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Effect of aging on the osteoinductive activity of recombinant human bone morphogenetic protein-2 in rats

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ARTICLE INFO

Article history:
Received 14 April 2014
Received in revised form
16 October 2014
Accepted 26 January 2015
Available online 30 January 2015

Keywords:
Bone morphogenetic protein (BMP)
Osteoinduction
Aging
Rats

ABSTRACT

Background: Bone morphogenetic proteins may hold broad potential for use in the reconstruction of bone defects resulting from tumor resection or trauma and in assisting bone healing thanks to methods enabling the synthesis of recombinant human bone morphogenetic protein-2 (rhBMP-2).

Methods: rhBMP-2 was implanted with atelopeptide type I collagen as a carrier into the calf muscles of 3-, 8-, and 48-wk-old Wistar/ST male rats. After 21 d, the formation of ectopic neoplastic bone was examined in soft x-ray imaging, and the bone mineral content, bone area, and bone mineral density (BMD) were measured by dual-energy x-ray absorptiometry. In addition, hematoxylin-eosin and von Kossa staining and proliferation cell nuclear antigen immunostaining were performed.

Results: BMD values determined by dual-energy x-ray absorptiometry were 29.40 (standard deviation ±5.47), 24.15 (±2.33), and 19.01 (±2.02) mg/cm^2 in the 3-, 8-, and 48-wk-old rats, respectively, demonstrating that BMD significantly decreased with aging ($P < 0.05$). The von Kossa stain-positive area decreased significantly with aging ($P < 0.01$). The number of proliferation cell nuclear antigen-positive cells also decreased significantly with aging ($P < 0.01$).

Conclusions: The capacity of rhBMP-2 to induce ectopic bone formation decreases with aging. These findings will be of considerable benefit in the development of clinical treatments for the regeneration of cranio-maxillofacial bone in elderly patients.

1. Introduction

Bone morphogenetic proteins (BMPs) play important roles in the migration of osteoblast progenitor cells, the proliferation of mesenchymal cells, differentiation to chondrogenic and osteogenic cells, and bone remodeling [1,2]. BMPs may hold broad potential for use in the reconstruction of bone defects resulting from tumor resection or trauma and in assisting bone healing thanks to methods enabling the synthesis of recombinant human bone morphogenetic protein-2 (rhBMP-2) [3]. We previously evaluated the osteoinducing activity of rhBMP-2 combined with atelopeptide type I collagen as a carrier in a rodent model [4,5]. Although it is generally well known that the differentiation and proliferation activities of

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0022-4804/$ – see front matter
http://dx.doi.org/10.1016/j.jss.2015.01.050

cells decrease with aging, the relationship between aging and ectopic bone formation induced by rhBMP-2 is hardly clarified.

The aim of the present study therefore was to investigate the effect of aging on osteoinduction induced by rhBMP-2. To elucidate whether aging affects rhBMP-2–induced ectopic bone formation, we examined the histology, bone mineral content (BMC), bone area (BA), and bone mineral density (BMD) of new ectopic bone.

2. Materials and methods

Male Wistar/ST rats aged 3-, 8-, and 24-w were used in the study ($n = 12$ rats per group). In previous reports on BMP-2 using atelopeptide type I collagen carrier, only males were used for the experimental animal model [4–10] because the estrus cycle in female rats may influence osteoinduction. This animal study was carried out in accordance with the Guidelines for Animal Experimentation of Kansai Medical University and was approved by the Animal Experimentation Committee of Kansai Medical University. All the animals received humane care in compliance with the "Guide for the Care and Use for Laboratory Animals, Eighth Edition," as promulgated by the National Research Council and published by the National Academies Press, 2010.

