Biomaterials 32 (2011) 5726–5732

Contents lists available at ScienceDirect

Biomaterials

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

Enhancement of ectopic osteoid formation following the dual release of bone morphogenetic protein 2 and Wnt1 inducible signaling pathway protein 1 from gelatin sponges

Hiroshi Kohara, Yasuhiko Tabata*

Department of Biomaterials, Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University, 53 Kawara-cho Shogoin, Sakyo-ku, Kyoto 606-8507, Japan

ARTICLE INFO

Article history:
Received 1 April 2011
Accepted 18 April 2011
Available online 13 May 2011

Keywords:
Controlled release
BMP2
WISP1
Bone formation
Age-related change

ABSTRACT

Bone morphogenetic protein (BMP) 2-incorporated gelatin sponge is effective for *in vivo* osteoinduction. However, the modeling capacity of bone decreases with age. As atrial to stimulate effective bone formation for animals with decreased osteogenic potential, Wnt1 inducible signaling pathway protein (WISP) 1, an osteoblastic regulator, was combined with gelatin sponge incorporating BMP2. *Osteopontin* (*Opn*) geneexpression was increased *in vitro* for mouse bone marrow stromal cells (BMSC) cultured in gelatin sponges incorporating BMP2 and WISP1 compared with those incorporating BMP2 or WISP1 alone. *In vivo* synergistic effect of BMP2 and WISP1 on the ectopic osteoid formation was observed when gelatin sponges incorporating BMP2 and WISP1 were implanted subcutaneously into middle-aged mice with decreased bone formation potential. It is concluded that the scaffold incorporating multiple osteoinductive agents could be effective in inducing bone formation in those with age-related decreased potential of bone formation.

1. Introduction

It is well known that bone formation is impaired with aging [1,2]. The bone is composed of extracellular matrix proteins, including type I collagen, stiffened by the crystals of calcium hydroxyapatite, and bone loses its matrix and mineral composition with advancing age [1,3]. At the cellular level, bone formation is a function of the osteoblast cell lineage, whereas bone resorption is regulated by cells of the osteoclast linage [4,5]. It has been also reported that aging is accompanied by the alteration of the relationship between osteoblasts and osteoclasts [6,7]. In the rat model of ectopic bone formation, bone tissue induced ectopically by the subcutaneous implantation of BMP2 into rat back skin was reduced with increasing age [8].

The bone morphogenetic protein (BMP) 2 incorporated in porous biodegradable scaffold is useful for bone tissue engineering. BMP subfamily within the transforming growth factor- (TGF-) superfamily is a group of proteins with osteoinductive activity [9,10]. Among the BMP subfamilies, BMP2 was originally cloned from the bovine bone [11]. It has been also shown that recombinant human BMP2 induces ectopic bone formation in rats [12]. The advantage of BMP2 in bone tissue engineering is to the clinical application for bone regeneration at the bone defect [13]. It has been demonstrated that a biodegradable hydrogel of gelatin is used for the controlled release of BMP2 [14]. Generally, the direct injection of growth factor solution into the site to be regenerated is not therapeutically effective. This is because the water-soluble factors are rapidly excreted from the injected site and often digested or deactivated. BMP2 was retained in the gelatin hydrogel *in vivo* for more than 1 month and long-termed BMP2 release was achieved [14]. BMP2-incorporated three-dimensional porous structure of gelatin hydrogel, what has been referred to as “gelatin sponges”, is effective for osteoinduction both *in vitro* and *in vivo* [15,16].

As one of the trials to stimulate effective bone formation in the patients with decreased osteogenic potential, bone tissue engineering approach to combine scaffold with multiple osteoinductive agents is worth examining. Wnt signaling pathway is predicted to control bone mass because of the increased bone mass in human with mutations in the Wnt receptor low density lipoprotein receptor-related protein (LRP) 5/6 [17,18]. Wnt1 inducible signaling pathway protein 1 (WISP1; also known as CCN4) is a member of CCN family protein, originally cloned as the target gene of the Wnt1/Frizzled pathway in the breast cancer cells [19]. It has been shown that recombinant WISP1 promotes proliferation and osteogenic differentiation of human bone marrow stromal cells (BMSC) *in vitro* [20]. Interestingly, human BMSC co-transduced WISP1 and BMP2 had significantly greater ectopic bone formation than those

* Corresponding author. Tel.: +81 75 751 4128; fax: +81 75 751 4646.
E-mail address: yasuhiko@frontier.kyoto-u.ac.jp (Y. Tabata).

0142-9612/$ – see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2011.04.035

transduced with BMP2 alone when implanted into the back of mice [20].

