (mm).
The length of bone growth that increased upward along the implant from the reference point of the vertical defect site on the alveolar ridge was measured.
(B) Bone to implant contact in defect area around implant (%).
The bone-to-implant contact ratio in the area where the bone grew along the implant from the reference point of the implantation on the alveolar ridge was measured.
(C) Bone to implant contact in the existing bone area around implant (%).
The bone-to-implant contact ratio in the existing bone where the implant was implanted was measured.
(D) Intra-thread bone density in the existing bone area around implant (%).
The intra-thread bone density in the existing bone where the implant was implanted was measured.

The percentage of bone-to-implant contact (BIC, %) was measured, and the ratio of the area of bone formation on intra-threads of the implant to overall threads was calculated to measure intra-thread bone density (ITBD, %). The height level of the newly formed marginal bone induced by the installed implant was measured. Overall specimen images were captured at a magnification of ×12.5. A magnification of ×40 was used for the histometric analysis, and ×100 magnification lens was used for a precise assessment of BIC and ITBD. The criteria for histometric analysis included images that were captured at a magnification of ×100.

2.4. Statistical analysis

Data are presented as means ± standard deviations (SDs). Statistical comparisons were carried out *via* one-way analysis of variance (ANOVA) (Systat Software, Inc., Chicago, IL, USA) for the *in vitro* study. Differences were considered significant at $^{*}P<0.05$ and $^{**}P<0.01$. All analyses the *in vivo* study were performed using a computer-based statistical software (SPSS software, ver.12.0). The mean and SD of BIC and ITBD in the histological specimens were calculated for each group. The Shapiro–Wilk test was performed to test the normal distribution, and a one-way analysis of variance (ANOVA) was performed to compare differences in the BICs, ITBDs, and bone growth of the groups. A Bonferroni test was performed for the post-hoc test with a significance level of 95%.

3. Results

3.1. In vitro study

3.1.1. Surface characterization

We confirmed the surface morphologies in each group by SEM (Fig. 2A). The control and surface-modified groups had similar morphologies. Toluidine blue dye was utilized to quantify the amount of heparin conjugated to the Ti surface. The amount of surface-exposed heparin was 1.5 ± 0.3 μg/sample. XPS analysis was performed to evaluate the elemental chemical compositions of Ti, aminated-Ti, Hep-Ti, BMP-2/Ti, and

