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Comparison between heparin-conjugated fibrin and collagen sponge as bone morphogenetic protein-2 carriers for bone regeneration

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Abbreviations: ALP, alkaline phosphatase; BMP-2, bone morphogenetic protein-2; FDA, Food and Drug Administration; HCF, heparin-conjugated fibrin; micro-CT, microcomputed tomography

Abstract

Bone morphogenetic protein-2 (BMP-2) is used to promote bone regeneration. However, the bone regeneration ability of BMP-2 relies heavily on the delivery vehicle. Previously, we have developed heparin-conjugated fibrin (HCF), a vehicle for long-term delivery of BMP-2 and demonstrated that long-term delivery of BMP-2 enhanced its osteogenic efficacy as compared to short-term delivery at an equivalent dose. The aim of this study was to compare the bone-forming ability of the BMP-2 delivered by HCF to that delivered by clinically utilized BMP-2 delivery vehicle collagen sponge. An *in vitro* release profile of BMP-2 showed that HCF released 80% of the loaded BMP-2 within 20 days, whereas collagen sponge released the same amount within the first 6 days. Moreover, the BMP-2 released from the HCF showed significantly higher alkaline phosphatase activity than the BMP-2 released from collagen sponge at 2 weeks *in vitro*. Various doses of BMP-2 were delivered with HCF or collagen sponge to mouse calvarial defects. Eight weeks after the treatment, bone regeneration was evaluated by computed tomography, histology, and histomorphometric analysis. The dose of BMP-2 delivered by HCF to achieve 100% bone formation in the defects was less than half of the BMP-2 dose delivered by collagen sponge to achieve a similar level of bone formation. Additionally, bone regenerated by the HCF-BMP-2 had higher bone density than bone regenerated by the collagen sponge-BMP-2. These data demonstrate that HCF as a BMP-2 delivery vehicle exerts better osteogenic ability of BMP-2 than collagen sponge, a clinically utilized delivery vehicle.

Keywords: bone morphogenetic protein 2; collagen; drug delivery systems; fibrin; osteogenesis

Introduction

The application of bone morphogenetic proteins (BMPs) has recently emerged as an effective treatment in bone reconstruction surgery (Friedlaender, 2004; Termaat *et al*., 2005). BMPs induce bone formation by regulating the recruitment and differentiation of osteoprogenitor cells (Derner and Anderson, 2005; Bessa *et al*., 2008; Kanakaris and Giannoudis, 2008). Among BMPs, BMP-2 and BMP-7 are now being used clinically (Mont *et al*., 2004). However, there are several limitations in the clinical application of BMPs. Firstly; BMP-2 may lose its bioactivity due to its short half-life (Takahashi *et al*., 2005). Additionally, a large dose of BMP-2 is required for clinical treatment due to its rapid loss through diffusion (Carter *et al*., 2008). Such limitations require large doses of BMP-2 to be used clinically, resulting in high treatment costs and undesirable side effects including bone over-

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growth and immune responses (Shields *et al*., 2006).

To overcome these problems, it is necessary to develop an appropriate delivery vehicle that can release BMP-2 locally over an extended period and at sufficient concentrations (Issa *et al*., 2008; La *et al*., 2010). The osteogenic efficacy, bioactivity, and optimal dosage of BMP-2 are dependent on its delivery vehicle (Issa *et al*., 2008; La *et al*., 2010; Yang *et al*., 2010). Our previous study has demonstrated that long-term delivery vehicles of BMP-2 enhance osteogenic efficacy of the protein compared with short-term delivery vehicles at an equivalent dose (Jeon *et al*., 2008; Yang *et al*., 2010). Long-term delivery vehicles enable sustained release and retain the bioactivity of BMP-2 (Jeon *et al*., 2008). Additionally, bone formation by BMP-2 is known to be dose-dependent, and the dose-dependency relies on the BMP-2 delivery vehicle (Patel *et al*., 2008; La *et al*., 2010).

A number of vehicles have been designed for the delivery of BMP-2. Among the delivery vehicles, collagen sponge has proven to be effective for therapeutic applications (Liu *et al*., 2006); thus, it is used clinically as a delivery vehicle for BMP-2 (Liu *et al*., 2006; Yang *et al*., 2011). Although the BMP-2-loaded collagen sponge is used clinically, routine BMP-2 application has been hindered by inherent problems. Several studies have demonstrated that the currently used collagen sponges have a large initial burst release and retention of less than 5% after 14 days of implantation (Uludag *et al*., 1999; Haidar *et al*., 2009).

