Biomaterials 35 (2014) 1869–1881

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

The effect of dose on rhBMP-2 signaling, delivered via collagen sponge, on osteoclast activation and *in vivo* bone resorption

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ARTICLE INFO

Article history:
Received 20 September 2013
Accepted 8 November 2013
Available online 8 December 2013

Keywords:
rhBMP-2
High-dose
Osteoclast activation
RANKL
NFATc1
Bone formation

ABSTRACT

While recombinant human bone morphogenetic protein (rhBMP)-2-based bone therapy presents potential osteoinductivity, it also leads concern due to transient osteoclast activation during early healing periods, ultimately limiting its clinical use. Therefore, we investigated *in vivo* and *in vitro* rhBMP-2 signaling which mediates early bone resorbing effect, depending on the dose, and attempted to inhibit this resorption phenomenon using NFAT inhibitor as a target molecule. High-dose of rhBMP-2 (20 μg/defect) enhanced osteoclast activation and the expression of bone resorption markers, compared to low dose (5 μg/defect) at one week after surgery in collagen sponge-delivered rat calvarial defect models. Interestingly, this trend was also observed in the expression of bone formation markers. In particular, rhBMP-2 upregulated *RANKL* expression, while it downregulated *osteoprotegerin* (*OPG*) expression, resulting in a dose-dependent increase in the ratio of *RANKL* to *OPG*. NFAT inhibitor (150 μM) treatment *in vivo* suppressed the high-dose effect of rhBMP-2 on both resorption and formation. *In vitro* results of rhBMP-2 signaling and NFAT inhibitor effects in rat mesenchymal stem cells showed similar trends as *in vivo* results. Microcomputer tomography-based evaluation at 4 weeks showed that combined treatment of NFAT inhibitor with 20 μg rhBMP-2 *in vivo* increased bone volume (BV) more than 20 μg rhBMP-2 alone, which showed little difference in BV compared to 5 μg of rhBMP-2. These results demonstrated that rhBMP-2 implantation concurrently signalized into enhanced osteoclastogenesis and osteoblastogenesis *in vivo*, dose-dependently. Ratio of *RANKL/OPG* might be an index for early bone resorbing activity of implanted rhBMP-2. A local cocktail treatment of NFAT inhibitor and high-dose rhBMP-2 might be an alternative to overcome early bone resorbing effects, thereby accelerating bone formation.

1. Introduction

Bone morphogenetic proteins (BMPs) have the potential capacity to induce ectopic and orthotopic bone formation *in vivo* [1,2] and can induce mesenchymal stem cells (MSCs) to differentiate into chondrocytes and osteoblasts *in vitro* [3]. Among BMPs, recombinant human BMP (rhBMP)-2, to date, has been regarded as the most efficient osteoinducer and has been frequently used in clinical treatment for skeletal repair [4]. However, rhBMP-2 therapy results in unexpected side effects due to use of supra-physiological doses; these side effects include heterotopic bone formation [5], osteoclast activation [6], cyst-like bone void formation [7,8], and life-threatening cervical swelling [9]. To alleviate the adverse clinical effects associated with high doses and to enhance the clinical efficacy of BMP-2, numerous approaches avoid an initial burst release and employ a more sustained release from a carrier [10,11]. Although collagen sponges have been approved by the FDA for human use as an alternative to autologous bone grafts [12], collagen biomaterial rapidly releases BMP-2 [13] from the implant site before achieving a critical density of newly infiltrating cells

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http://dx.doi.org/10.1016/j.biomaterials.2013.11.029

[14]; these sponges therefore require high doses of BMP-2 for stable bone formation [15]. Recently, a collagen sponge variant conjugated with heparin has been developed to enhance sustained release of BMP-2, as BMP-2 has a binding site specific to heparin [10,16].

