Biochemical and Biophysical Research Communications xxx (2016) 1–7

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

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

Local administration of a hedgehog agonist accelerates fracture healing in a mouse model

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ARTICLE INFO

Article history:
Received 15 September 2016
Accepted 26 September 2016
Available online xxx

Keywords:
Hedgehog
Smoothened agonist
Bone fracture healing
Callus formation
Chondrocyte
Osteoblast

ABSTRACT

Bone fracture healing is processed through multiple biological stages including the transition from cartilaginous callus to bony callus formation. Because of its specific, temporal and indispensable functions demonstrated by mouse genetic studies, Hedgehog (Hh) signaling is one of the most potent signaling pathways involved in these processes, but the effect of Hh-signaling activation by small compounds on the repair process had not yet been addressed. Here we examined therapeutic effects of local and one shot-administration of the Hh agonist known as smoothened agonist (SAG) on bone fracture healing in a mouse model. A quantitative analysis with three-dimensional micro-computed tomography showed that SAG administration increased the size of both the cartilaginous callus and bony callus at 14 days after the surgery. A histological analysis showed that SAG administration increased the number of cells expressing a proliferation marker and a chondrocyte marker in cartilaginous callus as well as the cells expressing an osteoblast marker in bony callus. These results indicate that the SAG administration resulted in an enhancement of callus formation during bone fracture healing, which is at least in part mediated by an increase in chondrocyte proliferation in cartilaginous callus and the promotion of bone formation in bony callus. Therapeutic strategies with a SAG-mediated protocol may thus be useful for the treatment of bone fractures.

1. Introduction

Bone fractures are common among humans, and they undermine one's quality of life. Although the human body's repair of a bone fracture is well-orchestrated through multiple regenerative processes, effective therapeutic strategies remain to be developed due to complexity of the fracture repair processes, which involve multiple cell types. The use of the accumulated knowledge about embryonic development is a reasonable approach for designing therapeutic strategies for the regeneration of tissues such as bone, because common features are observed in many contexts between development and regeneration [1].

A greater understanding of bone development will provide insights into therapeutic strategies for bone fracture healing. The processes of bone healing seem to partly mimic those of endochondral ossification in development [1]. During endochondral ossification, skeletal progenitors are initially condensed and subsequently differentiate into chondrocytes to form cartilage. This cartilage is used as a mold which undergoes a cartilage-directed ossification. Similarly, key features of bone fracture healing are the formation of a cartilaginous callus followed by mineralization and replacement with bone. The healing process is likely regulated by a large number of factors, as bone development is. Several factors have been implicated in the healing process, including bone morphogenetic proteins (BMPs) [2], fibroblast growth factors (FGFs) [3,4], parathyroid hormone (PTH) [5], PTH-related peptides (PTHrP) [6], Notch [7], Wnt/β-catenin [8], and hedgehog (Hh) [9,10]. Among these factors, Hh is one of the most potent because of its specific, temporal and indispensable function in bone development [11].

Indian hedgehog (Ihh), one of the three ligands of Hh, is highly expressed in pre-hypertrophic chondrocytes and hypertrophic

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http://dx.doi.org/10.1016/j.bbrc.2016.09.134
0006-291X/

chondrocytes, and it acts as an initial switch to specify the skeletal progenitors to bone-matrix-secreting osteoblasts [12,13]. The deletion of *smoothened* (*Smo*), a transmembrane signaling transducer for Hh, as well as that of *Ihh* itself, caused no bone formation in mice [14]. These mutant mice lacked the expression of *Runx2*, a key determinant for osteoblasts, suggesting that the activation of Hh is required for the specification into Runx2+ osteoblast precursors, whereas it is dispensable for the osteoblast maturation beyond the precursor stage [15].

In addition to the functions of Hh signaling in bone development, we and others have revealed the involvement of Hh signaling in adult bone homeostasis [16–19] and fracture healing [18]. We confirmed the therapeutic effect of the smoothened agonist (SAG) on a bone defect model in rat femurs by using artificial bones loaded with SAG [20]. In the present study, we tested an application of SAG for bone fracture healing in a clinically relevant manner, i.e., an injection of SAG in a mouse model. We estimated the bone fracture healing with qualitative and quantitative methods and gained molecular-level insight into the therapeutic effects of the manipulation of Hh signaling on bone fracture healing.

2. Materials and methods

2.1. Animals

Eight-week-old male C57BL/6J mice were obtained from Charles River Japan (Kanagawa, Japan). Seven mice were used for each experimental group. All experiments were performed in accord with the protocol approved by the Animal Care and Use Committee of The University of Tokyo (#KA13-5).

2.2. Reagent preparation

Smoothened agonist (SAG) (566660, Calbiochem, Darmstadt, Germany) was dissolved in distilled water to make a 100 μM solution. For injection of the SAG solution into the mice, 30 G insulin syringes (#326638, BD Biosciences, San Jose, CA) were used. Distilled water was used as a vehicle control.

