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Enhanced bioactivity of bone morphogenetic protein-2 with low dose of 2-*N*, 6-*O*-sulfated chitosan *in vitro* and *in vivo*

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ABSTRACT

Bone morphogenetic protein-2 (BMP-2) has been widely used as an effective growth factor in bone tissue engineering. However, large amounts of BMP-2 are required to induce new bone and the resulting side effects limit its clinical application. Sulfated polysaccharides, such as native heparin, and heparan sulfate have been found to modulate BMP-2 bioactivity and play pivotal roles in bone metabolism. Whereas the direct role of chitosan modified with sulfate group in BMP-2 signaling has not been reported till now. In the present study, several sulfated chitosans with different positions were synthesized by regioselective reactions firstly. Using C2C12 myoblast cells as *in vitro* models, the enhanced bioactivity of BMP-2 was attributed primarily to the stimulation from 6-*O*-sulfated chitosan (6SCS), while 2-*N*-sulfate was subsidiary group with less activation. Low dose of 2-*N*, 6-*O*-sulfated chitosan (26SCS) showed significant enhancement on the alkaline phosphatase (ALP) activity and the mineralization formation induced by BMP-2, as well as the expression of ALP and osteocalcin mRNA. Moreover, increased chain-length and further sulfation on 26SCS also resulted in a higher ALP activity. Dose-dependent effects on BMP-2 bioactivity were observed in both sulfated chitosan and heparin. Compared with native heparin, 26SCS showed much stronger simultaneous effects on the BMP-2 bioactivity at low dose. Stimulated secreted Noggin protein failed to block the function of BMP-2 in the presence of 26SCS. The BMP-2 ligand bound to its receptor was enhanced by low dose of 26SCS, whereas weakened by the increasing amounts of 26SCS. Furthermore, simultaneous administration of BMP-2 and 26SCS *in vivo* dose-dependently induced larger amounts of ectopic bone formation compared with BMP-2 alone. These findings clearly indicate that 26SCS is a more potent enhancer for BMP-2 bioactivity to induce osteoblastic differentiation *in vitro* and *in vivo* by promoting BMP-2 signaling pathway, suggesting that 26SCS could be used as the synergistic factor of BMP-2 for bone regeneration.

1. Introduction

Bone morphogenetic protein-2 (BMP-2), which is a member of the transforming growth factor-β (TGF-β) superfamily of multifunctional cytokines, has remarkable ability to induce bone formation and bone tissue reconstruction, is secreted in adult vertebrates, and plays critical roles in osteogenesis and bone metabolism [1–5]. However, it was reported that half of BMP-2 was degraded within 1 h *in vitro* [6]. And more than 100-fold larger amounts of BMP-2 (up to milligram) are required to induce new bone formation in higher animals, such as monkeys and humans than in rodents [7]. In addition, high dose for implantation may also be associated with potential side effects such as the stimulation of bone resorption, excessive bone formation and nerve cell reactions in unintended areas [8–11]. To overcome these shortcomings, carriers, such as scaffolds or microspheres, have been established to sustain the release of BMP-2 in local bone defect areas [7,12–15]. Considerable researches have been attempted to identify specific agents or materials to enhance the bioactivity of BMP-2 *in vitro* and *in vivo* as a direct approach. In recent years, several sulfated polysaccharides such as heparin and heparin sulfate were reported to stimulate the biological activity of BMP-2 *in vitro* [16]. In the

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presence of heparin, half-life of BMP-2 in the culture medium was prolonged by nearly 20-fold and larger amount of bone formation was observed in the *in vivo* model [6]. However, some conflict viewpoints have also been published recently, which shows that exogenous heparin inhibits BMP-2 osteogenic bioactivity and reduces BMP-2 signaling [17–19]. On the other hand, from the well known clinical observation, short-term use of heparin after bone fracture can delay bone healing and long-term treatment with heparin can increase the risk of osteoporosis [20–23]. These drawbacks might be critical obstructions for the usage of heparin on clinic.