In this study, both BMP2 and WISP1 were incorporated into gelatin sponges, and the *in vitro* osteogenic activities of gelatin sponges incorporating BMP2 and/or WISP1 were investigated. Ectopic bone formation *in vivo* was investigated by the subcutaneous implantation of gelatin sponges incorporating BMP2 and/or WISP1 into mice of a middle age, in which the bone formation activity declines, and compared with that of sponges incorporating BMP2 or WISP1.

2. Materials and methods

2.1. Materials

A gelatin sample with an isoelectric point (IEP) of 9.0 was prepared through an acidic process of porcine skin collagen type I (Nitta Gelatin Co., Osaka, Japan). β-tricalcium phosphate(β-TCP) granules (2 μm in average diameter) were obtained from Taihei Chemical Industries, Nara, Japan. $Na^{125}I$ (740 MBq/ml in 0.1 N NaOH aqueous solution) was purchased from Du Pont NEN Research Products (Wilmington, MA, USA). Glutaraldehyde (GA), glycine, and other chemicals were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and used without further purification. The monoclonal antibodies 30-F11 (anti-CD45) and D7 (anti-Sca-1) were from eBioscience, Inc. (San Diego, CA, USA). All primers (Table 1), SuperScript VILO and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Invitrogen Corporation (Carlsbad, USA). RNeasy Plus Mini Kit was from Qiagen Inc. (Valencia, CA). Power SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA). Fetal bovine serums (FBS) were purchased from Hyclone (Logan, UT, USA). Propidium iodide was obtained from Dojindo Laboratories (Kumamoto, Japan). 2.4G2 hybridoma was purchased from American Type Culture Collection (Rockville, MD, USA). WISP1 was purchased from PeproTech (Rocky Hill, NJ, USA).

2.2. Mice

C57BL/6NCrSlc female mice were purchased at 4–12 weeks old from Shimizu Laboratory Supplies Co. (Kyoto, Japan) and were used between 6 and 38 weeks old. All animal experimentation was conducted in accordance with the guidance of the Institute for Frontier Medical Sciences, Kyoto University.

2.3. Preparation of gelatin sponges with β-TCP

Gelatin sponges incorporating β-TCP were prepared as described previously [15]. Briefly, 4.29 wt% aqueous solution of gelatin (70 ml) was mixed at 5000 rpm at 37 °C for 3 min by using a homogenizer (ED-12, Nihonseiki Co., Tokyo, Japan). After addition of and 10 wt% of β-TCP and 2.17 wt% of glutaraldehyde aqueous solution (30 ml), the mixed solution was further mixed for 15 s by the homogenizer. The resulting solution was cast into a polypropylene dish of 138 × 138 mm^2 and 5 mm depth, followed by leaving at 4 °C for 12 h for gelatin crosslinking. Then, the crosslinked gelatin hydrogels with β-TCP were placed into 100 mM of aqueous glycine solution at 37 °C for 1 h to block the residual aldehyde groups of glutaraldehyde. Following complete washing with double distilled water (DDW), the hydrogels were freeze-dried and cut into disks (5 mm diameter).

2.4. In vitro release study of BMP2 and WISP1 from gelatin sponges

BMP2 and WISP1 were radioiodinated through the conventional chloramine-T method as previously described [21]. Briefly, 5 ml of $Na^{125}I$ was added to 50 μl of 5 mg/ml BMP2 or 1 mg/ml WISP1 solution in 0.5 M potassium phosphate buffer (pH 7.5) containing 0.5 M NaCl. Then, 0.2 mg/ml chloramine-T in the same buffer (100 μl) was added to each solution mixture. After agitation at room temperature for 2 min, 100 μl of phosphate-buffered saline (PBS) solution (pH 7.5) containing 0.4 mg sodium metabisulfate was added to the reaction solution to stop the radioiodination. The reaction mixtures were passed through PD-10 columns to remove the uncoupled, free ^{125}I molecules from the ^{125}I-labeled BMP2 and WISP1. A PBS solution of ^{125}I-labeled BMP2 and/or WISP1 (20 μl) was dropped onto the freeze-dried sponges of gelatin (5 mm diameter), followed by leaving at 25 °C for 3 h to obtain hydrogel sponges incorporating ^{125}I-labeled BMP2, WISP1, or both. For the *in vitro* release test, hydrogels incorporating ^{125}I-labeled cytokines were agitated at 37 °C in 1 ml of PBS. The supernatant was removed 1, 2, 4, 6, 12, and 24 h later and replaced with the same volume of fresh PBS. The radioactivity of each supernatant was measured on a gamma counter (ARC-301B, Aloka, Tokyo, Japan) to evaluate the time profile of cytokines release ($n = 3$ at each time point).

Table 1
Primer sequences used for qRT-PCR.

