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Controlled release of BMP-2 using a heparin-conjugated carrier system reduces *in vivo* adipose tissue formation

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Abstract: There is growing concern about unwanted effects associated with the clinical use of recombinant human bone morphogenetic protein-2 (rhBMP-2) at high concentrations, including cyst-like bone formation and excessive fatty marrow formation. We, therefore, evaluated the induction of mineralized/adipose tissue formation and the bone-healing pattern associated with the controlled release of *E. coli*-derived rhBMP-2 (ErhBMP-2) by a heparin-conjugated fibrin (HCF) system using ectopic and orthotopic *in vivo* models, respectively. In the ectopic transplantation model, mineralized tissue formed at the most superficial layer of the transplanted area and on the surfaces of grafted materials, and most of the interstitial space within the transplanted area was filled with excessive adipose tissue specifically at sites that received ErhBMP-2. However, sites that received ErhBMP-2 and HCF showed significantly increased mineralized tissue formation and decreased adipose tissue formation compared to the normal fibrin system with ErhBMP-2. In the orthotopic (calvarial defect) model, controlled release of ErhBMP-2 induced by HCF significantly reduced adipose tissue formation within the defect area compared to the clinically approved absorbable collagen sponge. From these results, it can be concluded that the use of a HCF system loaded with ErhBMP-2 may reduce adipose tissue formation and enhance mineralized tissue formation. © 2014 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 00A:000–000, 2014.

Key Words: bone morphogenetic protein, bone regeneration, bone tissue engineering, *in vivo* test, adipogenesis

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INTRODUCTION

Early studies on the activities of bone morphogenetic protein-2 (BMP-2) focused only on its involvement in the formation of mineralized tissue in various experimental models,[1–3] but it has recently been shown that recombinant human BMP-2 (rhBMP-2) also induces the formation of other types of tissue. Song et al.[4] found that rhBMP-2 not only enhanced mineralized tissue formation by mesenchymal stem cells but also induced the formation of adipose tissue. Zara et al.[5] also demonstrated that the bone-regeneration process could be varied by the dose of rhBMP-2 applied to defects; sites receiving a high concentration of rhBMP-2 exhibited cyst-like bone marrow tissue with an abundance of adipose tissue, while denser bone formed at sites that received a lower concentration of rhBMP-2.

In other recent preclinical and clinical studies of sinus augmentation,[6,7] similar tissue reactions to rhBMP-2 were observed: marrow tissue formation at the central area of the graft site, and mineralized tissue formation at the periphery. This could be explained by two sides of effects associated with the presence of rhBMP-2 at a high concentration in the grafted sinus: (1) the induction of bone formation from the Schneiderian membrane and (2) seroma and adipose tissue formation as part of the healing process.[6] Even though the adipose tissue formation induced by rhBMP-2 cannot be considered a negative effect, this

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peculiar type of regenerated tissue can cause clinical problems due to the concomitant reduced bone density.

Tissue engineering using growth factors such as rhBMP-2 is based on mimicking developmental processes that occur naturally at the cellular level: differentiation, proliferation, and migration.[8] However, the concentrations of rhBMP-2 used in both research and clinical settings tend to be markedly higher than the endogenous BMP-2 concentration.[3] In addition, most of the carrier systems for BMP-2 produce a burst release of growth factor, resulting in an instant and explosive increase in concentration in the early healing phase, with a subsequent decrease in biologic activity in the later phase.[9–11] The initial burst increase in the concentration of rhBMP-2 in the early healing phase may modify the natural healing processes in various ways, sometimes with unwanted or abnormal complications. Wikesjö and coworkers[12] reported the occurrence of abnormal extensive swelling or seroma formation at some sites receiving rhBMP-2, despite the eventual enhanced bone formation at most experimental sites.[13] Therefore, optimum carrier systems with a spontaneous and controlled release profile should be developed for the field of tissue engineering, to encompass the handling advantages and control the complications associated with grafting with rhBMP-2.

Various carrier systems have already been designed to this end: collagen sponge/gel, gelatin, hydroxyapatite, poly-L-lactic acid scaffold, fibrin gel, hyaluronic acid, microsphere, and the heparin-conjugated systems.[14] Among these, the recently developed microspheres[15,16] and heparin-conjugated systems[17–19] have focused on the slow/controlled release of growth factor. Heparin-conjugated systems mimic the physiologic role of heparin-regulating growth factors by binding to proteins, including vascular endothelial growth factor, fibroblast growth factor, and BMP-2.[20] Previous studies have demonstrated that heparin-conjugated fibrin (HCF) systems enable a slower and more controlled release of rhBMP-2 compared to normal fibrin,[21] and even compared to absorbable collagen sponge (ACS).[22] This could increase bone formation markers *in vitro*, and produce extensive bone formation *in vivo* with reduced concentrations of rhBMP-2 that produce only limited bone formation when using conventional carrier systems. It may, therefore, be expected that clinical use of a heparin-conjugated carrier system may enable a reduction in the minimum concentration of rhBMP-2 in grafts to almost physiologic levels, thus reducing the induction of unwanted healing process, such as adipose tissue formation, that would occur with higher rhBMP-2 concentrations.

The aims of this study were twofold: (1) to elucidate the effects of controlled release of rhBMP-2 by a heparin-conjugated carrier system on the ectopic induction of mineralized/adipogenic tissue formation by rhBMP-2 in an *in vivo* ectopic transplantation model, and (2) to quantify the bone-healing processes in an orthotopic (critical-sized calvarial bone defect) model using carrier systems with different release profiles and loaded with rhBMP-2 at various concentrations.

MATERIALS AND METHODS

Expression of rhBMP-2 in *Escherichia coli*

The rhBMP-2 used in this study was provided by the Research Institute of Cowellmedi (Pusan, Korea). The rhBMP-2 was expressed in *E. coli* (ErhBMP-2), as described previously.[2] The total RNA of human osteosarcoma cells was reverse transcribed. Complementary DNA for BMP-2 protein was amplified using the polymerase chain reaction and subcloned into a pRSET(A) vector (Invitrogen, Paisley, UK). The subcloned vector [pRSET(A)/hBMP-2] was transformed to the *E. coli* BL21(DE3) strain, and high-cell-density cultivation of *E. coli* was accomplished using a bioreactor (KoBioTec, Incheon, Korea), as described by Tabandeh.[23] The cultured biomass was harvested and crushed twice in a French press, and then centrifuged. Following resuspension and centrifugation, the inclusion bodies (pellets) were resuspended in a solubilization buffer [6-*M* guanidine-HCl, 0.1-*M* Tris-HCl (pH 8.5), 0.1-*M* dithiothreitol, and 1-m*M* EDTA] and incubated overnight at room temperature with constant stirring.