From the uncertain bioactivity of heparin and clinical requirements on enhancing BMP-2 bioactivity, it's rather important to seek for such kind of agents which can synergistically work with BMP-2. Over the years, chemical modification on sulfated chitosan has attracted great interest because it promotes the structure similarity to that of heparin and achieves higher degree of sulfation than any natural sulfated polysaccharides. In fact, sulfated chitosans have been applied as effective blood anticoagulant (a typical clinical function for heparin) [24]. On the other hand, low cytotoxicity raises its feasibility of application on developing specific anti-HIV agents [25]. However, the direct role of sulfated chitosans on BMP-2 activity has not been reported till now. In the present study, chitosans modified with different sulfation were synthesized, and we describe for the first time that dose-dependent effects of 2-*N*, 6-*O*-sulfated chitosan on the bioactivity of BMP-2 were observed *in vitro* and *in vivo*. The availability of sulfated chitosan to enhance the bioactivity of BMP-2 was demonstrated, which provided a direct and effective approach to reduce costs and risks of BMP-2 implantation.

2. Materials and methods

2.1. Materials

All reagents used are available from commercial sources, used as received, unless otherwise noted. Two kinds of highly deacetylated (>90%) chitosans with M_w of $20–30 \times 10^4$ and $5–8 \times 10^4$ were purchased from Carbo and Weikang Biologics (Shanghai, China), respectively. Heparin derived from porcine intestine was purchased from Shanghai Yuanju Biotechnology Co. Ltd. Chlorosulfonic acid was from Sinopharm Chemical Reagent Co. (Shanghai, China), pyridine-sulfur trioxide (SO_3-Pyd) from Acros Chem. (New Jersey, USA). Recombinant human BMP-2 was generous gift from Shanghai Rebone Biomaterials Co. Ltd. (Shanghai, China). Water used in all experiments was purified using a Millipore Synergy 185 System (≥18 MΩ resistance).

2.2. Synthesis and characterization of sulfated chitosan

2.2.1. 2-N-Sulfated chitosan (2SCS)

Low molecular weight chitosan ($5–8 \times 10^4$, 1 g) was dispersed in 75 mL of water under gentle agitation. Then, 2 g of Na_2CO_3 was added to maintain alkaline environment (pH>9). 3 g SO_3-Pyd was added to this viscous solution to start the reaction. The mixture was maintained at 60 °C for 24 h under argon atmosphere to form a yellow mixture [26].

2.2.2. 6-O-Sulfated chitosan (6SCS)

6-*O*-Sulfated chitosan was prepared according to Nishimura's method [25] with minor modifications. Briefly, high molecular weight chitosan ($20–30 \times 10^4$, 2 g) was added to 50 mL of formamide, 5 mL of concentrated sulfuric acid (98%) was added to maintain high acidity reaction environment (pH < 1). With the protection of argon, 10 mL of $HClSO_3$ was added dropwise with mechanical stirring and cooling. The reaction was run at 0–4 °C for 3 h to obtain a pale yellow mixture.

2.2.3. 2-N, 6-O-Sulfated chitosan (26SCS)

2-*N*, 6-*O*-Sulfated chitosan was prepared using the similar method as reported [27]. In short, sulfating reagent was prepared firstly, $HClSO_3$ was added dropwise to 50 mL of *N*,*N*-dimethylformamide (DMF) cooled at 0–4 °C, the mixture was stirred for stabilization for 15 min and then transferred to a three-necked bottomed flask containing 2.5 g chitosan ($20–30 \times 10^4$) in 50 mL of formamide and 2 mL of formic acid. Under mechanical agitation and argon atmosphere, the reaction was maintained at 45–55 °C for 2 h, a homogeneous pale yellow solution was obtained finally.

After the three reactions described above, the following post-processing treatments were performed: 500–1000 mL of EtOH was added to precipitate the products. The precipitation was filtered under vacuum, washed with EtOH for at least 3 times, and then redissolved in water. The pH value was adjusted to 7–8 with 1 N HCl or 1 N NaOH. The mixture with undissolved substance was centrifuged at 5000 rpm for 15 min, the supernatant was obtained and dialyzed against water for 3 d with a 14 000 Da M_w cut-off dialysis membrane. Consequently, all kinds of sulfated chitosans were then obtained by lyophilization.

2.2.4. Characterization of sulfated chitosan

The FTIR spectrum of sulfated chitosan was measured by ATR on Nicolet 380 (Thermo, USA). Laser light scattering was carried out to measure molecular weight on Dawn Heleos light scattering instrument (ALV/CGS-5022F). As all the sulfated chitosans obtained in this paper were water-soluble, 0.1 N $NaNO_3$ aqueous solution was used as solvent at a flow rate of 0.5 mL/min. Sulfur content (%) was measured by Elementar (Vario Macro, Germany). The sulfur content of heparin used in this paper is 10.23%.