Gene	Primer sequence
Runx2	(S) CCAAGTAGCCAGGTTCAACG
	(AS) TGGGGAGGATTTGTGAAGAC
Opn	(S) GCTTGGCTTATGGACTGAGG
	(AS) AGGTCCTCATCTGTGGCATC
18S	(S) ACTCAACACGGGAAACCTCA
	(AS) AACCAGACAAATCGCTCCAC

2.5. In vitro culture of BMSC in gelatin sponges

To isolate BMSC, fresh bone marrow cells were harvested from femurs and tibias of the euthanized mice (7 wk old), and were suspended in DMEM supplemented with 2% FBS and penicillin/streptomycin. The red blood cells were hemolyzed in BD Pharm Lyse Lysing Buffer (BD Bioscience, Rockville, MD, USA) under the manufacturer's guidance. The cell suspensions were filtered through a cell strainer (BD Bioscience) to remove debris. The filtrates were pelleted by centrifugation for 5 min at 4 °C. The bone marrow cells were resuspended at 5×10^6 cells/ml in DMEM supplemented with 10% FBS and penicillin/streptomycin, and plated onto 100-mm culture dish. The adherent cells were passaged two times before use.

The BMSC at passage two were incubated with 1 ml of the 0.25% trypsin/EDTA solution for 5 min at 37 °C, until the cells detach from the dishes. The cells were washed by centrifugation, and seeded onto the gelatin sponges in which BMP2 and/or Wips1 were incorporated as described above. BMSC were resuspended in DMEM supplemented with 10% FBS, 50 μg/ml ascorbic acid-2- phosphate, 10 mM β-glycerophosphate, 10^{-8} M dexamethasone. The cell suspensions were seeded into the gelatin sponges by an agitated seeding method, by which cells were seeded homogeneously throughout 3-dimensional porous scaffolds [15].

2.6. Quantitative, real-time polymerase chain reaction with reverse transcription (qRT-PCR)

The total RNA was extracted from BMSC cultured for 7 days in gelatin sponges incorporating BMP2 and/or Wips1 by using RNeasy Kit according to the manufacturers' instructions. Reverse transcription reaction was performed with SuperScript VILO. Real-time PCR was performed on a Prism 7500 real-time PCR thermal cycler (Applied Biosystems, Foster City, CA, USA) from 10 ng of cDNA in a total volume of 25 ml containing Power SYBR Green PCR Master Mix and 10 mM of each primer (Table 1). The reaction mixture was incubated for the initial denaturation at 95 °C for 10 min, followed by 40 PCR cycles. Each cycle consisted of the following three steps; 94 °C for 15 s, 57 °C for 15 s and 72 °C for 1 min. Each mRNA level was normalized by the expression level of 18S ribosomal RNA as an internal control ($n = 3$ at each treatment combination).

2.7. In vivo osteogenic ectopic assay

Gelatin hydrogels incorporating BMP2 and/or WISP1 as described above were implanted into the back subcutis of mice at various ages (6 mice/experimental group). The BMP2-free, WISP1-free empty gelatin hydrogel were used as controls. The skin tissue including the hydrogel-implanted or injected site was taken out for following flow cytometric and histological assays 10 days later.

2.8. Flow cytometric analysis

Gelatin implants harvested were digested as described previously (Matsuzaki Stem cells). Briefly, Gelatin implants were harvested and digested in a solution containing 400 U/mL collagenase D (Roche, Basel, Switzerland) for 40 min at 37 °C. The resulting single-cell suspensions were blocked by anti-CD16/32 antibody, washed and stained with monoclonal antibodies and its secondary reagents in PBS containing 2 vol% FBS and 0.1 vol% sodium azide. Propidium iodide was used to distinguish dead cells from viable cells. The immunostained cells were analyzed on FACSCanto II flow cytometer. Analysis was performed by BD FACSDiva software (BD Bioscience) and FLOWJO software (Tree Star, San Carlos, CA).

2.9. Histological evaluation of bone tissue ectopically induced by gelatin implants

Gelatin implants were fixed in 4% paraformaldehyde and equilibrated in 30% sucrose/PBS. Fixed samples were embedded in OCT medium (Sakura) and frozen in liquid nitrogen. Sections of undecalcified samples were generated via Kawamoto's film method (Cryofilm transfer kit; FINETEC). The 7 mm thick cryostat sections were and stained by hematoxylin and eosin (H&E) to view on an optical microscope (AX-80, OLYMPUS, Japan).

2.10. Colony forming unit-fibroblast (CFU-F) assay

CFU-F assay was performed as described previously (Benedetto AD, J cell science, 2010). In brief, single-cell suspensions from gelatin hydrogel implants were suspended in αMEM supplemented with 2% FBS, penicillin and streptomycin using a 18-gauge needle, and then filtered through a 70 μm cell strainer (Falcon) to remove