The solubilized ErhBMP-2 was dimerized by incubation in a renaturation buffer [0.5-*M* guanidine-HCl, 50-m*M* Tris-HCl (pH 8.5), 0.75-*M* 2-(cyclohexylamino) ethanesulfonic acid, 1-*M* NaCl, 5-m*M* EDTA, and 3-m*M* total glutathione] for 72 h. Active ErhBMP-2 (dimer) was purified with a Heparin Sepharose 6 Fast Flow column (GE Healthcare, IL), and eluted/separated via a stepped NaCl gradient (0.15, 0.3, and 0.5 *M*).

Preparation of heparin-conjugated fibrinogen loaded with ErhBMP-2

The HCF gel that was used in this study as the ErhBMP-2 carrier system was provided by Professor Byung-Soo Kim from the School of Chemical and Biological Engineering, Seoul National University, Seoul, Republic of Korea. The HCF was incorporated as described previously.[21,22] Briefly, 100 mg of heparin (molecular weight = 4000–6000; Sigma) was dissolved in a 100-mL buffer solution (pH 6) containing 0.05-*M* 2-morpho-linoethanesulfonic acid (Sigma-Aldrich, St. Louis). *N*-hydroxysuccinimide (0.04 m*M*; Sigma) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (0.08 m*M*; Sigma) were added to the solution to activate the heparin's carboxylic acid groups. After allowing the chemical reaction to proceed overnight at 48°C, the solution was stirred to produce a homogeneous state, and the product was lyophilized. Fibrinogen (100 mg) was dissolved in 20 mL of phosphate-buffered saline (PBS; pH 7.4) at 48°C, and reacted with the activated carboxyl acid groups of the heparin (60 mg) under the same conditions for 3 h. The product was lyophilized again, and the white powder product was completely dissolved in PBS and dialyzed over 1 day through a porous membrane bag to remove any residual heparin. The heparin-conjugated fibrinogen was then lyophilized, and HCF was formed by mixing it (40 mg/mL) with normal fibrinogen (60 mg/mL) together with factor XIII, aprotinin (100 KIU/mL), calcium chloride (6 mg/mL), and thrombin (500 IU/mg). The ErhBMP-2 was loaded onto the HCF by adding the prefabricated ErhBMP-2 solutions at the required concentrations to the HCF during the final mixing process.

Animals

Twenty-five immunodeficient mice (body weight 20–25 g) and 50 male Sprague-Dawley rats (200–300 g) were used for this study. Throughout the experimental period the animals were housed in plastic cages in a room with a 12-h day/night cycle at 21°C, and with *ad libitum* access to water and a laboratory pellet diet. The animal selection, management, surgical protocols, and preparation procedures were approved by the Institutional Animal Care and Use Committee, Yonsei Medical Center, Seoul, Korea.

Subcutaneous transplantation into an ectopic model

The experimental materials were implanted subcutaneously into both the right and left dorsal regions of 25 immunodeficient mice, which were divided randomly into the following five groups ($n = 5$ per group):

- BCP Control: biphasic calcium phosphate (BCP) particles only.
- Fibrin/BMP(–): normal fibrin-coated BCP without ErhBMP-2.
- Fibrin/BMP(+): normal fibrin-coated BCP with ErhBMP-2.
- HCF/BMP(–): HCF-coated BCP without ErhBMP-2.
- HCF/BMP(+):HCF-coated BCP with ErhBMP-2.

A normal fibrin system (Tissel, Baxter Healthcare, Deerfield, IL) was used as a control carrier, and ErhBMP-2 was loaded onto the HCF and control fibrin carriers at a dose of 0.01 mg of ErhBMP-2 in a total volume of 200 μL for ectopic transplantation. BCP particles (500–1000 μm in diameter, 80 mg; MBCP, Biomatlante, Vigneux, France), which were used as a space-maintaining scaffold in this model, were mixed with each experimental material before solidification. The experimental animals received one of the five different types of biomaterial depending on the experimental group to which they had been randomly allocated. The animals were sacrificed 8 weeks after the surgery, and the implanted material, including the surrounding tissues, was harvested and immediately fixed in 4% buffered formalin for 10 days.

Implantation in a critical-sized orthotopic (calvarial defect) model

HCF implants were constructed by solidifying them in a cylindrical mold, as described previously.[24] The thus-formed disk-shaped HCF implant was 3-mm thick and 8 mm in diameter, with a total volume of 200 μL, which included 50 μL of ErhBMP-2 solution at one of the various concentrations. ACS (Collacote, Zimmer Dental, Carlsbad, CA), which is currently the only carrier system for rhBMP-2 that has been approved by the US Food and Drug Administration, was used as a control carrier system in this model. A piece of ACS (3-mm thick and 8 mm in diameter) was soaked for 30 min with 50 μL of ErhBMP-2 solution at one of various concentrations (depending on the experimental group) and 50 μL of buffer solution. Fifty male rats were randomized into the following ten groups ($n = 5$ animals per group): (1) ACS control without ErhBMP-2; (2) ACS loaded with 0.0025 mg, (3) 0.005 mg, (4) 0.01 mg, or (5) 0.02 mg of ErhBMP-2; (6) HCF control without ErhBMP-2; and (7) HCF loaded with 0.0025 mg, (8) 0.005 mg, (9) 0.01 mg, or (10) 0.02 mg of ErhBMP-2.

