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The effect of crosslinking heparin to demineralized bone matrix on mechanical strength and specific binding to human bone morphogenetic protein-2

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Abstract

Demineralized bone matrix (DBM) is a collagen-based scaffold, but its low mechanical strength and limited BMP-2 binding ability restrict its application in bone repair. It is known that heparin could be immobilized onto scaffolds to enhance their binding of growth factors with the heparin-binding domain. Here, we crosslinked heparin to DBM to increase its BMP-2 binding ability. To our surprise, the mechanical strength of DBM was also dramatically increased. The compression modulus of heparin crosslinked DBM (HC-DBM) have improved (seven-fold increased) under wet condition, which would allow the scaffolds to keep specific shapes *in vivo*. As expected, HC-DBM showed specific binding ability to BMP-2. Additional studies showed the bound BMP-2 exerted its function to induce cell differentiation on the scaffold. Subcutaneous implantation of HC-DBM carrying BMP-2 showed higher alkaline phosphatase (ALP) activity (2 weeks), more calcium deposition (4 and 8 weeks) and more bone formation than that of control groups. It is concluded that HC-DBM has increased mechanical intensity as well as specific BMP-2 binding ability; HC-DBM/BMP-2 enhances the osteogenesis and therefore could be an effective medical device for bone repair.

Keywords: Heparin; Demineralized bone matrix; Bone morphogenetic protein-2; Ectopic bone formation

1. Introduction

Demineralized bone matrix (DBM) is one of the extensively used scaffolds for bone repair. Its porous structure is suitable for bone growth and its matrix proteins, such as collagen, provide the osteoconductive matrix [1]. Studies have demonstrated the clinical potential of DBM implants in the treatment of bone defects [2–4]. However, the bone inducing growth factors are often eliminated during the preparing process, thus it does not have enough osteoinductivity. On the other hand, the elimination of mineral component decreases the mechanical strength of DBM, which is often critical for bone repair.

To establish the osteoinductivity of DBM, bone morphogenetic protein-2 (BMP-2) was often employed to activate the scaffold for its multi-function on bone induction, maintenance, and repair [5]. However, studies have shown that the repair was limited and unhomogeneous because the simple addition of BMP-2 to the DBM usually leads to a rapid diffusion of BMP-2 from the implant site [6,7]. Thus, modifications of DBM to allow it to possess a high BMP-2 binding capacity represent a major challenge.

Heparin, a sulfated polysaccharide which plays a critical role in the regulation of various biological processes, has been shown to have the binding affinity with a number of biologically important proteins, including bFGF, VEGF and BMP-2 [8]. Heparin has been incorporated into biomaterials to immobilize growth factors by its growth factor binding domain [9–12]. Such heparin-containing systems showed some

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advantages in making controlled release for these growth factors [13,14]. Biological activities of the heparin-binding growth factors loaded in scaffolds were also preserved by the specific binding via the heparin-binding domain [15]. The results by Steffens et al. showed loading of VEGF to heparinized collagen matrices leaded to an increased angiogenic potential when compared to loading the same amount of VEGF to non-heparinized collagen matrices [11]. Heparin-crosslinking PLGA loaded with BMP-2 also promoted enhanced bone formation [15,16]. In this work, scaffolds and heparin molecule were crosslinked using *N*-hydroxysuccinimide (NHS) and *N*-(3-di-methylaminopropyl)-*N*9-ethylcarbodiimide (EDC), respectively. Then EDC and NHS reacted with each other to link the scaffold and heparin covalently, which had been demonstrated to be non-cytotoxic *in vitro* as well as *in vivo* after completely washing [17–19].

In this study, we presented a bone defect replacement matrix using the heparin crosslinked DBM plus BMP-2. We tested the effects of crosslinking of heparin to DBM on the mechanical intensity of DBM and its specific binding ability to BMP-2. We also tested the bioactivity of the functional biomaterial composed with BMP-2 and heparin-crosslinking DBM (HC-DBM) both *in vitro* and *in vivo*.

2. Materials and methods

2.1. Preparations of DBM and heparin-crosslinking DBM (HC-DBM)

DBM was obtained from Zhenghai Biotechnology Inc. (Shandong, China), which was prepared from bovine sponge bones. The methods of preparing DBM were the same as the reference described before [7,20].

Incorporation of heparin to the DBM was performed with a routine procedure that was a little modified from Yao et al. [6]. Briefly, 2 mg of heparin (H-4784; Sigma, USA) was dissolved with 2 mg EDC/1.2 mg NHS (39391 and 14405, Sigma, USA) in 1 ml of 0.05 M MES buffer (pH 5.6). DBM was then immersed into this solution and the mixtures were evacuated at 200 Pa for about 30 s to remove the air within the DBM. The reaction was proceeded for 4 h at 37 °C, after which the DBM was taken and extensively washed with 0.1 M Na_2HPO_4 (2 h), 4 M NaCl (three times in 24 h) and distilled water (three in 24 h), respectively. After the modification procedure, matrices were frozen at −80 °C overnight and lyophilized. For cell culture and animal study, scaffolds were sterilized by C_{60} irradiation.