Recent research into BMP signaling routes has revealed a complex role in bone metabolism. BMPs exhibit biphasic function encompassing bone formation as well as bone resorption which is regarded as a regulatory mechanism to control bone mass through balance between bone formation and resorption [17–19]. The Smad-dependent and Smad-independent p38 MAPK pathways play a central role in BMP signaling by accelerating osteoblastogenesis [20,21]. However, Smad pathways activated by BMPs also activate the bone resorption process, representing a classic feedback inhibition loop [22]. This feedback inhibition involves the expression of BMP antagonists such as Noggin and Sclerostin (SOST), both of which are also secreted from osteoblasts. While Noggin prevents BMPs from binding their receptors [23], the regulatory route of *SOST* expression involves the interplay between BMP and Wnt signaling. *SOST* is up-regulated by BMP through the Smad pathway, and it then inhibits Wnt signaling by binding to the co-receptors, low-density lipoprotein receptor-related proteins 5 and 6 (LRP5 and LRP6) [24]. *SOST* eventually enhances resorption by triggering receptor activator of nuclear factor-kappa B ligand (RANKL)-osteoprotegerin (OPG) pathway-induced osteoclastogenesis, leading to a decrease in bone mass [18,25]. The RANKL/RANK/OPG system has been considered a central signaling pathway governing osteoclastogenesis [26]. RANKL mediates osteoclast differentiation via TNF receptor-associated factor 6-activated c-Jun N-terminal kinase upregulation and subsequent activation of c-Jun and c-Fos signaling coupled with nuclear factor of activated T cells (NFAT) [27,28]. Several studies also report that BMPs directly stimulate osteoclast differentiation *in vitro* [19,29]. Although BMP-2 and NFAT each play a crucial role in osteoclastogenesis, current understanding lacks the evidence about direct interaction between BMP-2 and NFAT in osteoclastic bone resorption while it is reported that NFAT controls osteoblastic bone formation via complex formation with Osterix which is a responsive gene of BMP-2 [30].

This dual function of BMP signaling is reflected in bone regeneration using rhBMP-2. High rhBMP-2 dosing using collagen sponges led to a dose-dependent and transient osteoclastic resorption of peri-implant bone in bilateral cortico cancelous defective femora of sheep, even though rhBMP-2 accelerated bone regeneration over long-term observation [6,31]. Similarly, several studies reported the osteoclastic resorbing effect of rhBMP-2 in dental wounds [32,33]. For example, Miyaji et al. reported dentin resorption with high-dose of BMP-2 and cementum-like tissue induction with low dose of BMP-2 at dentin surfaces [34], suggesting the importance of applying the correct concentration of BMP-2. Taken together, further investigation is required to elucidate how bone resorption and formation are modulated by BMP-2 signaling during early healing periods, after high-dose rhBMP-2 in association with a scaffold is administered into the local bone area. Therefore, this study was designed to compare the dose effect of rhBMP-2 *in vivo* between 5 and 20 μg using an 8 mm-critical-sized calvarial defect model and *in vitro* between 50 and 200 ng/mL using rat MSCs (rMSCs), respectively. The major purposes of this study were threefold: (a) to investigate dose-dependent signaling by evaluating the *in vivo* expression of both bone-forming and bone-resorbing markers as well as osteoclast activation; (b) to investigate the relevance of the NFAT pathway to BMP-2-mediated signaling *in vivo* and *in vitro*; and (c) to investigate the effect of NFAT inhibitor on new bone regeneration as an alternative to overcome early bone resorption mediated by high-dose rhBMP-2 and enhance the efficiency on bone regeneration using micro-computed tomography (micro-CT)-based quantitative analysis.

2. Materials and methods

2.1. In vivo model for the osteogenic effect of rhBMP-2

2.1.1. Preparation of rhBMP-2/NFAT inhibitor-loaded scaffold

Escherichia coli-derived rhBMP-2 powder was provided from Daewoong Co. (Seoul, Korea), and dissolved with stabilizing buffer which was obtained by manufacture, at a concentration of 1 mg/mL. NFAT inhibitor (Sigma–Aldrich, St. Louis, MO, USA) was prepared by dissolving 50% acetic acid at a concentration of 5 mM. Each collagen sponge was prepared from the cross-reaction of chondroitin-6-sulfate (CS; Sigma Chemical Company, St. Louis, MO, USA) and type I collagen as described previously [35]. A type I atelocollagen powder (KOKEN Corp., Osaka, Japan) was dissolved in 0.05 M acetic acid, at a concentration of 5.0 mg/mL, which was co-precipitated by the dropwise addition of CS while stirring in a homogenizer. The resulting collagen–CS solution was placed in a freezer at −80 °C for 6 h, and was then lyophilized by freeze-drying at −80 °C for 48 h, yielding a collagen sponge. Subsequently, the collagen sponges were placed in a vacuum oven and subjected to a vacuum of 1.3 in. of Hg at 105 °C for 24 h. After a series of treatments with 40% ethanol containing 50 mM 2-morpholineoethane sulfonic acid (MES; Fluka Chemie, Buchs, Switzerland) (pH 5.5), and washings, collagen sponges are finally rinsed with distilled water, lyophilized by freeze-drying and sterilized with γ-irradiation at 10 kGy. The collagen disks were 8 mm in diameter and 1 mm thickness for the calvarial defect model. Cocktail solution which contains rhBMP-2, NFAT inhibitor or stabilizing buffer was rapidly loaded onto each scaffold before *in vivo* implantation at a total volume 30 μl which was controlled at an amount not exceeding 0.1% of the total scaffold volume. For control experiments, the scaffold was loaded with buffers which are dissolving solutions of rhBMP-2 or NFAT inhibitor.