The animals were anesthetized by intramuscular injection (5 mg/kg) of a 4:1 solution of ketamine hydrochloride (Ketalar, Yuhan, Seoul, Korea) and xylazine (Rompun, Bayer Korea, Seoul, Korea). The surgical sites were shaved and scrubbed with iodine. Standardized calvarial defects were initiated by performing an incision in the middle of the cranium, and elevation of a full-thickness flap to expose the calvarial bone. A standardized, circular, 8-mm-diameter transosseous defect was created on the cranium with a trephine drill (Dentium, Seoul, Korea). After removal of the trephined calvarial disk, the prepared ACS and HCF implants, with or without ErhBMP-2 at various concentrations, were placed according to the group allocation of individual animals. All surgical sites were sutured for primary closure with 4-0 Monosyn (Glyconate absorbable monofilament, B-Braun, Aesculap, PA). The animals were sacrificed at 8 weeks after the experimental surgery by anesthetic overdose. Block sections including the experimental site and the surrounding tissues were removed and immediately fixed in 10% buffered formalin for 10 days.

Radiographic observation

Microcomputed tomography (Skyscan 1072, Skyscan, Aartselaar, Belgium) images of these block specimens were taken at a resolution of 35 μm (achieved using 100 kV and 100 μA). The scanned CT images were processed in DICOM format and three-dimensionally (3D) reconstructed with PC-based software (On-Demand3D, Cybermed, Seoul, Korea). Before 3D reconstruction, radiopaque areas that were suspicious of newly formed bone, and which was distinguishable from the preexisting bone defect margin due to its different radiopaque density, were identified at all sections. The area and volume of the identified areas were calculated from the 3D reconstructed images.

Histologic/histomorphometric analysis

In the ectopic transplantation model, the fixed samples were decalcified with 5% EDTA and 4% sucrose, and dehydrated using a graded ethanol series. The specimens were embedded in paraffin and cut at the central-most section at a thickness of 5 μm. Histologic analyses were performed on hematoxylin/eosin (H-E)-stained slides, and images were digitally acquired using light microscopy (BX50, Olympus, Tokyo, Japan). Histomorphometric analyses were performed on the obtained images using a PC-based image-analysis system (Image Pro Plus, Media Cybernetics, Silver Spring, MD). The total transplanted area (in mm^2) was measured by identifying the newly formed bone/grafted biomaterials or outer fibrous connective tissue capsulation. The area of residual transplanted materials was measured, and