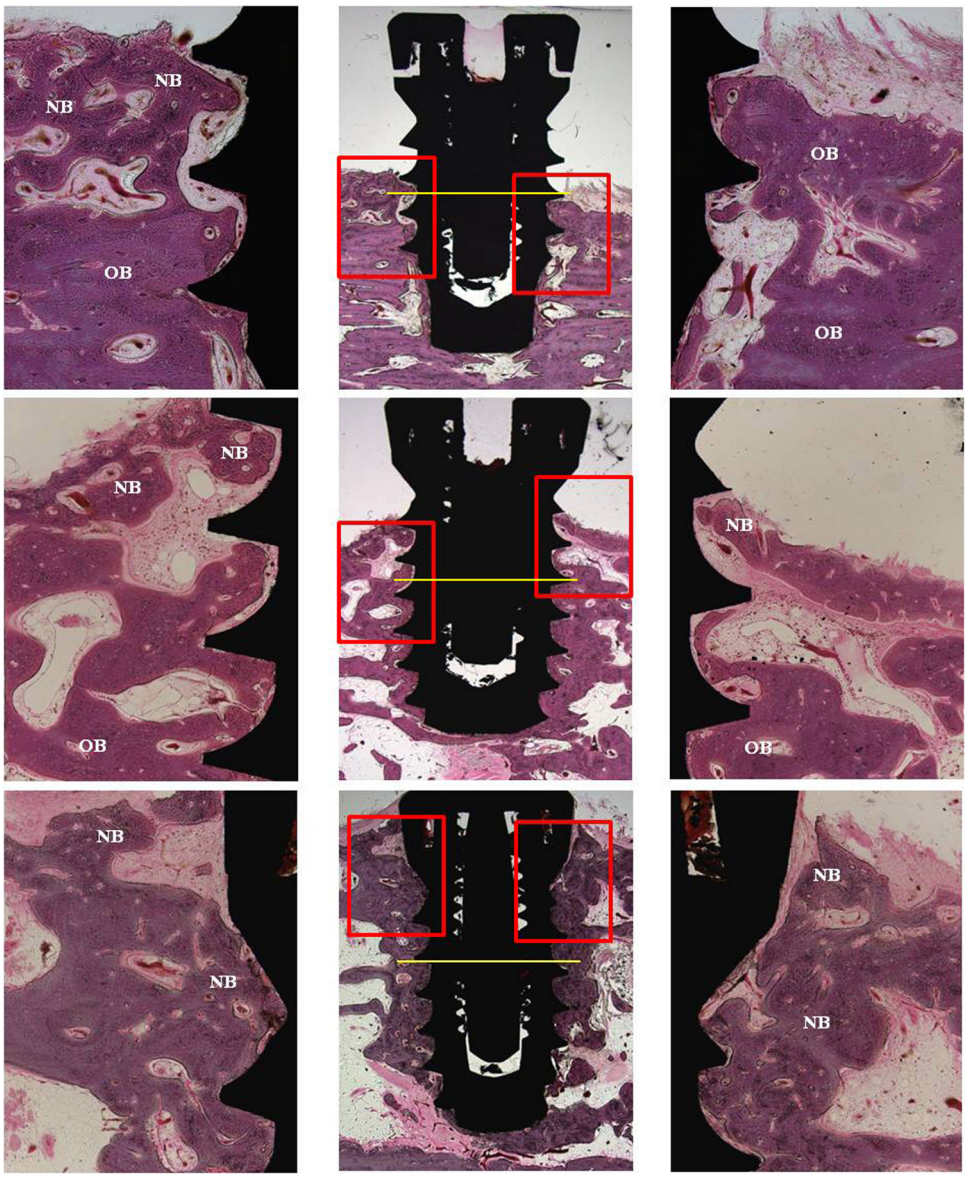

Fig. 5. Histological specimens of the three groups ((a)–(c) Ti, (d)–(f) BMP-2/Ti, (g)–(i) BMP-2/Hep-Ti). Note pronounced peri-implant bone remodeling and vertical bone growth in the BMP-2/Ti and BMP-2/Hep-Ti groups. Yellow lines are the level of implant insertion ((b), (e) and (h) ×12.5 magnification). The histological specimens of the BMP-2/Ti and BMP-2/Hep-Ti groups in the new bone area ((a), (c), (d), (f), (g) and (i) ×40 magnification). Note pronounced peri-implant bone re-modeling and vertical bone growth in the BMP and Hep-BMP groups. In particular, the bone to implant contacts in the Hep-BMP group was detected partially in a new bone area.

BMP-2/Hep-Ti. Fig. 2B and Table 1 show the XPS wide-scan spectra of the Ti, aminated-Ti, Hep-Ti, BMP-2/Ti, and BMP-2/Hep-Ti. Successful DOPA modification on the surface of Ti was assessed by an increase of N1s content from 1.9 ± 0.1% to 6.2 ± 0.2% and a decrease of O1s content from 36.5 ± 0.4% to 16.8 ± 0.6% compared to those of the Ti substrates. Additionally, heparin adhesion on the aminated-Ti surface was confirmed by a decrease in N1s content from 6.2 ± 0.2% to 4.7 ± 0.2% and an increase in S content from 0% to 0.6 ± 0.1% compared to aminated-Ti substrates. Moreover, successful immobilization of BMP-2 on the surface of Ti or heparinized-Ti was demonstrated by an increase in N content compared with Ti or heparinized-Ti (Table 1). The contact angle decreased after surface modification of Ti with DOPA, heparin, and BMP-2 compared to that of Ti. The means ± SDs of the contact angles on Ti, aminated-Ti, heparinized-Ti, BMP-2/Ti, and BMP-2/Hep-Ti were 79.3° ± 3.1, 75.6° ± 7.6, 57.2° ± 6.1, 47.8° ± 4.2, 43.8° ± 3.8, respectively.