2.3. Evaluations of sulfated chitosan on the bioactivity of BMP-2 in vitro

2.3.1. Cell culture

C2C12, a myoblastic precursor cell with osteoblastic potential, was purchased from the American Type Culture Collection (ATCC). C2C12 cells were cultured in 37.5 cm^2 flasks with growth medium (Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) containing 10% fetal calf serum (Sijiqing, Hangzhou, China), antibiotics (100 U/mL penicillin-G, and 100 μg/mL streptomycin)) at 37 °C in humidified atmosphere of 5% CO_2/95% air until confluence, then detached with 0.25% trypsin/0.03% ethylenediamine tetraacetic acid (EDTA) and the cell density was calculated and used at the desired density in later experiments.

2.3.2. Determination of alkaline phosphatase (ALP) activity

To detect the ALP activity induced by BMP-2, C2C12 cells were seeded at a density of 10×10^4/mL to 96-well plates. After 24 h incubation in growth medium, cells were washed with PBS twice and refreshed with DMEM containing 2% fetal calf serum (called maintenance medium) in the presence of BMP-2 with or without sulfated chitosan or heparin. At the end of 72 h incubation, 50 μL 1% Nonidet P-40 (NP-40) solution was added to each well at room temperature (RT) for 1 h to obtain cell lysate, to which 50 μL of 1 mg/mL *p*-nitrophenylphosphate (Sangon, Shanghai, China) substrate solution (pH = 9) composed of 0.1 mol/L glycine, 1 mmol/L $MgCl_2 \cdot 6H_2O$ was added and incubated for 15 min at RT. The reaction was quenched by adding 100 μL of 0.1 N NaOH, the absorbance of ALP was quantified at the wavelength of 405 nm using a microplate reader (SPECTRAmax 384, Molecular Devices, USA). To determine the ALP activity histochemically, cells were fixed with 1% glutaraldehyde for 10 min on ice, washed with PBS, and incubated in a mixture of 0.5 mg/mL naphthol AS-BI phosphate (Sigma, St. Louis, USA), 2% (V/V) DMF, 0.05 mol/L $MgCl_2$, 0.01 mol/L levamisole, and 1 mg/mL fast blue BB salt (Yuanju, Shanghai, China) in 0.2 mol/L Tris–HCl (pH = 9.0) for 30 min at RT. Stained cells were visualized and photographed with inverted light microscope (TE2000U, Nikon Corp., Japan).

2.3.3. Semi-quantitative reverse transcription polymerase chain reaction

Cells were cultured to 80% confluency in 10-cm Corning® dishes with 0.8 μg/mL BMP-2 in the presence of indicated concentrations of 26SCS for 48 h. Total RNAs were extracted from the cells using the mRNA Purification Kit (Shanghai Shenergy Biocolor Bioscience & Technology Company, China) according to the manufacturer's guidelines. The concentrations of RNA were determined by A_{260nm}/A_{280nm}. Complementary first strand DNA (cDNA) was synthesized from 2 μg of total RNA using the reverse transcriptase MMLV (Promega, USA) and Oligo (dT) (Takara, Japan) according to the manufacturer's protocol. One tenth of the cDNA products were used for PCR amplification with targeted primers designed as reported to amplify fragments corresponding to ALP, osteocalcin and Noggin mRNAs [16]. PCR was carried out as follows: an initial denaturation of 3 min at 94 °C was followed by 32 cycles of 45 s at 94 °C, 45 s at 52 °C, and 1 min at 72 °C, followed by 10 min of final elongation at 72 °C. Control PCR amplifications were performed with β-actin specific primers which were purchased from Waston Biotech (Shanghai, China). Oligonucleotide sequences for primers used in this study are listed in Table 1. Amplification products were analyzed through 1% agarose gel electrophoresis and following ethidium bromide staining. For an estimation of the relative expression, integrated optical densities of the bands were scanned and quantified by Total 2.01 (Nonlinear Dynamics Ltd, USA), and normalized by that of β-actin.

2.3.4. Immunofluorescence assay of BMP-2 localized on the surface of C2C12 cells

To observe the interactions of BMP-2 and receptors on cell layers, cells were precultured on chamber slides with 0.01% poly-L-lysine (PLL) for attachment for 24 h. Medium was supplemented with 0.8 μg/mL BMP-2 and/or 26SCS. After 4 h incubation, cells were placed on ice for 5 min, washed twice with ice cold PBS. Cells were fixed with 1% glutaraldehyde for 15 min at 4 °C. 1% BSA solution was