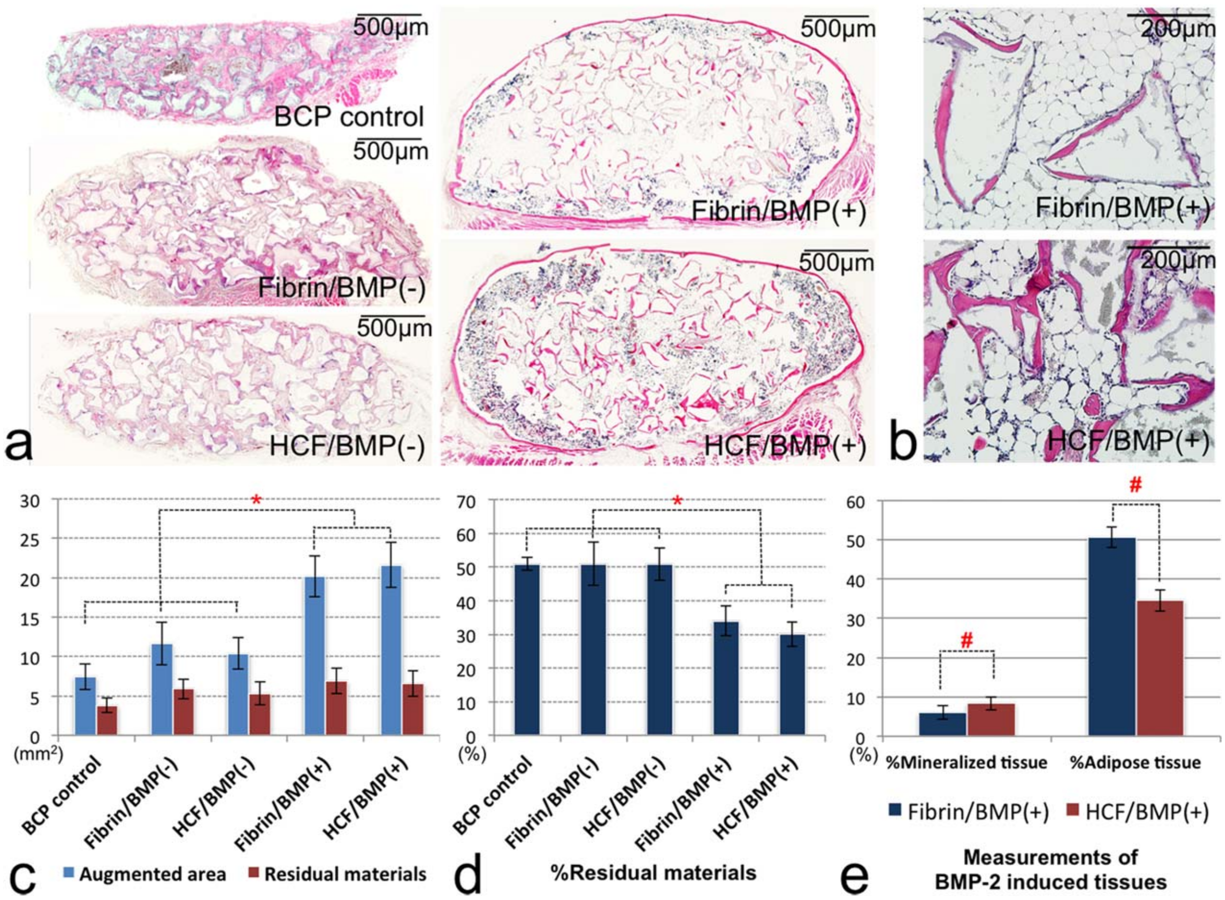

FIGURE 1. Histologic views with low magnification (A) demonstrate different appearances between ectopically transplanted sites received with and without ErhBMP-2. Significantly increased size of the transplanted area, mineralized tissue at the most superficial layer of the transplanted and on the surfaces of residual graft materials, and excessive adipose tissue within interstitial space can be observed at sites that received ErhBMP-2, but small size of cross-sectional area, and residual materials encapsulated by dense connective tissue without any mineralized/adipose tissue at sites without ErhBMP-2. Histologic views with high magnification (B) reveal more thickened mineralized tissue on the surface of materials at sites that received HCF and ErhBMP-2 compared to normal fibrin and ErhBMP-2. Results of histomorphometric analyses show statistical difference in augmented area (C) and proportion of residual materials in total augmented area (D). Comparison of BMP-2-induced specific tissues (mineralized and adipose tissue) between HCF and normal fibrin with ErhBMP-2 reveals statistically significant reduction of adipose tissue formation and increase of mineralized tissue (E). BCP control, biphasic calcium phosphate (BCP) particles only; Fibrin/BMP(–), normal fibrin-coated BCP without ErhBMP-2; Fibrin/BMP(+), normal fibrin-coated BCP with ErhBMP-2; HCF/BMP(–), HCF-coated BCP without ErhBMP-2; HCF/BMP(+), HCF-coated BCP with ErhBMP-2. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

calculated as a proportion of the total transplanted area. At the sites of the specimens that received ErhBMP-2, the percentage of the area occupied by BMP-2-induced specific tissues (mineralized or adipose tissues) relative to the total transplanted area was calculated.

In the calvarial defect model, harvested specimens were decalcified in 5% formic acid for 14 days and then embedded in paraffin. Serial sections were cut at a thickness of 5 mm through the center of the circular calvarial defects. From each of the harvested blocks, the three central-most sections were selected for staining with H-E (two histologic slides) and Masson's trichrome (one histologic slide). Histologic observation was performed with the aid of light microscopy, and the following histomorphometric measurements were made: augmented area (in mm^2), identified as newly formed woven bone and grafted materials within the defect; and the proportions of the total augmented area occupied by each composite (i.e., newly formed bone, fibrovascular/connective tissue, and adipose tissue; in %).

Statistical analysis

Histomorphometric and radiographic measurements in both experimental models were used to calculate group means and standard deviations based on animal means. One-way analysis of variance (ANOVA) was used to compare the measured parameters between the experimental groups in the ectopic transplantation model. However, the Mann–Whitney *U* test was selectively used to compare BMP-2-induced specific tissues (i.e., adipose and mineralized tissue) between the Fibrin/BMP(+) and HCF/BMP(+) groups. Two-way ANOVA was used to analyze the effect of type of carrier (i.e., HCF vs. ACS) and applied dose of ErhBMP-2 on both the histomorphometric and radiographic analyses in the calvarial defect model. The *post hoc* Bonferroni test was used to analyze the

TABLE I. Results of Histomorphometric Analyses in Both *In Vivo* Experimental Models

	Ectopic Transplantation Model				
Group	Augmented Area	Residual Material Area	%Residual Material	%Mineralized Tissue	%Adipose Tissue
BCP control	7.44 ± 1.63[a,b]	3.81 ± 0.95	50.90 ± 1.91[a,b]	–	–
Fibrin/BMP(–)	11.62 ± 2.70[a,b]	5.88 ± 1.24	50.97√6.39[a,b]	–	–
HCF/BMP(–)	10.38 ± 2.03[a,b]	5.33 ± 1.47	50.89 ± 4.87[a,b]	–	–
Fibrin/BMP(+)	20.16 ± 2.59	6.91 ± 1.60	34.03 ± 4.45	6.06 ± 1.69[b]	34.55 ± 2.55[b]
HCF/BMP(+)	21.62 ± 2.86	6.57 ± 1.65	30.07 ± 3.63	8.36 ± 1.71	50.57 ± 2.71

Calvarial Defect Model							
		Radiographic Analyses		Histomorphometric Analyses			
Carrier	Dose of rhBMP-2	Bone Regeneration Area	Bone Regeneration Volume	Augmented Area	%Newly Formed Bone	%FVCT	%Adipose Tissue[d]
ACS	0	9.41 ± 2.86	12.57 ± 1.81	4.06 ± 2.90	42.57 ± 29.76	54.00 ± 34.01	1.39 ± 3.11
	0.0025	57.38 ± 9.66[c]	55.93 ± 3.51[c]	8.37 ± 2.32[c]	69.16 ± 10.40[c]	16.29 ± 7.14[c]	14.55 ± 7.92[c,d]
	0.005	54.22 ± 1.96[c]	37.84 ± 4.05[c]	8.75 ± 2.50[c]	74.83 ± 7.90[c]	9.80 ± 2.99[c]	15.37 ± 5.23[c,d]
	0.01	47.31 ± 13.44[c]	35.49 ± 9.38[c]	7.73 ± 3.89[c]	69.57 ± 8.14[c]	18.23 ± 9.28[c]	12.20 ± 6.95[c,d]
	0.02	44.78 ± 13.74[c]	34.79 ± 6.90[c]	7.65 ± 1.17[c]	72.26 ± 16.55[c]	14.93 ± 16.14[c]	12.81 ± 2.92[c]
HCF	0	10.09 ± 1.09	13.48 ± 5.60	3.09 ± 1.28	49.83 ± 19.41	44.15 ± 16.65	0.00 ± 0.00
	0.0025	50.86 ± 6.60[c]	30.92 ± 6.21[c]	6.84 ± 1.46[c]	70.53 ± 7.52[c]	25.44 ± 10.03[c]	3.05 ± 3.68[c]
	0.005	55.74 ± 5.82[c]	42.33 ± 8.44[c]	7.11 ± 2.18[c]	70.00 ± 11.98[c]	20.66 ± 10.49[c]	9.09 ± 8.49[c]
	0.01	49.05 ± 8.78[c]	33.42 ± 7.96[c]	8.91 ± 2.72[c]	63.90 ± 13.39[c]	26.97 ± 12.04[c]	7.53 ± 7.91[c]
	0.02	59.41 ± 6.76[c]	38.31 ± 12.84[c]	9.65 ± 1.93[c]	67.55 ± 7.04[c]	18.01 ± 13.37[c]	13.73 ± 6.60[c]

[a]Significantly different from Fibrin/BMP(+).
[b]Significantly different from HCF/BMP(+).
[c]Significantly different from group of the same carrier without loading of ErhBMP-2.
[d]Significantly different between groups that received ACS and HCF.

differences between the groups in both experimental models. The level of significance was set at 5%.