2.1. Implants and implanting procedure

Recombinant human BMP-2 (batch number OPB21001) was purchased from Osteogenetics GmbH (Wuerzburg, Germany). rhBMP-2 (5 μg) was dissolved in 1 mL of a solution containing 3 mg/mL of atelopeptide type I collagen (Cellmatrix I-C; Nitta Gelatin Inc, Osaka, Japan). Atelopeptide type I collagen served as a carrier and was purified from fresh porcine skin; the telopeptide was removed by proteolytic digestion. The solutions were lyophilized (EYELA FDU-2200; Tokyo Rikakikai Inc, Tokyo, Japan) and the resulting dried material was compressed in a syringe (SS-01T2613S; Terumo Corporation, Tokyo, Japan) to make a discoid implant, 4.2 mm in diameter and 1.5 mm in thickness. rhBMP-2 was added to 12 implants in each group. Rats were lightly anesthetized with isoflurane (2%) and then deeply anesthetized with sodium pentobarbital (30 mg/kg, intraperitoneal injection). After removal of the calf hair of each rat with hair-removal cream, the skin and muscular fascia were cut to produce a muscle pouch, into which the disc implant was inserted. The fascia and the skin were then tightly sutured, layer by layer.

2.2. Radiologic examination

Three weeks after the implantation, rats were euthanized with an overdose of CO_2 gas. Palpable implants were excised and exposed to a special film (XIFR; Fujifilm, Tokyo, Japan) using a soft x-ray system (conditions used: 20 kV, 2.5 mA, 90 s, 60 cm from the x-ray tube; TRS-1005; Sofron, Tokyo, Japan). Subsequently, the BMC and BA of the implants were measured by dual-energy x-ray absorptiometry (DXA, DSC-600EX-IIIR; Aloka, Tokyo, Japan). The BMD was calculated as BMC per unit of BA.

2.3. Histologic examination

After radiologic examination, the bone specimens were fixed in 10% neutral-buffered formalin solution and frozen in Optimal Cutting Temperature compound (4583, Tissue-Tek O.C.T. Compound; Sakura Finetek Japan, Co, Ltd, Tokyo, Japan) (frozen section embedding medium). Frozen sections (7 μm) were stained with von Kossa reagent and visualized under a BZ-9000 microscope (Keyence, Osaka, Japan). The von Kossa-stained areas ($n = 12$ per group, 1 field in each slide) were identified using Hybrid Cell Count image analysis program (Keyence). Subsequently, a subsample was removed from each frozen specimen, embedded in paraffin, decalcified, and sliced, after which it was subjected to hematoxylin-eosin (H-E) staining and proliferation cell nuclear antigen (PCNA) immunostaining. Images of the H-E and PCNA slides were captured and converted into digital slides using a NanoZoomer 2.0-HT digital slide scanner (C9600-13; Hamamatsu Photonics K.K., Shizuoka, Japan). The PCNA-positive cells among osteoblasts ($n = 12$ per group, 1 field in each slide) were identified using Hybrid Cell Count image analysis program (Keyence).

2.4. Statistical analysis

The Mann–Whitney *U*-test was used to compare differences between groups, with significance set at either $^{*}P < 0.05$ or $^{**}P < 0.01$. Data are presented as the mean ± standard deviation.

3. Results

All 36 rats survived throughout the experimental period. No animals showed signs of local infection when the wound was opened to extract the implant after 21 d.

3.1. Radiologic evaluation

A radio-opaque oval shadow was observed in all samples from all groups. Typical soft x-ray images for each group are shown in Figure 1. The oval shadows observed in the 48-wk-old rats were a little narrower and exhibited lower radio-opacity than those observed in the 8- and 3-wk-old rats. In addition, the oval shadows observed in the 8-wk-old rats were a little narrower and exhibited somewhat lower radio-opacity than those observed in the 3-wk-old rats.

DXA results and calculated BMC, BA, and BMD values are shown in Figure 2. The application of x-rays of two different energies to a measurement site in DXA enables the distinguishing of bone components from other tissues, with a minimum of error. Thus, DXA is used as a standard method for bone mass measurement. As shown in Figure 2A, the BMC values were 9.0 ± 4.77, 3.39 ± 1.00, and 1.49 ± 0.79 mg in the 3-, 8-, and 48-wk-old rats, respectively, demonstrating that BMC decreased significantly with aging ($P < 0.01$). In Figure 2B, the BA values were indicated in 0.29 ± 0.11, 0.14 ± 0.03, and 0.08 ± 0.03 cm^2 in the 3-, 8-, and 48-wk-old rats, respectively, that showed BA significantly decreased paralleled with aging ($P < 0.01$). Figure 2C shows that the BMD values calculated from the BMC and BA data were 29.40 ± 5.47, 24.15 ± 2.33, and 19.01 ± 2.02 mg/cm^2 in the 3-, 8-, and 48-wk-old rats,