We previously reported that heparin-conjugated fibrin (HCF) worked well as an injectable delivery vehicle for BMP-2 (Yang *et al*., 2010). The previous results indicated that BMP-2 released from HCF significantly increased alkaline phosphatase (ALP) activity of cultured osteoblasts (Yang *et al*., 2010). HCF releases BMP-2 over a long-term period, while protecting its bioactivity (Yang *et al*., 2010) because heparin enables BMP-2 release over a long-term period with activity retention through the ionic interactions between heparin and BMP-2 (Zhao *et al*., 2006). These results suggested that HCF could be utilized to deliver BMP-2 over a long-term period for bone regeneration. The aim of the present study was to compare the efficacy of HCF as a sustained release BMP-2 delivery vehicle to that of collagen sponge, which is the delivery vehicle currently used for clinical purposes of orthotopic bone formation. We hypothesized that bone regeneration relies on the dose of BMP-2 delivered by HCF and that HCF-mediated delivery of BMP-2 enhances bone regeneration compared with collagen sponge-mediated delivery of BMP-2.

Results

in vitro BMP-2 release

The profiles of *in vitro* release of BMP-2 from HCF and collagen sponge were compared (Figure 1). It was observed that the HCF delivery vehicle maintained a sustained release of BMP-2 compared with the collagen sponge vehicle. The amounts of released BMP-2 differed dramatically depending on the delivery vehicles during the first 10 days of BMP-2 release. When compared to the HCF vehicle, the collagen sponge vehicle showed an initial BMP-2 burst during the first 3 days. Approximately 80% of the BMP-2 loaded on the collagen sponge was released over a period of 6 days, whereas the same dose of BMP-2 was released from the HCF over a period of 20 days.

The ALP activity of cultured rat osteoblasts was significantly higher in the group of BMP-2 released from the HCF than in the group of BMP-2 released

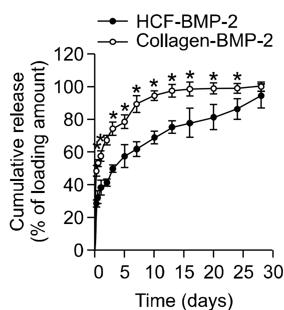

Figure 1. Profiles of BMP-2 release from HCF and collagen sponge. Open circles indicate BMP-2 release from the collagen sponge delivery vehicle, and closed circles indicate BMP-2 release from the HCF delivery vehicle. The values represent the mean ± standard deviation (n = 8). (Collagen: collagen sponge, *$P <$ 0.05 compared with HCF).

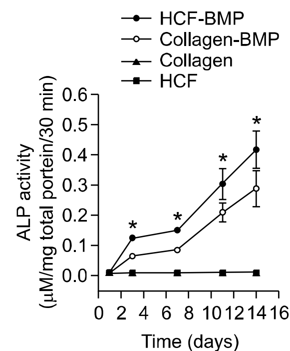

Figure 2. The bioactivity of BMP-2 released from the delivery vehicles, as assessed by measuring ALP activity of rat calvarial osteoblasts cultured with the different delivery systems (n = 5, *$P <$ 0.05 between HCF and collagen).

from the collagen sponge for 14 days (Figure 2). The groups of carriers without BMP-2 showed no ALP activity for 14 days.

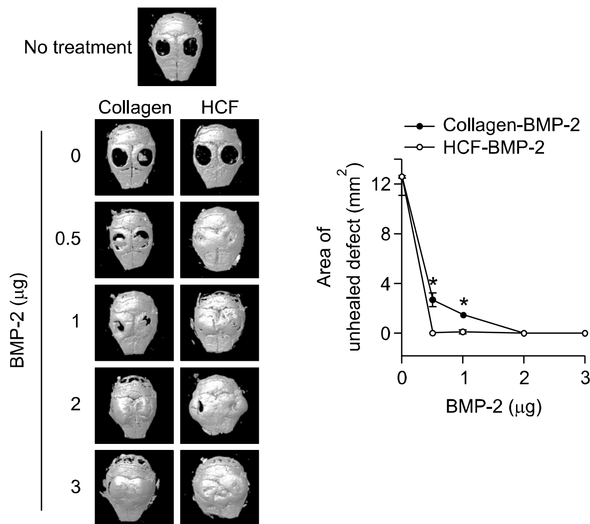

Figure 3. Representative micro-CT images of mouse skulls treated with the different doses of BMP-2 delivered by either collagen or HCF for 8 weeks. Defects were treated with 0.0, 0.5, 1.0, 2.0, and 3.0 μg of BMP-2 delivered with either collagen sponge or HCF vehicle. The area of unhealed defect was determined from the micro-CT images.