RESULTS

The ectopic transplantation model

The healing during the postoperative observation period was uneventful in all of the experimental animals. There were noticeable differences in the histologic appearance between transplanted sites that had received and not received ErhBMP-2 [Fig. 1(A)]. At sites transplanted with ErhBMP-2, a thin band of newly formed bone tissue surrounded the transplanted biomaterials, regardless of the type of carrier, and the bony layer appeared to segregate the transplanted implant from the recipient connective tissue. Conversely, sites without ErhBMP-2 exhibited congregated residual biomaterials encapsulated by dense connective tissue that could be distinguished from the recipient loose connective tissue. No mineralized or adipose tissues could be observed in specimens that had not been treated with ErhBMP-2.

The transplanted area appeared to be larger and the residual biomaterial particles were more dispersed at sites that had received ErhBMP-2 compared to those without ErhBMP-2. In both the Fibrin/BMP(+) and HCF/BMP(+) groups, the inner area of the transplanted sites was filled with adipose tissue, residual BCP particles, and mineralized tissue. However, most of residual fibrin or HCF could not be found. The mineralized tissue had formed on the residual biomaterial particles, and cuboidal cells could be observed linearly around mineralized tissue or densely studded in the spaces between adipose cells [Fig. 1(B)]. At the other sites that had not been treated with ErhBMP-2, residual biomaterial particles were observed surrounded by dense connective tissue with densely accumulated fibroblast-like cells; multinucleated cells could also be observed in some areas.

Histomorphometric measurements (Table I and Fig. 1) of the total transplanted area revealed a significantly increased size of ErhBMP-2-induced tissues compared to the transplanted tissues at sites without ErhBMP-2 ($p < 0.001$): 20.16 ± 2.59, 21.62 ± 2.86, 11.62 ± 2.70, 10.38 ± 2.03, and 7.44 ± 1.63 mm^2 (mean ± SD) in the Fibrin/BMP(+), HCF/BMP(+), Fibrin/BMP(–), HCF/BMP(–), and BCP Control groups, respectively. The measured areas of residual materials were similar within the transplanted area in all groups, but the proportion of residual materials was significantly decreased at the two experimental sites that received ErhBMP-2 [$p < 0.001$; Fig. 1(D)]. However, measurements of mineralized and adipose tissues could be performed on the histologic sections only at those sites that received ErhBMP-2, due to the absence of these tissues at sites without ErhBMP-2. The proportions of mineralized tissue at the total transplanted site were 8.36 ± 1.71% and 6.06 ± 1.69% at sites that received HCF and the fibrin carrier loaded with ErhBMP-2, respectively; the corresponding values for adipose tissue were 34.55 ± 2.55% and

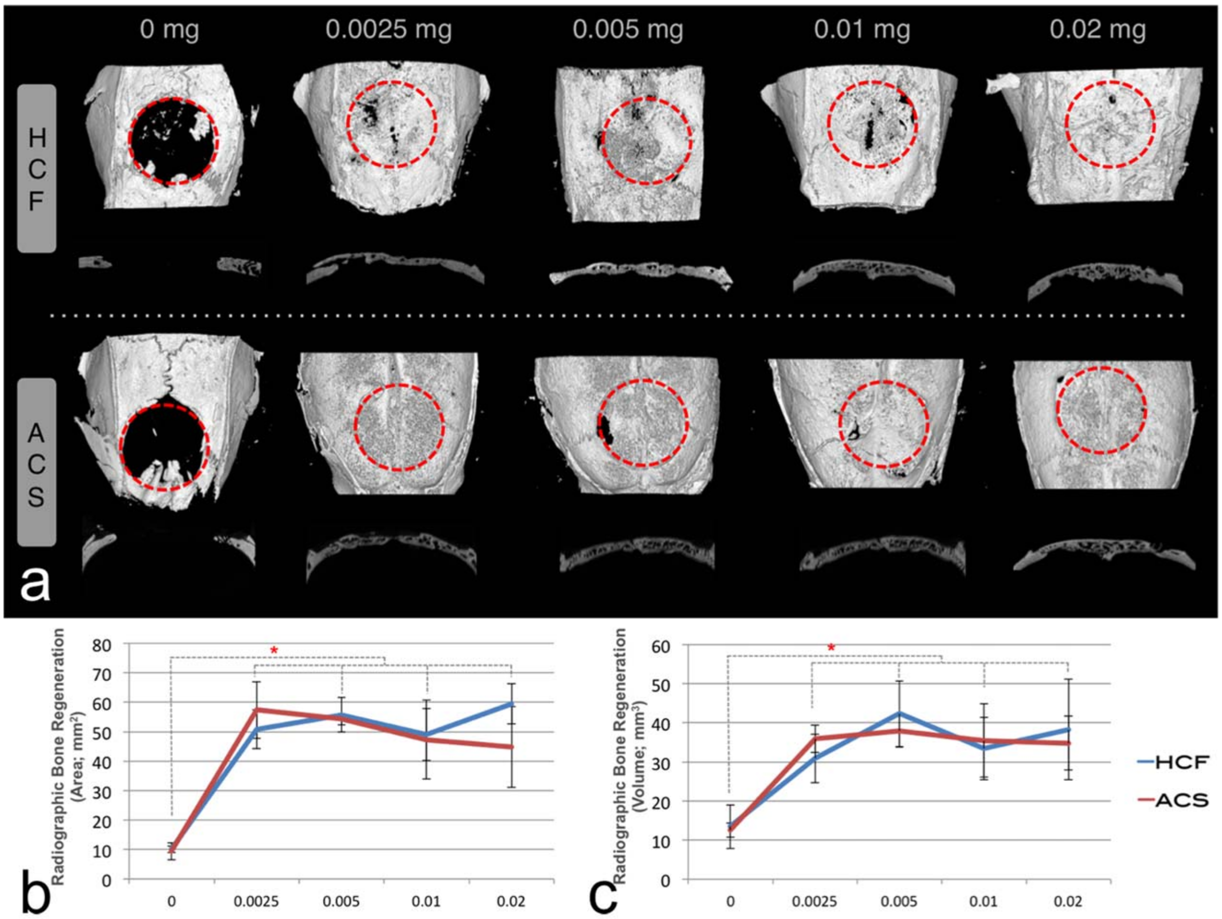

FIGURE 2. Three dimensionally reconstructed and cross-sectional views from microcomputed tomography of calvarial defects (A) demonstrate complete defect resolution at sites with ErhBMP-2, regardless of carrier system or applied dose of ErhBMP-2. Cross-sectional views also reveal reduced marrow formation and increased bone density at sites with ErhBMP-2 carried by HCF than absorbable collagen sponge (ACS). In histomorphometric analyses (B and C), sites received ErhBMP-2 shows statistically significant increase of bone regeneration area (defect resolution) and volume, but without difference according to the types of carrier system: HCF and ACS. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