3.1.2. *In vitro BMP-2 release test*

At predetermined time intervals, the release kinetics of BMP-2 immobilized on the heparinized-Ti surface was analyzed by ELISA (Fig. 2C). The amount of BMP-2 released on the first day from BMP-2/Ti and BMP-2/Hep-Ti was approximately 6.4 ± 1.3 ng and 4.1 ± 2.0 ng, respectively. Over a release period of 28 days, 10.7 ± 1.4 ng and 14.8 ± 1.3 ng BMP-2 were released from BMP-2/Ti and BMP-2/Hep-Ti, respectively.

3.1.3. *Cell adhesion and cell proliferation*

The adhesion of MG-63 cells was observed after seeding onto the surface of Ti, BMP-2/Ti, and BMP-2/Hep-Ti by *F*-actin staining. Almost all MG-63 cells were alive after 24 h adhesion to the Ti and surface-modified Ti substrates (Fig. 3A). The cell proliferation of MG-63 cells on the surface of each group was examined for 7 days (Fig. 3B). MG-63 cell proliferation cultured on each group increased for the culture period for up to 7 days. There were no significant differences between cells proliferation on each group after 1 day

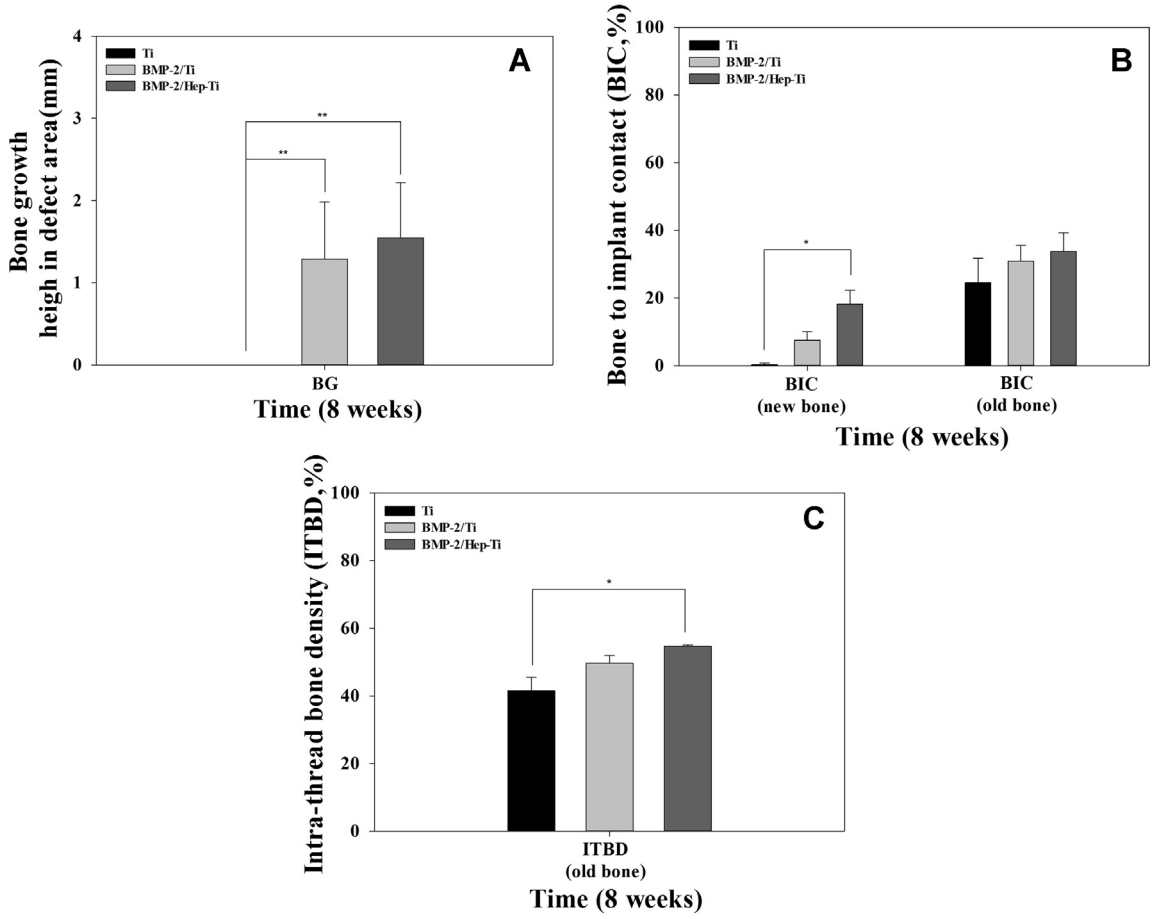

Fig. 6. (A) Vertical bone growth height in the 2.5 mm defect area (mesial and distal area). (B) Bone to implant contact ratio in the new bone area (defect area) and old bone area (existing bone area). (C) Intra-thread bone density (%, bone volume) in old bone area. ($^{*}P < 0.05$ and $^{**}P < 0.001$).

of culture. However, cell proliferation was significantly different between MG-63 cells in Ti substrates containing BMP-2 and in the Ti group ($^{*}P < 0.05$ and $^{**}P < 0.001$) at 3 and 7 days of culture. Significant differences in cells proliferation were observed between the MG-63 cells grown on BMP-2/Ti substrates and those grown on BMP-2/Hep-Ti substrates at 3 and 7 days ($^{*}P < 0.05$ and $^{**}P < 0.001$).

3.1.4. Osteogenic differentiation

The ALP activity of the MG-63 cells was investigated after 3, 7, 10, and 14 days on the surfaces of each group. As shown in Fig. 4A, significant differences were observed between the ALP activity of MG-63 cells cultivated on Ti containing BMP-2 and those cultivated on Ti at the time of analysis ($^{*}P < 0.05$ and $^{**}P < 0.001$). The ALP activity of MG-63 cells cultured on the BMP-2/Hep-Ti was significantly higher than those cultured on Ti at throughout the culture period ($^{*}P < 0.05$ and $^{**}P < 0.001$). MG-63 cells grown on BMP-2/Hep-Ti had significantly higher ALP activity than those grown on BMP-2/Ti for 7, 10, and 14 days ($^{*}P < 0.05$ and $^{**}P < 0.001$).

