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Modulation of osteogenic activity of BMP-2 by cellulose and chitosan derivatives

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ABSTRACT

Polysaccharides with structure and potential bioactivity similar to heparin were synthesized based on cellulose which was regioselectively sulfated, carboxylated or carboxymethylated, and chitosan that was sulfated only. Osteogenic activity of these derivatives was studied in cooperation with BMP-2 using C2C12 myoblast cells as a model system measuring alkaline phosphatase (ALP) activity and the expression of the genes Osterix, Noggin and Runx-2. It was found that highly sulfated chitosan showed the strongest osteogenic activity of all polysaccharides, but only at lower concentrations, while higher concentrations were inhibitory. By contrast, cellulose with a low or intermediate degree of sulfation showed increasing ALP activity and expression of Osterix and Noggin with rising concentrations. Lower sulfated cellulose with a high degree of carboxylation was less osteogenic, but had a positive effect on cell viability, while carboxymethylated cellulose had almost no osteogenic activity. The results indicate that regioselectively sulfated as well as carboxylated cellulose and chitosan possess an osteogenic activity, which makes them interesting candidates for application in tissue engineering of bone.

1. Introduction

Worldwide, many patients suffer from traumatic bone lesions, osteoporosis and osteolytic bone metastases. The gold standard in bone replacement therapy is still autograft from the iliac crest of patients. However, they can only be obtained in limited quantities, which reduce their therapeutic potential. Hence the regeneration of bone by tissue engineering approaches has attracted broad attention [1]. Besides the use of hormones and other bone formation-stimulating factors, the family of bone morphogenic proteins (BMP) is well known for their capability to stimulate bone regeneration [2]. BMP also play critical roles during embryonic development [3], which makes them interesting for application in regenerative medicine. Among them, BMP-2 is one of the most effective inducers of osteogenic differentiation [4] and is used in various fields of bone regeneration [5]. However, the application of BMP in solution has several drawbacks. Besides their fast proteolytic degradation [6], the use of high doses can lead to adverse side effects such as bone resorption by increasing osteoclastic activity [7] or an exaggerated ossification of tissues [8]. To overcome these limitations, methods have recently been established using micro- and nanospheres or scaffolds as carrier systems for long-term controlled release of growth factors [9].

Certain glycosaminoglycans (GAG), such as heparin, have been used as components of controlled release systems, because of their ability to bind growth factors selectively [10]. But, the application of GAG from natural sources also has significant drawbacks, such as limited accessibility, relatively high costs and large batch-to-batch variability. For example, heparin and heparan sulfates have an unpredictable derivatization pattern, dependent on the source of isolation [11]. Hence, it is not surprising that the application of heparins from different sources may enhance, but may also diminish, the activity of BMP-2 [6,12]. Therefore, chemical routes to modify polysaccharides such as cellulose or chitosan in a defined way to achieve structural and functional similarity to heparin may provide significant advantages. Since chemical synthesis can be run with large amounts of these abundant polysaccharides in a reproducible manner, large quantities of well-defined products with the desired bioactivity could be possibly obtained.

So far, mostly native and rarely sulfated chitosans have been used as components of scaffolds [13], and micro- or nanoparticles for the release of growth factors such as BMP-2 [14] or FGF-2 [15]. Sulfated celluloses have been applied only for encapsulation of cells and enzymes so far [16,17], but have expressed anticoagulant activity *in vitro* [18]. Composites of chitosan and cellulose have also been used as drug release systems [19]. It should be emphasized that all these compounds were homogeneously sulfated with no site-specific modifications [16,17]. It is also interesting to note that studies on the direct effects of sulfated chitosan on the bioactivity of BMP-2 were rarely done. One recent study showed superior

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activity of sulfated chitosan in comparison with heparin [20]. However, no attempts to investigate the potential of cellulose derivatives to modulate the osteogenic activity of BMP-2 have been published so far. Indeed, it was shown recently that regioselectively sulfated cellulose derivatives possess mitogenic activity in cooperation with fibroblast growth factor 2 which far exceeds that of heparin [21].

Hence, the current study investigates for the first time the effect of cellulose derivatives on BMP-2-induced osteogenic activity in comparison with sulfated chitosan and heparin as lead substances. A range of cellulose derivatives with different degrees of site-specific sulfation and carboxylation or carboxymethylation and one chitosan sulfate were synthesized. The osteogenic activity of derivatives in the presence of BMP-2 was studied with C2C12 cells, which can be converted into osteoblast lineage by BMP-2 [6,22] and not with osteoblastic or osteoprogenitor cells, because their osteogenic activity *per se* that would make the detection of the osteogenic potential of derivatives more difficult, The results show that not only sulfated chitosans, but also sulfated and carboxylated cellulose lead to higher BMP-2-induced osteogenic activity in C2C12 cells than heparin does. The results are reported herein.

2. Materials and methods

2.1. Materials

Microcrystalline cellulose (MCC) with an average molecular weight (M_W) of 5×10^4 was received from J. Rettenmaier & Söhne GmbH (Rosenberg, Germany). Native cellulose (AC, with 97.0% alpha cellulose) with an average M_W of 21×10^4 was purchased from Buckeye Technologies Inc. (Memphis, USA). Cellulose-2.5-acetate (C2.5A) with a degree of substitution by acetyl groups of 2.5 was received from M&G Group (Verbania-Pallanza, Italy). Chitosan with a degree of deacetylation of 95.7% and viscosity of 145 mPa was purchased from Heppe Medical Chitosan GmbH (Halle, Germany). Dimethylformamide (DMF) was freshly distilled before use, and all chemicals were of analysis grade and used as received. Heparin from porcine intestinal mucosa was obtained from Calbiochem (Gibbstown, USA) and recombinant human BMP-2 was purchased from Invitrogen (Karlsruhe, Germany). Freshly deionized water was applied during chemical synthesis, while MilliQ water was used during the biological experiments.