$50.57 \pm 2.71\%$, respectively [Table I and Fig. 1(E)]. Statistical analyses revealed significantly increased levels of mineralized tissue formation and significantly decreased adipose tissue formation in the HCF/BMP(+) group compared to the Fibrin/BMP(+) group.

The critical-sized calvarial defect model

Limited bone formation was observed only in the area around the defect margin at sites that received ACS or HCF without ErhBMP-2, on both radiographic (Fig. 2) and histologic images (Fig. 3). Histologically, thin and dense connective tissue could be observed within the defect area in the calvaria that received ACS or HCF without ErhBMP-2; however, limited remnants of HCF remained in some of the calvarial defects. All ErhBMP-2-loaded sites exhibited extensively increased bone formation, regardless of the carrier or the applied dose of ErhBMP-2. The surgically induced calvarial defects were almost completely filled with newly formed bone in histologic images. Radiographic analyses also revealed a significantly increased volume of bone regeneration with ErhBMP-2 loading, while the volume did not differ between the groups that received ErhBMP-2 (i.e., regardless of the dose applied).

The histomorphometric measurements are presented in Table I and Figure 4. Despite the similar defect resolution in the groups that received either HCF or ACS loaded with ErhBMP-2, histologic adipose tissue formation in the regenerated area appeared to be greater in the ACS group than in the HCF group, especially at sites that had received lower doses of ErhBMP-2. Statistical analysis also revealed a significant difference in adipose tissue formation between sites that received ErhBMP-2-loaded HCF and ACS. The level of adipose tissue formation tended to increase with the dose of ErhBMP-2 in HCF groups, and was similar at the sites that received HCF and ACS loaded with 0.02 mg of ErhBMP-2. Regardless of the carrier, sites that received ErhBMP-2 exhibited larger augmented areas and proportions of new bone proportion, and decreased fibrovascular connective tissue formation compared to those that did not. However, neither the type of carrier nor the dose of ErhBMP-2 had any statistically significant effect on these measurements.

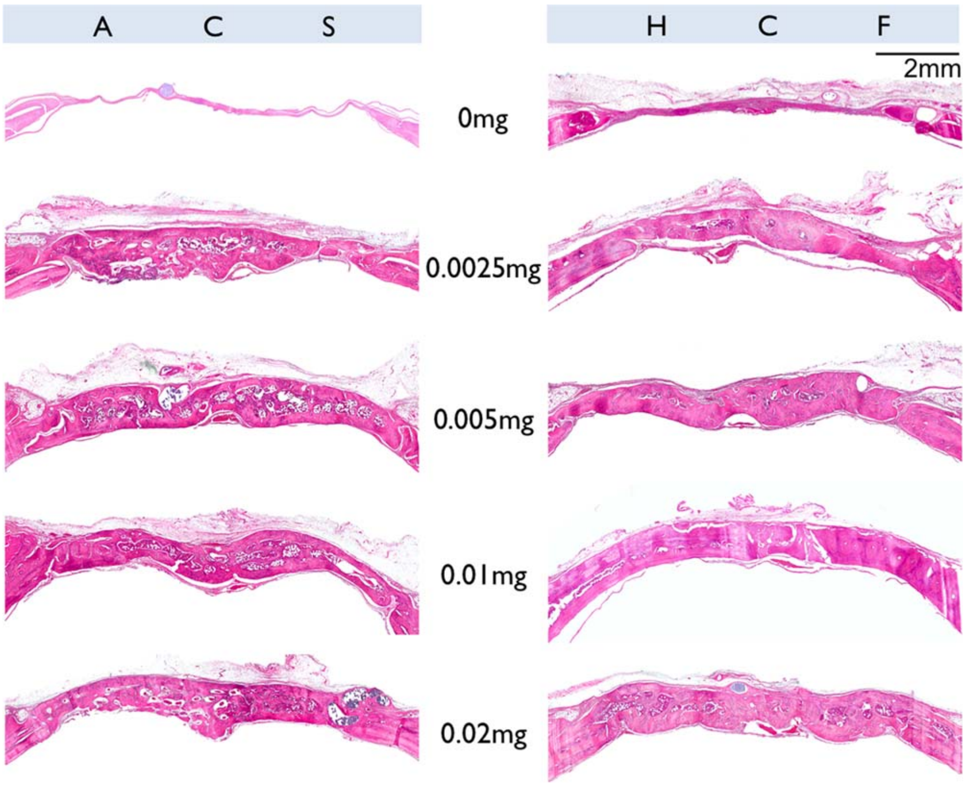

FIGURE 3. Histologic views of the experimental sites using calvarial defect model shows complete bone regeneration in all sites that received ErhBMP-2, despite limited bone formation at sites with HCF or ACS only. At sites that received ACS with ErhBMP-2, extensive adipose tissue formation can be observed even at sites containing the lowest dose of ErhBMP-2. However, minimal adipose tissue is shown at the sites with HCF and low dose of ErhBMP-2, although there is an increasing tendency of adipose tissue formation with increasing dose of ErhBMP-2. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DISCUSSION

The working hypothesis in this study was that the reduction in the initial burst release of ErhBMP-2 induced by the HCF carrier system would reduce the induction of adipose tissue formation by ErhBMP-2 and increase the bone density in the bone-healing process. To this end, two types of *in vivo* (rather than *in vitro*) experimental models were used, to avoid the possible discordance of the carrier release profile and tissue responses between *in vivo* and *in vitro* experimental approaches. These two experimental approaches are representative of orthotopic (calvarial defect) and ectopic (transplantation) models. The ectopic transplantation model has been used to demonstrate induction from recipient progenitor cells to various types of tissue by the implanted rhBMP-2.[25] The calvarial defect model is the most representative critical-sized defect model,[26] and was used to demonstrate the histologic changes in bone healing induced by controlled release of rhBMP-2 at various concentrations from the carrier.