Bone formation *in vivo*

After 8 weeks of implantation in the mouse calvarial defect models, all samples were analyzed by X-ray microcomputed tomography (micro-CT) examinations (Figure 3). The analysis revealed a BMP-2 dose-dependent increase in bone regeneration. The results of the micro-CT evaluations were verified with histological analysis with Goldner's trichrome staining (Figure 4). No bone formation was observed in groups in which BMP-2 treatment was not administered. However, fiber-like tissue was observed in calvarial defect sites (Figure 4). Most of the implanted collagen and HCF resorbed at 8 weeks. Beginning at a BMP-2 dose of 0.5 μg, however, the HCF delivery vehicle showed more extensive bone formation. Mature bone with lamellar structures and osteocytes in lacunae was observed in groups with BMP-2 doses equal to or higher than 0.5 μg in HCF groups. In contrast, the collagen sponge vehicle group showed bone formation to a much lesser extent. Histology revealed excessive areas of bone formation in samples that received high doses of BMP-2. When compared with the collagen sponge delivery vehicle, the HCF delivery vehicle resulted in higher bone density at the same BMP-2 dose.

Bone formation area and bone density were quantified with histomorphometric analysis (Figure 5). The group in which no treatment was ad-

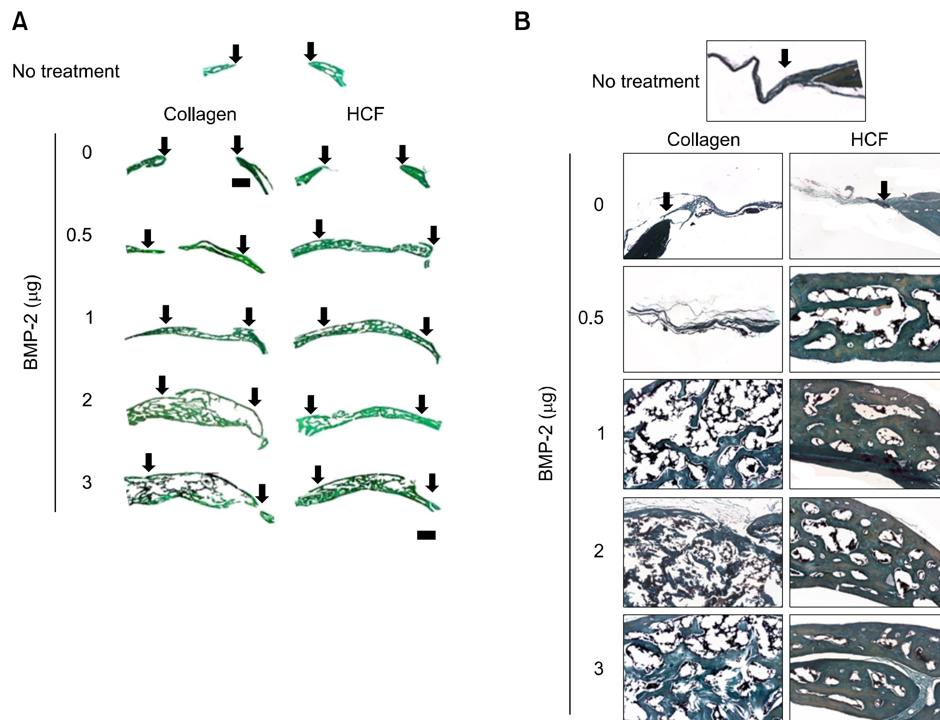

Figure 4. Histological analysis with Goldner's trichrome staining of mouse calvarial defects treated with various doses of BMP-2 with two types of delivery vehicles, collagen sponge and HCF. Calvaria were retrieved 8 weeks after treatment. Arrows indicate defect margins. All photographs were taken at either × 40 in (A) or × 100 in (B).