2.2. Calculating the amount of heparin crosslinked on the HC-DBM

The amount of heparin crosslinked to the DBM was determined with the toluidine blue method [21]. Fifty mg of toluidine blue was dissolved in 0.01 N hydrochloric acid containing 0.2 wt % NaCl, then 20 mg HC-DBM was added to the 1 ml toluidine blue solution and the mixtures were agitated with a vortex mixer. After removing the scaffolds from solution, the unreacted toluidine blue in the water phase was determined by absorption at 620 nm with a plate reader (TECAN, SUNRISE, Austria). The amount of heparin crosslinked to DBM was calculated based on reference standards.

2.3. Mechanical measurement

After DBM and HC-DBM were soaked into PBS overnight, the compression test for wet DBM and HC-DBM was done at room temperature on an autograph machine (Tinius olsen H5KS, Hong Kong, China) at a crosshead speed of 1 mm/min. The compression modulus was calculated from the slope of the initial linear portion of the stress–strain curve which was automatically made from the machine. The experiment was performed for six samples independently.

2.4. Morphological observation

The morphology of DBM and HC-DBM was observed with a scanning electron microscope (SEM, S-3000N; Hitachi, Tokyo, Japan). The samples were dried by freeze drier and coated with gold on an ion sputterer (E-1010; Hitachi), then viewed by SEM at a voltage of 15 kV.

2.5. In vitro bioactivity assay

BMP-2 was produced from our lab, and $His_{\times 6}$ was fused to purify and detect BMP-2 [7]. The experiment was manipulated under sterilized condition. Ten mg of DBM and HC-DBM were placed into each 96-well cell culture plate (Costar 3599, Corning Inc., USA), respectively. BMP-2 solutions with an amount of 55.0 μg, 18.3 μg, 2.0 μg and 0.7 μg (100 μl/well) were added to those 96-well plates and incubated at 37 °C for 1 h. Then the solutions were collected to perform enzyme linked immunosorbent assay (ELISA) to measure the bound BMP-2 on DBM and HC-DBM.

The scaffolds were extensively washed with PBS for three times, then mouse C2C12 cells were added at a density of 5000 cells/well and maintained in 150 μl Dulbecco's Modified Eagle's Medium (DMEM, Gibco, Grand Island, NY, USA) containing 2% (v/v) fetal bovine serum (Gibco, Grand Island, NY, USA) at 37 °C with 5% (v/v) CO_2 for 3 days, then the intracellular alkaline phosphatase (ALP) activities of cells on plates and scaffolds were determined, respectively.

2.5.1. BMP-2 bound to DBM and HC-DBM

BMP-2 solutions of different concentration after mixed with DBM or HC-DBM were collected. To measure BMP-2 in the solution, ELISA was performed. The primary antibody was anti-poly-histidine antibody (1:1000, Sigma, USA), and an alkaline phosphatase (ALP)-conjugated goat anti-mouse IgG (1:10,000 dilution, Sigma, USA) was utilized as the secondary antibody. The procedures were based on the method described before [7]. According to the reference standards under same condition, BMP-2 in solutions was calculated, and then the bound BMP-2 on scaffolds could be gained.

2.5.2. ALP activity assay

The bioactivity of BMP-2 bound on DBM or HC-DBM *in vitro* was assessed by determining its ability to stimulate the intracellular alkaline phosphatase (ALP) activity of C2C12 cells on the scaffolds [22].

After 3 days stimulation, scaffolds were taken from the wells and cells on the scaffolds were independently washed with PBS and lysed with 0.1% TritonX-100/PBS and repeatedly frozen/thawed for three times to disrupt the cell membranes. Lysates were then cleared by centrifugation for 10 min at 12,000*g*. Subsequently, 50 μl solution was utilized to determine the protein content which was measured by the BCA protein assay reagent (Pierce Chemical, Rockford, IL, USA), and other 50 μl solution was incubated with 50 μl *p*-nitrophenylphosphate (2 mg/ml in AP buffer, Sigma) at 37 °C for 30 min. The reaction was stopped by adding 100 μl 2 N NaOH. The absorbance of the samples was read at 405 nm using an ELISA plate reader.

2.6. In vivo experiment – assessment of the ectopic bone formation

Subcutaneous implantation was carried out to estimate the bone formation of DBM and HC-DBM carrying BMP-2. During these animal experiments, Chinese Ministry of Public Health (CMPH) guidelines for the care and use of laboratory animals have been observed. Male Sprague-Dawley (SD) rats weighing 200–225 g were anesthetized by an intraperitoneal injection of sodium pentobarbital (4 mg/100 g body weight). When the dorsal hair was shaved, two independent incisions were made subcutaneously on each dorsolateral side of the rat. Then the sample measuring 8 × 5 mm area and 2 mm thickness was inserted. There were four groups employed: (1) DBM loading