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Leading opinion

The promotion of osteoclastogenesis by sulfated hyaluronan through interference with osteoprotegerin and receptor activator of NF-κB ligand/osteoprotegerin complex formation

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ABSTRACT

In order to improve bone regeneration, in particular in aged and multimorbid patients, the development of new adaptive biomaterials and their characterization in terms of their impact on bone biology is warranted. Glycosaminoglycans (GAGs) such as hyaluronan (HA) are major extracellular matrix (ECM) components in bone and may display osteogenic properties that are potentially useful for biomaterial coatings. Using native and synthetically derived sulfate-modified HA, we evaluated how GAG sulfation modulates the activity of two main regulators of osteoclast function: receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG). GAGs were tested for their capability to bind to OPG and RANKL using surface plasmon resonance (SPR), ELISA and molecular modeling techniques. Results were validated in an *in vitro* model of osteoclastogenesis. Sulfated GAGs bound OPG but not RANKL in a sulfate-dependent manner. Furthermore, OPG pre-incubated with different GAGs displayed a sulfate- and dose-dependent loss in bioactivity, possibly due to competition of GAGs for the RANKL/OPG binding site revealing a potential GAG interaction site at the RANKL/OPG interface. In conclusion, high-sulfated GAGs might significantly control osteoclastogenesis via interference with the physiological RANKL/OPG complex formation. Whether these properties can be utilized to improve bone regeneration and fracture healing needs to be validated *in vivo*.

1. Introduction

To date, bone grafts are one of the most demanded implant materials [1]. The increasing age of implant recipients has created new challenges for biomaterial design, since these implants are used in defective or osteoporotic bone or in patients with comorbidities that may impair osseointegration, such as diabetes mellitus [2]. Successful osseointegration of these implants depends on the characteristics and regenerative potential of the host bone as well as the material properties.

Physiological bone regeneration is a coordinated process, maintained by bone-forming osteoblasts and bone-resorbing osteoclasts (OC). The activity and differentiation of OCs are controlled by receptor activator NF-κB ligand (RANKL) and osteoprotegerin (OPG), two factors produced by bone-forming osteoblasts. RANKL binding to its receptor RANK on monocytic/macrophagic precursor cells induces osteoclastogenesis. In this system, OPG acts as a soluble decoy receptor that interferes with RANKL/RANK signaling by binding RANKL.

Recently, we reported that the sulfate modification of GAGs, a key component of the native ECM of bone, may be a promising tool to regulate biological processes at the bone/biomaterial interface [3]. The sulfate modification of GAGs led to a profound inhibition of OC differentiation and their resorptive capacity. Therefore, GAGs hold an interesting potential for the development of innovative

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biomimetic materials for bone tissue engineering applications and regenerative medicine [4].

Local GAGs in bone consist of about 90% chondroitin sulfate and small amounts of hyaluronan (HA), and dermatan sulfate [5]. These GAGs are linear polymers of repetitive disaccharide units whose chemical structures differ with respect to monosaccharide composition and posttranslational modification. Bound to a small core protein sulfated GAGs are subunits of proteoglycans. Because the properties of GAGs largely determine the properties of the entire proteoglycan molecule [6] and the lower immunogenicity of GAGs compared to proteoglycans, GAG composites are currently developed and evaluated for a wide range of applications in tissue engineering [6].

Previous studies have suggested that GAGs, such as heparin (HEP) and dermatan sulfate, may inhibit OC functions through direct interactions with osteoclastic mediators such as RANKL, OPG, and cathepsin K [7,8], though the precise mechanisms have not been identified. Moreover, some of the data are inconsistent, which is in part due to different sources and formulations of the GAGs employed [6]. To circumvent this issue we investigated the effects of chemically sulfated HA derivatives with defined chain length and sulfation degrees in comparison to native HEP and non-sulfated HA. We decided to use chemically sulfated HA derivatives since their interaction with several mediator proteins relevant to bone healing was found to be sulfation dependent in previous studies [9,10].