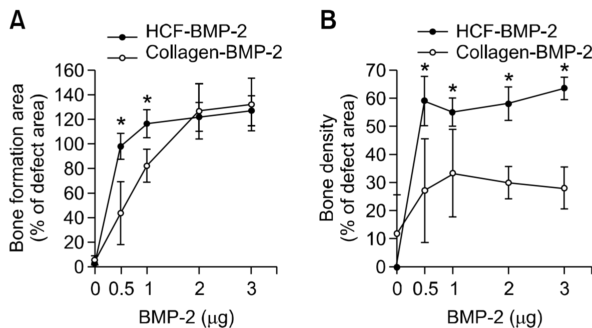

Figure 5. (A) Bone formation area and (B) bone density as determined with histomorphometry analysis (n = 4). Open circles indicate BMP-2 delivery with collagen sponge, and closed circles indicate BMP-2 delivery with HCF. The area of bone formation was calculated as bone area/defect area $\times$ 100 (*$P < 0.05$ between HCF and collagen), and bone density was calculated as new bone area / (new bone area + fibrous tissue area + residual biomaterials area) $\times$ 100 (*$P < 0.05$ between HCF and collagen).

ministered had almost no bone regeneration. In the BMP-2-treated groups, bone regeneration area increased with the BMP-2 dose. At 0.5 μg of BMP-2, the bone formation area in the HCF group was nearly 100% of the defect area (Figure 5A). However, to achieve 100% of bone regeneration area, the collagen sponge delivery vehicle needed more than 1 μg/defect of BMP-2. The BMP-2 dose required by the HCF delivery vehicle to result in 100% of the bone regeneration area without excessive bone formation was approximately one-third of that required by the collagen sponge delivery vehicle. Furthermore, the HCF delivery vehicle resulted in significantly higher bone density than the collagen sponge delivery vehicle (Figure 5B). Taken together, micro-CT, histology, and histomorphometric analysis showed that the HCF delivery vehicle for BMP-2 resulted in much more extensive bone formation and higher bone density at lower BMP-2 dosage than the collagen sponge delivery vehicle.

Discussion

The aim of this study was to compare HCF and collagen sponge, which is a product that is currently used in clinical applications, as BMP-2 delivery vehicles for bone formation. HCF was utilized for sustained delivery of BMP-2 for bone regeneration (Yang *et al*., 2010). Upon comparing the *in vitro* release kinetics of BMP-2, the HCF delivery vehicle was superior to the collagen sponge vehicle, more effectively providing a sustained release of BMP-2 (Figure 1). BMP-2 delivery with the HCF vehicle is achieved by electrostatic interactions between the positive charges of the amino acid residues in the BMP-2 protein and the negative charges of the heparin in HCF (Zhao *et al*., 2006), whereas the collagen sponge relies mostly on adsorption and desorption of BMP-2 protein (Boerckel *et al*., 2011). These features of HCF allow for the sustained release of BMP-2 protein.

The *in vivo* bone formation area may depend on the BMP-2 release period (Fu *et al*., 2008; Issa *et al*., 2008; Yang *et al*., 2010). For clinical treatment, high doses of BMP-2 are required because BMP-2 is released rapidly from the delivery vehicle of collagen sponge (Haidar *et al*., 2009). However, high doses of BMP-2, coupled with a quick release from the delivery vehicle could lead to side effects such as excessive bone formation and immune responses (Shields *et al*., 2006). These side effects could be reduced by facilitating BMP-2 delivery using HCF. The enhanced bone formation may be a result of not only the sustained delivery of BMP-2 by the HCF, but also the higher ALP activity of the BMP-2 released from the HCF, compared with that released from the collagen sponge (Figure 2).

In addition, bone tissue regenerated with BMP-2 delivery using HCF had a higher bone density than the bone tissue regenerated with BMP-2 delivery using the collagen sponge. This could be due to the different characteristics of HCF and collagen. Tissues other than bone, such as fibrous tissues, could grow easily into collagen sponge because the porous nature of the collagen sponge provides structure and space appropriate for fibrous tissue ingrowth. However, because HCF degrades more quickly than collagen sponge, the ingrowth of fibrous tissue is prevented (Yang *et al*., 2011).

In conclusion, the present study shows that BMP-2 can be delivered with an HCF vehicle for its sustained release over a sufficiently long period and that HCF as a BMP-2 delivery vehicle resulted in enhanced bone formation compared to the collagen sponge vehicle. In addition, the HCF vehicle used a reduced dose of BMP-2, which was suitable for efficient bone regeneration and could diminish the potential side effects caused by the excessive use of BMP-2. Therefore, the HCF delivery vehicle could improve the current BMP-2 therapy for bone regeneration.

Methods

Preparation of HCF

Heparin-conjugated fibrinogen was prepared by covalently bonding heparin (molecular weight = 4,000-6,000; Sigma, St.

Louis, MO) to plasminogen-free fibrinogen (Sigma) as previously described (Yang *et al*., 2010). HCF was formed by mixing heparin-conjugated fibrinogen (40 mg/ml) and normal fibrinogen (60 mg/ml) with factor XIII, aprotinin (100 KIU/ml), calcium chloride (6 mg/ml), and thrombin (500 IU/mg).