2.1.2. Rat calvarial defect model

Eight-week-old Sprague–Dawley rats (total $n = 42$) were used. The experimental protocol was approved by the Animal Care and Use Committee of Seoul National University. After disinfection of the calvarial skin with 10% betadine (Potadines; Sam-Il Pharm, Seoul, Korea) and subcutaneous injection of 2% lidocaine containing 1:100,000 epinephrine (Lidocaine HCL Injs.; Yuhan, Seoul, Korea), an incision was made along the sagittal suture. The periosteum was elevated and an 8-mm-diameter calvarial bone defect was created with a trephine burr without dural perforation. A rhBMP-2-soaked collagen disk was then implanted into the defect area. The experimental groups were implanted with buffer-loaded scaffold alone ($n = 10$), rhBMP-2 (5 μg/defect) ($n = 12$), rhBMP-2 (20 μg/defect) ($n = 12$), or rhBMP-2 (20 μg/defect)+NFAT inhibitor (150 μM; 7.5 μg/defect) ($n = 10$). Half rats in each group was evaluated for RT-PCR at one week after implantation, and the other half was analyzed after four-weeks healing periods using micro-CT and histological observation to evaluate the new bone formation in calvarial defects. Collagen disks removed at one week was cut in half for RT-PCR analysis and TRAP staining to draw consistent results for the effect of BMP-2 with or without NFAT inhibitor on osteoclast activation.

2.2. In vivo osteogenesis evaluation

2.2.1. Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR

The collagen disks removed from calvarial defect was washed with PBS solution and chopped into small pieces. After adding 0.5 mL of TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) directly to the chopped sponge, total RNA was extracted and then subsequently treated as prescribed in the manufacturer's instructions. One microgram of RNA from each sample was subjected to cDNA synthesis using SuperScript™ Reverse Transcriptase II and oligo $(dT)_{12-18}$ primer (Invitrogen) in a 20 μL reaction volume according to the manufacturer's instructions, with an additional step of removing RNA complementary to the cDNA using *E. coli* RNase H (Invitrogen). One microliter of cDNA was then subjected to PCR with the following amplification profile: predenaturation at 95 °C for 40 s, amplification (denaturation at 95 °C for 40 s, annealing at 60 °C for 40 s, and extension at 72 °C for 1 min) for a duration of 30 cycles, with a final extension at 72 °C for 10 min. PCR was performed in a DNA thermal cycler (model PTC-200; MJ Research, Waltham, MA, USA). For each of the PCR products, 10 μL was electrophoresed on a 1.5% agarose gel in the presence of ethidium bromide and visualized by the Gel documentation system (Vilber Lourmat, Marne-la-vallee Cedex, France).

For quantitative real-time RT-PCR, total RNA isolation and cDNA synthesis were carried out by the same method as RT-PCR. SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used to detect accumulation of PCR product during cycling with the ABI Prism 7700 sequence Detection system (Applied Biosystems). The thermocycling conditions were as follows: predenaturation at 95 °C for 10 min, amplification with denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min for a duration of 30 cycles, with a final dissociation cycle at 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Real-time RT-PCRs were carried out in triplicate with three independent experiments ($n > 3$–5). Oligonucleotide primers for real-time RT-PCR were designed for product sizes under 200 bp using real-time PCR system Sequence Detection Software v1.3 (Applied Biosystems), and their sequences are provided in Table 1. Fold differences of each gene were