In the present study, the ErhBMP-2-loaded HCF carrier system significantly reduced the amount of adipose tissue formation in both the ectopic transplantation and calvarial defect models relative to the non-heparin-conjugated carrier system (ectopic transplantation model) and the control (ACS) carrier loaded with ErhBMP-2 (calvarial defect model). This study used two different control carrier systems in different experimental models. Because space maintenance is necessary for an ectopic experimental model, calcium phosphate particles and gel-type ErhBMP-2-carrying systems with/without heparin-conjugation (HCF or normal fibrin) were used in ectopic transplantation. Conversely, HCF was compared to ACS in the calvarial defect model mimicking the clinical bone defect, because ACS is the only rhBMP-2-carrying system approved by the FDA for clinical use.[27]

Previous *in vitro* studies have demonstrated an rhBMP-2-induced, dose-dependent increase in mineralized tissue formation,[28–30] and the findings of *in vivo* studies suggested that species-specific osteoinductive dose requirements should be increased in animals that are at a higher level in the zoological hierarchy.[31] However, the present results in the calvarial defect model revealed that the amounts of augmentation and bone regeneration were similar at all sites that received ErhBMP-2, regardless of the dose, despite the application of lower doses in this experiment compared to previous studies.[32] This indicates that quantitative improvements in bone healing cannot be achieved by increasing the dose of ErhBMP-2 beyond a certain threshold, and that this threshold could be lower than previously suggested concentration.[32] This finding concurs with other studies using long-bone defect models that have found that bone healing increased in a dose-dependent manner for concentrations up to a certain level, but with no difference in bone healing

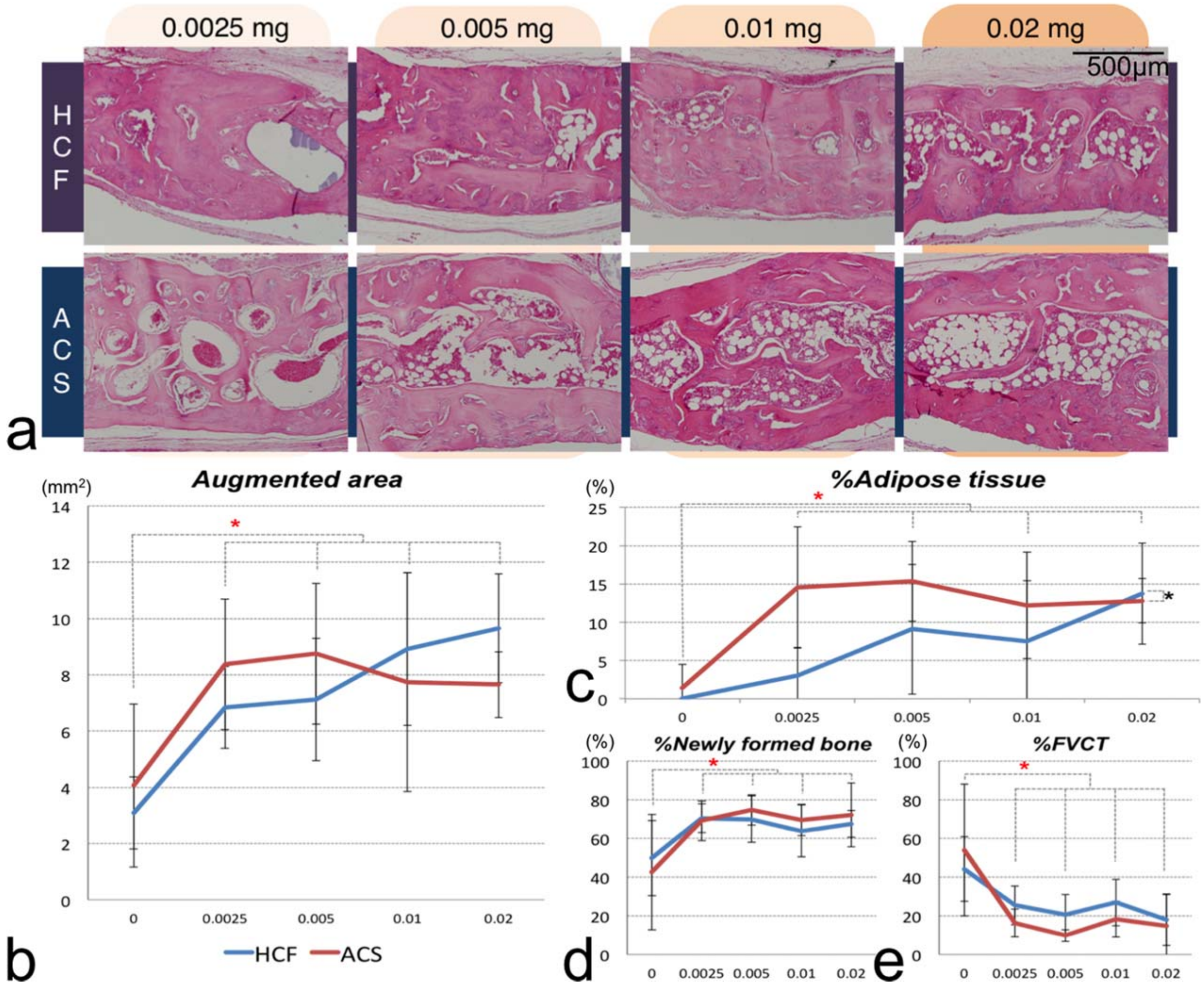

FIGURE 4. Histologic views with high magnification (A) of sites received HCF and ACS with ErhBMP-2 in calvarial defects show significant increase of adipose tissue formation within the regenerated bone induced by ErhBMP-2 at sites used ACS as a carrier compared to HCF. Histomorphometric results demonstrate no effects of type of carrier for ErhBMP-2 on augmented area in calvarial defect healing (B), but two-way ANOVA tests reveal statistically significant effects on adipose tissue formation by applied dose of ErhBMP-2 and the type of carrier system (C). However, proportion of newly formed bone and fibrovascular connective tissue does not differ according to the type of carrier system, despite the significant difference between sites with and without ErhBMP-2 (D and E). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

for higher concentrations.[33] Conversely, some clinical and preclinical studies have found deteriorated bone quality or complications such as increased inflammatory reactions at sites receiving a high concentration of rhBMP-2.[5]