Kinetics of *in vitro* BMP-2 release

The kinetics of BMP-2 (Cowell Medi Co., Busan, Korea) release from HCF and collagen sponge delivery vehicles (Integra Life Science Co., Plainsboro, NJ) were determined with an enzyme-linked immunosorbent assay (ELISA). Each delivery vehicle containing BMP-2 (1 μg, n = 8 per group) was immersed in a 2-ml microcentrifuge tube containing 1.5 ml of PBS, and the tubes were incubated at 37°C with continuous agitation. At various time points, the supernatant was collected, and the microcentrifuge tubes were replenished with fresh buffer. The amounts of BMP-2 in the supernatants were determined with an ELISA kit (Human BMP-2 Quantikine®; R&D vehicle, Minneapolis, MN).

Bioactivity of *in vitro*-released BMP-2

Calvarial osteoblasts were isolated from calvaria of neonatal (less than 1 day old) Sprague-Dawley rats (SLC, Tokyo, Japan) by a digestive enzymatic process. The bioactivity of BMP-2 released from the delivery systems *in vitro* was assessed by determining their ability to stimulate alkaline phosphatase (ALP) activity of cultured rat calvarial osteoblasts. Rat calvarial osteoblasts (3×10^4 cells per well) were plated in each well of six-well tissue culture plates (Corning, NY). The HCF and the collagen sponge containing BMP-2 (1 μg) were placed on the culture insert (Transwell®; Corning) in the culture plates. The medium was Dulbecco's modified Eagle's medium (Gibco, NY) containing 10% (v/v) fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Pen Strep®; Gibco). The medium was changed every 3 days. ALP activity was determined using p-nitrophenol phosphate (Anaspec®; San Jose, CA) as the substrate. Rat calvarial osteoblasts were rinsed twice with PBS and lysed in alkaline lysis buffer, followed by three freeze-thaw cycles at -70°C and 37°C. The aliquots were incubated in glycine buffer containing 2 mg/ml of p-nitrophenol phosphate. After 30 min, 3N NaOH was added to stop the reaction. The absorbance of p-nitrophenol was measured at 405 nm. Total cellular protein was determined using the Bradford reagent (Sigma). The enzyme activity was normalized to the total cellular protein. The experiments were performed in triplicate.

BMP-2 delivery in mouse calvarial defect model

Six-week-old mice from the Institute of Cancer Research (Orient Bio Co., Gyeonggi-Do, Korea) were anesthetized with xylazine (20 mg/kg) and ketamine (100 mg/kg). After shaving the scalp hair, a longitudinal incision was made in the midline of the cranium from the nasal bone to the posterior nuchal line, and the periosteum was elevated to expose the surface of the parietal bones. Using a surgical trephine bur (Ace Surgical Supply Co., Brockton, MA) and a low-speed micromotor, two circular, transosseous defects with a diameter of 4 mm were produced in the skull. The drilling site was irrigated with saline and bleeding points were electrocauterized. Each animal had two defects, and eight animals were used for each group. The calvarial defects were filled with BMP-2 loaded HCF and collagen sponge. The doses of implanted BMP-2 were 0, 0.5, 1, 2, and 3 μg per defect. The animal study was approved by Institutional Animal Care and Use Committee of Seoul National University (SNU-100203-5).

Bone formation analysis

Eight weeks after the implantation, the animals were euthanized by CO_2 asphyxiation and the skulls were harvested for analysis. Bone formation was evaluated with micro-CT scans (n = 8 per group) and histological analysis (n = 8 per group). CT images were obtained with a micro-CT scanner (SkyScan-1172; Skyscan, Kontich, Belgium). After the micro-CT examination, the implants were used for histological analysis. All samples were decalcified in 10% (vol/vol) formic acid for 7 days, embedded in paraffin, and sectioned transversely at the middle part of the defects at a thickness of 4 μm. The sections were stained with Goldner's trichrome staining. The percentage of bone formation area in the defect area was calculated as (new bone area / bone defect area) × 100. Bone density was calculated as [new bone area / (new bone area + fibrous tissue area + residual biomaterial area)] × 100 (Lee *et al*., 2010).

Statistical analysis

Quantitative data were expressed as the mean ± standard deviation. Statistical analysis was performed with one way analysis of variance (ANOVA) with Tukey's honestly significant difference post-hoc test using SPSS software (SPSS Inc., Chicago, IL). A value of $P < 0.05$ was considered statistically significant.

Acknowledgements

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