Furthermore we observed that the regulation of osteoclastogenesis by sulfated HA and chondroitin sulfate was depending on GAG sulfation rather than the monosaccharide composition [3]. In addition the inexpensive accessibility of HA makes sulfated HAs highly attractive model substances with a comparable polymer structure to the naturally sulfated HEP consisting of repeating disaccharide units of uronic acid (D-glucuronic and L-iduronic acid) and D-glucosamine.

A thermally degraded HA was used as a reference instead of full-length HA since its molecular weight is known to significantly decrease during the sulfation process [10].

In the present study, we aimed to determine, if these HAs directly interact with OPG and RANKL using ELISA, SPR and molecular modeling techniques. Utilizing an *in vitro* model of osteoclastogenesis we then analyzed whether this translates in increased or decreased OC differentiation and activity that could be beneficial for biomaterial applications.

2. Materials and methods

2.1. Materials

HA (from Streptococcus, MW = 1.1 × 10^6 $gmol^{-1}$) was purchased from Aqua Biochem (Dessau, Germany). Recombinant human OPG (185-OS-025/CF), monoclonal mouse anti-human OPG (MAB8051), biotinylated polyclonal goat anti-human OPG (BAF805) antibodies and the recombinant human RANKL (6449-TEC-10/CF) were obtained from R&D Systems (Wiesbaden-Nordenstadt, Germany). The Series S Sensor Chips C1™ and CM3™, the Amine Coupling Kit and HBS-EP (10×) were purchased from GE Healthcare Europe GmbH (Freiburg, Germany). Methyl vinyl ether/maleic acid copolymer and adipic acid dihydrazide were purchased from Fisher Scientific (Nidderau, Germany). HEP from porcine intestinal mucosa, sodium cyanoborohydride, bovine serum albumin (BSA), Tween 20, sucrose, and 3,3′,5,5′-tetramethylbenzidine liquid substrate were available from Sigma–Aldrich (Schnelldorf, Germany).

2.2. Preparation of HA derivatives

The low molecular weight HA and the sulfated HA derivatives (sHA1, sHA3), were synthesized and characterized as previously described [10,11]. ^{13}C NMR investigations [10] on the distribution of sulfate groups within the disaccharide repeating unit of the synthesized HAs have revealed that the low-sulfated HA (sHA1) is sulfated exclusively at the C6 position of the N-acetyl-D-glucosamine (GlcNAc) sugar ring. The high-sulfated derivative sHA3 is also completely sulfated at the C6 position of the GlcNAc sugar, whereas the C4 position of the same sugar unit and the C2 as well as the C3 position of the D-glucuronic acid (GlcA) unit are partly sulfated to a comparable extent. Characteristics of the prepared HAs are summarized in Table 1.

2.3. Covalent coupling of GAGs to MaxiSorp™ 96-well ELISA plates

A 96-well microtiter plate (Nunc MaxiSorp™) was coated with GAGs via their reducing ends as previously described [12]. In brief, each well was incubated for 30 min at room temperature (RT) with methyl vinyl ether-maleic anhydride copolymer in DMSO, which was removed and adipic acid dihydrazide was added for 2.5 h. Then, 55 μg HA was dissolved in 25 mM citrate-phosphate buffer pH 5.0. The molar concentrations of the other GAG, including sulfated HA derivatives and HEP, were the same in relation to the molecular weight of their disaccharide units. Schiff bases were obtained via reaction of the reducing ends of GAG with the previously introduced hydrazino groups. 50 μL of GAG solution were applied to each well. The previously untreated wells for the negative control were filled with 50 μL TBS (10 mM Tris, 50 mM NaCl, pH 7.4)/2% BSA. The plate was incubated overnight (o/N) at RT. The next day 10 μL of a 1% sodium cyanoborohydride solution in methanol, which reduces the Schiff bases to stable alkylamine bonds [12], were added to each well. The respective wells were then washed three times with TBS, followed by a blocking step for 2 h with 300 μL/well TBS/2% BSA. Then, 0, 50 and 100 ng/ml OPG in 1% BSA/PBS was incubated on the prepared surfaces o/N at 4 °C.