2.3. Fracture model

Eight-week-old C57BL6/J male mice were used for the fracture model. Each mouse was anesthetized with 3% isoflurane in O_2. Under a sterilized condition, the skin, subcutaneous tissue, and fascia in the left limb were incised along the anterior part of the leg, and the tibia was exposed. A transverse osteotomy was performed using a diamond cutting disc at the mid-diaphysis as described [18]. The bone marrow cavity of the fractured tibia was internally stabilized by insertion of a 23 G spinal needle (SN-2370, Terumo Clinical Supply, Kakamigahara, Japan). After irrigation with saline, the skin was stitched up with 4-0 nylon sutures. On postoperative day 1 (POD1), 10 μL of the 100 μM SAG solution or vehicle was slowly injected into the fracture site. On POD 14, the fractured tibiae were harvested from the euthanized mice.

2.4. Sample preparation

All mice underwent perfusion fixation with 4% paraformaldehyde under 3% isoflurane anesthesia in O_2. The fractured tibiae were carefully dissected and additionally fixed in 4% paraformaldehyde/phosphate-buffered saline (PBS) for 1 h at 4 °C. The tibiae were stored in PBS until further processing. Intramedullary spinal needles were removed before three-dimensional micro-computed tomography (3D-μCT) scanning. After the 3-D μCT scanning, the tibiae were decalcified with 0.5 M ethylenediaminetetraacetic acid (EDTA) for 21 days. Standard 10-μm frozen sections were made for immunostaining. The CryoJane tape-transfer system (39475205, Leica Biosystems, Nussloch, Germany) was used to obtain frozen sections for hematoxylin and eosin (H&E) and Alcian blue double staining (10-μm thickness). Paraffin sections were prepared for Masson's trichrome staining (8-μm thickness).

2.5. Radiological analysis

The 3-D μCT scanning was performed using a microfocus X-ray CT system (SMX-90CT, Shimadzu, Kyoto, Japan) under the following conditions: tube voltage, 90 kV; tube current, 110 μA; and field of view (XY), 13.5 mm. The resolution of one CT slice was 512 × 512 pixels. We used the three-dimensional construction software package TRI/3D-BON (Ratoc System Engineering, Tokyo) for bone morphometric analysis. The images of callus and bone in Fig. 2A were created in a 5 × 5 × 4-mm cuboid at callus where a fracture line was adjusted to be centered. Bone area and callus were defined as different levels of bone mineral density (BMD) values as described [21]: BMD value > 700 = bone area; 700 > BMD value > 350 = highly mineralized callus; 350 > BMD value > 200 = less mature callus. The following parameters were quantified in the defined callus regions: total callus volume, total bone volume (BV_T), high-density bone volume (BV_H), low-density bone volume (BV_L), total bone mineral density (BMD_T), highly mineralized bone density (BMD_H), total bone mineral contents (BMC_T), highly mineralized bone mineral contents (BMC_H), and low-density bone mineral contents (BMC_L).

2.6. Histological analysis

Vehicle- and SAG-injected samples were stained with H&E and Alcian blue as described [18]. To assess new bone formation, we performed Masson's trichrome staining according to the standard protocol [22]. To detect Sp7 and Sox9 expressions in fractured tibiae, we performed immunostaining. Briefly, the sections were incubated with antibodies against SP7 (1: 2000; ab22552, Abcam, Cambridge, MA), SOX9 (1: 500; AB5535, Millipore, Bedford, MA), and proliferating cell nuclear antigen (PCNA; 1:1000; 2586s, Cell Signaling Technology, Beverly, MA) as primary antibodies.

For the Sp7 and Sox9 immunostaining, Anti-Rabbit IgG (H&L) horseradish peroxidase (HRP)-conjugated secondary antibody (W402B, Promega, Madison, WI) were used as secondary antibodies. For the PCNA immunostaining, the CSAII Biotin-free Tyramide Signal Amplification System (K1497, Dako, Glostrup, Denmark) was combined with the primary antibody. Antigen-antibody complex was visualized by diaminobenzodine (DAB), which gave a brown signal. Methyl green was used for counterstaining. Histological images were captured by a fluorescence microscope (BZ-X710, Keyence, Osaka, Japan), and we quantified the immunostaining data by counting stained cells in the middle of the soft callus (200 × 200 μm) using ImageJ software (U.S. National Institutes of Health, http://rsbweb.nih.gov/ij/download.html). The percentage of positive cells was calculated by the number of positive cells per total cells in the determined area.

2.7. Statistical analysis

Significant differences in values were identified by Student's t-test. *P*-values <0.05 were considered significant.

Please cite this article in press as: M. Kashiwagi, et al., Local administration of a hedgehog agonist accelerates fracture healing in a mouse model, Biochemical and Biophysical Research Communications (2016), http://dx.doi.org/10.1016/j.bbrc.2016.09.134