These complications can be explained by the findings of the present ectopic transplantation model, in which ErhBMP-2 induced a significantly increased formation of "adipose tissue filled mass." Even though similar amounts of residual materials were observed in cross-sectional histologic views in all groups, significantly increased volumes containing specific tissues (mineralized and adipose tissues) were induced only at the sites with ErhBMP-2 [Fig. 1(A)]. Several previous studies have also demonstrated cyst-like bone formation in *in vivo* experimental models using high concentrations of ErhBMP-2,[5,34] which indicates that overdose of rhBMP-2, may potentially create an unwanted adverse effect. The present results in calvarial defect model also showed a similar tendency for the adipose tissue formation to increase with the concentration of ErhBMP-2. This can be explained at both the cellular and molecular levels, according to the pleiotropic effects of BMP-2 on the differentiation of stem cells: commonly osteoblastogenesis and adipogenesis.[32,35] A recent study demonstrated that while ErhBMP-2 induced the enhancement of both adipogenic and osteogenic differentiation of periodontal ligament stem cells *in vitro*, *in vivo* tests revealed discriminative effects of ErhBMP-2 application to periodontal stem cells on two other differentiation-related processes: down-regulation of mineralized tissue formation and increase in adipose tissue formation.[4] Other studies also found that rhBMP-2 increased adipogenesis rather than osteogenesis via the activation of peroxisome proliferator-activated receptor gamma, which induced bone marrow stem cells toward adipogenic differentiation,[36,37] or suppressed osteogenesis via one of the BMP signaling pathways.[38] These findings might explain the present results of an antithetical response in the tissue formation process in the transplantation model, or an increase in adipose tissue formation in calvarial bone defect healing. In addition, the present finding of increase in adipose tissue formation was related to not only the increase in ErhBMP-2 concentration but also the type of its carrier system. Given the release kinetics demonstrated in previous studies,[21,22] a decreased release of ErhBMP-2 at the early phase by HCF might reduce the ErhBMP-2-induced adipogenic differentiation in the bone-healing process.

This study was based on preliminary experimental data that were consistent with the release profile of HCF found in previous studies demonstrating a reduction in the initial burst of rhBMP-2 release by HCF compared to normal fibrin and ACS.[21,22] The initial burst releases for the first 6 h from fibrin and fibrin-containing free heparin were $44.1 \pm 2.2\%$ and $45.1 \pm 4.0\%$, respectively, whereas that from HCF was only $28.4 \pm 2.2\%$. During the first 3 days, $83.7 \pm 7.6\%$ and $81.1 \pm 3.8\%$ of the loaded BMP-2 was released from fibrin and fibrin-containing heparin, respectively. This was due to the biological activities of heparin on the binding of the growth factors and the regulation of their activities in the experimentally grafted area, as demonstrated by their normal physiological characteristics. Due to the amount of heparin being fixed in each experimental model, increased doses of rhBMP-2 would have overcome the binding capacity of the applied heparin, shifting the course of the healing process to adipose tissue formation. The lower ratio of conjugated heparin to fibrin would result in a faster release of BMP-2, which would, in turn, reduce bone formation and increase adipose tissue formation.

Osteogenic/adipogenic processes in *in vivo* environments could also be affected by degradation of the carrier biomaterials, as well as their rhBMP-2 release profile. It was previously reported that the *in vivo* degradation of HCF was significantly slower than that of fibrin: 50% of HCF degraded over a 6-day period, whereas 72% of fibrin degraded over the same time period.[39] Therefore, the *in vivo* controlled release of rhBMP-2 could be maximized by HCF. In addition, HCF degradation would positively affect bone formation, given that it was reported that gel degradation promoted osteogenesis by mesenchymal stem cells.[40] However, a possible disadvantage of HCF relative to other BMP-2 release systems such as hydroxyapatite and poly-l-lactic acid scaffolds is its inferior mechanical properties, which might make HCF inappropriate for use in regions subjected to significant mechanical forces.

Adipose tissue formation itself does not necessarily represent a problem or limitation in the process of bone tissue regeneration as this tissue is well known to contain a pool of multipotent stem cells.[41–43] However, the formation of excessive adipose tissue in the bone tissue engineering setting can be disadvantageous to clinicians as it affects the mechanical strength of the concomitantly regenerated bone, and mechanical strength is the most important prerequisite property of regenerated bone tissue in most clinical situations, such as augmentation for dental implant placement or spinal fusion. In the field of dentistry, the findings of several previous studies support this in both the clinical setting and in preclinical animal models that mimic the clinical setting. One recent human study suggested that excessive adipose tissue formation was a "negative effect" of rhBMP-2 application in sinus grafts,[7] and in preclinical animal studies, seroma or cyst-like bone tissue formation was observed in the early healing phase of vertical ridge augmentation and in the sinus graft model.[6,12]

The results of the present study show that there is a reduction in adipose tissue formation following the controlled release of rhBMP-2 at both ectopic sites and bone defects. Although the adipose tissue formation at high concentrations of ErhBMP-2 was comparable in both experimental conditions including ACS and HCF, the amount was less at sites that received controlled carrier system (HCF) with lower concentrations of ErhBMP-2. In addition, ectopic transplantation of the same controlled carrier system showed enhancement of mineralized tissue formation and reduced adipose tissue formation. It can, thus, be concluded that HCF systems loaded with ErhBMP-2 may reduce adipose tissue formation and enhance mineralized tissue formation, and can ultimately be expected to increase bone density in rhBMP-2-engineered bone tissue. However, further studies should be carried out to overcome the lack of space-maintaining property and rapid degradability for clinical applications.

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