The following day 150 μL PBS/1% BSA was added to each well. The resulting 200 μL were collected into tubes, the wells were flushed with 200 μL PBS/1% BSA, which were then also transferred into the respective tube and stored frozen at −80 °C for a following sandwich ELISA.

2.4. Enzyme linked immunosorbent assay (ELISA)

All procedures were performed at RT and on an orbital shaker. In a direct ELISA, the wells, which had previously been coated with GAG derivatives and incubated with OPG, were blocked for 1 h with 300 μL/well blocking buffer (1% (w/v) BSA, 5% (w/v) sucrose in PBS). Afterward they were washed three times with 300 μL/well washing buffer (0.05% (v/v) Tween 20 in PBS) for approx. 1 min each and 100 μL/well detection antibody (goat anti-human OPG, 0.05 μg/mL PBS/1% BSA) were applied. The plate was covered with parafilm and incubated for 2 h. After several washing steps, 100 μL/well streptavidin-HRP (R&D systems, dilution 1:400 in PBS/1% BSA) were applied. The streptavidin-HRP was aspirated 20 min later and the plate was washed as previously described. Via addition of 100 μL/well 3,3′,5,5′-tetramethylbenzidine liquid substrate HRP enzyme activity was detected. The reaction was stopped after 5 min with 50 μL/well 1 M H_2SO_4 and absorption was measured at 450 nm (Tecan Genios).

The amount of unbound OPG was determined by sandwich ELISA using a 96-well plate (Nunc MaxiSorp™) coated with 100 μL/well capture antibody (mouse anti-human OPG, 1 μg/mL PBS) and incubated o/N at 4 °C. The plate was washed three times with 300 μL/well washing buffer for approx. 1 min and blocked for 1 h with 300 μL/well blocking buffer. In the meantime, the samples to be tested were thawed and further diluted with 600 μL 1% BSA/PBS, resulting in an overall dilution of 20 times. Also a standard curve was prepared from 12.5 ng OPG/mL 1% BSA/PBS. After washing, 100 μL/well of sample or standard were incubated in the wells for 2 h. The plates were washed and the procedure was continued as described for the direct ELISA starting with the addition of detection antibody.

2.5. Immobilization of OPG and RANKL on Series S Sensor Chips CM3™ and C1™

Interaction analysis of OPG, RANKL and GAGs were performed using a Biacore™ T100 instrument (GE Healthcare). OPG was immobilized onto a Series S Sensor Chip CM3™ via its amine groups at 25 °C. Following the instructions of the manufacturer (GE Healthcare) for amine coupling, the carboxyl groups of the chips dextran matrix were activated to succinimide esters with a freshly prepared mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS), which was injected over the flow cell for 7 min at 10 μL/min. Next 2.5 μg/mL of OPG diluted in sodium acetate buffer pH 5.5 were injected at

Table 1
Synoptic table of the characteristics of HEP, HA, and synthesized sulfated HA derivatives (degree of sulfation: D.S.), weight-average molecular weight: Mw (as determined by gel permeation chromatography equipped with laser light scattering detection or *as specified by the distributor).

Sample-ID	HA	sHA1	sHA3	HEP
Mw [g mol^{-1}]	48 000	31 000	21 000	18 000*
D.S.	–	1.0	2.9	2.0
Sulfate position determined by NMR	–	C6 (GlcNAc)	C4, C6 (GlcNAc), C3, C2 (GlcA)	n.d.
Designation of tetrasaccharides for *in silico* modeling	HA	HA6	HA463′	–