2.2. Synthesis and characterization of sulfated, carboxylated and carboxymethylated cellulose and sulfated chitosan

For acetosulfation, 10 g native cellulose (M_W 21×10^4) or MCC (M_W 5×10^4) was swollen in 500 ml DMF and converted into cellulose sulfate by dropwise addition of different amounts of chlorosulfuric acid or sulfuric acid and acetic anhydride for 5 h at different temperatures. For direct sulfation, native cellulose (10 g) was swollen, while C2.5A (10 g) was dissolved in 500 ml DMF. Then chlorosulfuric acid was added dropwise. The reaction was carried out at room temperature (RT) for different times. The products obtained by both reactions were isolated by pouring the mixture into 500 ml of a saturated solution of anhydrous sodium acetate in ethanol. The precipitate obtained from acetosulfation was deacetylated with 1 M ethanolic solution of sodium hydroxide for 15 h. The pH value was adjusted to 8.0 with acetic acid/ethanol (50/50, w/w). After washing with ethanol, the product was collected through centrifugation, then dissolved in water, filtered, dialysed against deionized water and finally lyophilized. The precipitate obtained from direct sulfation was washed with 4% sodium acetate solution in ethanol and collected by centrifugation. The product was dissolved in water and neutralized with acetic acid/ethanol (50/50, w/w). The solution was subsequently filtered, dialysed against deionized water and lyophilized as well.

Carboxylation and carboxymethylation of cellulose sulfates were conducted as described below. Briefly, for the homogeneous oxidation, 1 g of cellulose sulfate with low degree of sulfation was dissolved in 60 ml water. Then, the oxidation agent consisting of 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO)/NaBr/NaClO system was applied in a ratio of 0.1/3/16 mol per mol hydroxyl groups of cellulose sulfates. The pH was kept constant at 10.5 with NaOH for 4 h. After the addition of 5 ml methanol, the pH value of the solution was adjusted to 7.5 using 0.5 M aqueous HCl solution. The product was precipitated with 300 ml ethanol and collected by centrifugation. After washing with ethanol/water (80/20, v/v) and further treatment as described above for cellulose sulfates, the product was obtained.

Synthesis of carboxymethylated cellulose sulfates was achieved by dissolving lower sulfated celluloses in 175 ml DMSO. Then 22 ml NaOH aqueous solution (3.75 M) was added to the suspension and, after 3 h, 3.85 g chloroacetic acid in solid state was added. The reaction mixture was kept at 55 °C for 5 h, then cooled to RT and precipitated in five volumes of ethanol. The precipitate was collected by centrifugation again, then dissolved in water and adjusted to pH value 7.5 with acetic acid/water (50/50, v/v). After precipitating the solution in 5 volumes of ethanol, the product was finally purified as described above.

Synthesis of chitosan sulfates was carried out by activation of chitosan in a non-homogeneous sulfation reaction. Briefly, 1 g chitosan was dissolved in 1% aqueous acetic acid, followed by addition of 100 ml of methanol. After addition of 100 ml of 4% sodium hydrogen carbonate, 2 h stirring and washing, the activated chitosan was dispersed in 50 ml DMF for subsequent sulfation. Chlorosulfuric acid in DMF was added, and the mixture was kept at 50 °C for 3 h. After cooling to RT, the yellow to brown solution was poured into 600 ml saturated alkaline ethanolic solution of anhydrous sodium acetate. The obtained precipitate was dissolved in water after washing with ethanol/water mixture (8/2, v/v). The pH value of the solution was adjusted to 7.5, and the solution was filtered, dialysed against deionized water and finally lyophilized.

2.3. Characterization of derivatives

The ^{13}C NMR spectra of the samples in D_2O were recorded at RT with a Bruker DFX 400 spectrometer (Bruker) at a frequency of 100.13 MHz, 30° pulse length, 0.35 acquisition time and a relaxation delay of 3 s. Up to 20,000 scans were accumulated. The quantity of carboxymethyl groups of the cellulose derivatives was measured by ^1H NMR with a 200 Bruker Ultrashield 500 Plus spectrometer (Bruker Optik GmbH) after hydrolysis of the products with 25% D_2SO_4/D_2O and an accumulation of 16 scans.

The contents of carbon, hydrogen and nitrogen were determined with Elemental Analyser vario El from Elementar (Hanau, Germany). The sulfur content was measured with elemental analyser Eltra CS 500 (Neuss, Germany). The 2-*N*-sulfation of chitosan sulfate was measured with solid-state CP/MAS ^{15}N NMR, using a Bruker Avance 400 WB spectrometer (Bruker Optik GmbH). The degree of sulfation at the 3-*O*-position of chitosan sulfate was determined by subtraction of the degree of sulfation at the 2-*N*- and 6-*O*-position from the overall sulfur content.

2.4. Measurement of binding affinity of the polysaccharides to BMP-2

The affinity of cellulose derivatives and heparin to BMP-2 was examined in a competitive assay using heparin agarose beads (Fluka, Saint Louis, USA) according to the method described recently [23]: 25 ng of BMP-2 was mixed with 50 μl heparin agarose beads