As shown in Fig. 4B, the amount of calcium deposited by MG-63 cells grown on each group was investigated after 21 days of culture. The results of the amount of calcium deposition clearly show that MG-63 cells cultivated on the Ti containing BMP-2 had significantly higher amounts than those cultivated on Ti ($^{*}P < 0.05$ and $^{**}P < 0.001$). Significant differences were observed in calcium deposition between cells cultured on BMP-2/Hep-Ti and those cultured on BMP-2/Ti ($^{**}P < 0.001$).

The results of OCN and OPN mRNA expression by MG-63 cells were assessed by real-time PCR after 21 day incubation. As shown in Fig. 4C-a and -b, significant differences were observed between OCN and OPN gene expression by MG-63 cells grown on the Ti containing BMP-2 and those grown on Ti ($^{*}P < 0.05$ and $^{**}P < 0.001$). The OCN and OPN expression by MG-63 cells cultivated on BMP-2/Hep-Ti was significantly greater than those cultivated on BMP-2/Ti ($^{**}P < 0.001$).

3.2. In vivo study

3.2.1. Histological findings

Jaw quadrants in the BMP-2/Ti and BMP-2/Hep-Ti groups exhibited bone formation approaching the first thread and bone remodeling around the implants (Fig. 5). Vertical bone loss was detected in some Ti group specimens. The Ti group showed failure of osseointegration in the defect area between the fixture and the bone (Fig. 5a and c). The BIC in the new bone area of BMP-2/Ti and BMP-2/Hep-Ti was significantly greater than that of Ti ($^{*}P < 0.05$), and no significant difference was observed between BMP-2/Ti and BMP-2/Hep-Ti ($^{*}P > 0.05$). The BMP-2/Hep-Ti showed successful bone regeneration in the supra-defect area (Fig. 6A). The peri-implant bone re-modeling and vertical bone growth was detected in BMP-2/Ti and BMP-2/Hep-Ti. The BIC and ITBD within the old bone area at 8 weeks after surgery were not different between the groups (Fig. 6B). No significant differences were observed between the groups between the intra-thread bone density of all groups in old bone area (Fig. 6C). Pronounced peri-implant bone re-modeling and vertical bone growth was observed in the BMP-2/Ti and BMP-2/Hep-Ti groups (Fig. 6A and B). In particular, bone to implant contacts was detected partially in the new bone area of the BMP-2/Hep-Ti group (Fig. 6B).

4. Discussion

We modified Ti substrates with DOPA to prepare the Hep-Ti substrates, and heparin was conjugated by an EDC-mediated reaction between the carboxylic groups of heparin and the amine groups of DOPA on the surface of the Ti substrates. DOPA modifies the surfaces of inorganic, organic, and metallic substrates due to the *ortho*-dihydroxyphenyl (catechol) functional group in dopamine (Fan, Lin, Dalsin, & Messersmith, 2005; Lee, Scherer, & Messersmith, 2006; Lee, Dellatore, Miller, & Messersmith, 2007). Our previous studies demonstrated that Ti surfaces modified with DOPA easily immobilize heparin (Kim et al., 2013; Lee et al., 2012). BMP-2 was sequentially immobilized onto the heparinized-Ti surfaces through electrostatic interactions between positively charged BMP-2 and negatively charged heparin. Ti and surface-modified Ti substrates were characterized by XPS and contact angle measurement, indicating that the surface-modified Ti substrates were successfully prepared.

Heparin was used in this study to create an effective delivery system for growth factors and ensure release for a sufficient time period and in appropriate amounts. Heparin is composed of many sulfate and carboxyl groups. Heparin also has good binding affinities for various growth factors, such as VEGF, bFGF, transforming growth factor-β (TGF-β), and BMP-2 (Im & Lee, 2010; Kim et al., 2011; Sasisekharan et al., 1997; Seif-Naraghi, Horn, Schup-Magoffin, & Christman, 2012). Our release study showed sustained release of BMP-2 from Hep-Ti with no high initial burst release pattern compared to that from Ti, due to the strong electrostatic interactions between the highly negatively charged heparin and the highly positively charged BMP-2.

The clinical success of dental implants is highly dependent on cell adhesion, matrix production, anti-inflammatory, and mineralization properties of the implant materials. In the present *in vitro* and *in vivo* studies, we observed an increase in osteoblast proliferation when osteoblasts were cultured on all substrates. Cell proliferation on the Ti substrate containing BMP-2 increased significantly compared to that on Ti. Moreover, to confirm the osteogenic differentiation of MG-63 cells on all substrates, we evaluated ALP activity and calcium deposition. ALP activity and calcium deposition are widely used as markers for early and late differentiation of osteoblast cells, respectively (Turksen, Bhargava, Moe, & Aubin, 1992; van den Beucken et al., 2006), which indicate that BMP-2 stimulates early and late osteoblast differentiation. We also investigated the osteogenic differentiation of MG-63 cells on all substrates by measuring OCN and OPN gene expression using real time-PCR. OCN and OPN gene expression levels by MG-63 cells cultured on Ti containing BMP-2 substrates were significantly higher than those on Ti substrates. Significant differences were observed in OCN and OPN gene expression of MG-63 cells grown on BMP-2/Hep-Ti and those on BMP-2/Ti. Previous studies have reported that OCN and OPN gene expression is upregulated on the substrates with BMP-2 compared to substrates without BMP-2.

If the timing and degeneration rate of the BMP-2 used to coat the implant could be controlled, a more favorable outcome would be expected. How long such growth factors should be released when an implant is coated with a bioactive material can be ascertained by further studies, but the potential of implants coated with a bioactive material is believed to be great.

5. Conclusions

A Ti surface was successfully functionalized *via* a chemical method involving conjugation of heparin and subsequent immobilization of BMP-2 on the surface of heparinized Ti through electrostatic interactions. The BMP-2 released from BMP-2/Hep-Ti showed sustained BMP-2 release profiles compared to that from BMP-2/Ti. We demonstrated that BMP-2/Hep-Ti substrates significantly enhanced osteogenic differentiation from the results of ALP activity, calcium deposition, and gene expression levels compared to those of Ti substrates. BMP-2/Hep-Ti substrates induced new bone formation at the defect area. Thus, a BMP-2/Hep-Ti substrate seemed to be a suitable delivery system to improve osteoblast function and new bone formation at defect areas around implants.

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