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Sulfated hyaluronan/collagen I matrices enhance the osteogenic differentiation of human mesenchymal stromal cells in vitro even in the absence of dexamethasone

U. Hempel [a,*], S. Möller [b], C. Noack [a], V. Hintze [c], D. Scharnweber [c], M. Schnabelrauch [b], P. Dieter [a]

[a] Institute of Physiological Chemistry, Carl Gustav Carus, Faculty of Medicine, Technische Universität Dresden, Fiedlerstrasse 42, D-01307 Dresden, Germany
[b] Biomaterials Department, INNOVENT e.V., Prüssingstrasse 27 B, D-07745 Jena, Germany
[c] Max Bergmann Center of Biomaterials, Technische Universität Dresden, Budapester Straße 27, D-01069 Dresden, Germany

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ABSTRACT

Glycosaminoglycans (GAG) are multifunctional components of the extracellular matrix (ECM) involved in different steps of the regulation of cellular differentiation. In this study artificial extracellular matrices (aECM) consisting of collagen (Col) I and different GAG derivatives were used as a substrate for human mesenchymal stromal cells (hMSC) to study osteogenic differentiation in vitro. hMSC were cultured on aECM containing col and hyaluronan sulfates (HyaS) with increasing degrees of sulfation (DS_S) and were compared with aECM containing col and the natural GAG hyaluronan or chondroitin 4-sulfate. hMSC were analyzed for osteogenic differentiation markers such as calcium phosphate deposition, tissue non-specific alkaline phosphatase (TNAP) and expression of runt-related transcription factor 2 (*runx2*), osteocalcin (*ocn*) and bone sialoprotein II (*bspII*). Compared with aECM containing Col and natural GAG all Col/HyaS-containing aECM induced an increase in calcium phosphate deposition, TNAP activity and *tnap* expression. These effects were also seen in the absence of dexamethasone (an established osteogenic supplement). The expression of *runx2* and *ocn* was not altered and the expression of *bspII* was diminished on the col/HyaS-containing aECM. The impact of the Col/HyaS-containing aECM on hMSC differentiation was independent of the DS_S of the HyaS derivatives, indicating the importance of the primary (C-6) hydroxyl group of *N*-acetylglucosamine. These results suggest that Col/HyaS-containing aECM are able to stimulate hMSC to undergo osteogenic differentiation even in the absence of dexamethasone, which makes these matrices an interesting tool for hMSC-based tissue engineering applications and biomaterial functionalizations to enhance bone formation.

1. Introduction

Human mesenchymal stromal cells (hMSC) are widely used to study osteogenic differentiation and to gain an understanding of bone healing and for tissue engineering approaches [1,2]. In the bone marrow niche hMSC are surrounded by an extracellular matrix (ECM) consisting mainly of collagen (Col) types I, III, IV, V and VI and fibronectin [3]. A characteristic of the ECM in the bone marrow niche is the high content of heparan sulfate and heparan sulfate proteoglycans in comparison with bone [3]. Within the bone marrow niche ECM the sulfated glycosaminoglycans (GAGs) are of physiological importance because they interact with many regulatory growth factors, chemokines and cytokines (e.g. basic fibroblast growth factor (bFGF), granulocyte–macrophage colony stimulating factor, interleukin-3, and interleukin-8), and with structural proteins such as collagens and fibronectin [4]. Many of these proteins are stabilized by interaction with GAGs, protected from hydrolytic degradation, presented to their receptors or sequestered in the ECM [5]. Sulfated GAGs and the non-sulfated, but highly charged hyaluronan are involved in water and extracellular cation homeostasis as well as in cell adhesion and signalling processes [6–8]. On following the osteogenic differentiation route hMSC exchange the bone marrow niche ECM for the solid bone ECM. In contrast to the bone marrow niche ECM, the solid bone ECM is rich in inorganic minerals (calcium phosphate/hydroxyapatite) and contains about 20% Col I, non-collagenous proteins such as osteocalcin

Abbreviations: aECM, artificial extracellular matrix; bFGF, basic fibroblast growth factor; BM, basic medium for hMSC; BMP, bone morphogenetic protein; BSP II, bone sialoprotein II; C4S, chondroitin-4-sulfate; Col, collagen I; Dex, dexamethasone; DMEM, Dulbecco's minimum essential medium; DSS, degree of sulfation; ECM, extracellular matrix; GAG, glycosaminoglycan; GlcNAc, *N*-acetylglucosamine; GPC, gel permeation chromatography; hMSC, human mesenchymal stromal cells; Hya, hyaluronan; HyaS, hyaluronan sulfate; HyaS1, low-sulfated hyaluronan; HyaS2, medium-sulfated hyaluronan; HyaS3, high-sulfated hyaluronan; LLS, laser light scattering; OCN, osteocalcin; OMDex, osteogenic differentiation medium; OM, osteogenic differentiation medium without dex; PBS, phosphatebuffered saline; PD, polydispersity; RI, refraction index; *Runx2*, runt-related transcription factor 2; TCPS, tissue culture polystyrene; TNAP, tissue non-specific alkaline phosphatase.

* Corresponding author. Tel.: +49 351 458 6430.
E-mail address: ute.hempel@tu-dresden.de (U. Hempel).

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(OCN) and bone sialoprotein (BSP II) and various growth factors such as bone morphogenetic proteins (BMPs), transforming growth factor-β1 and insulin-like growth factors [3]. The GAG content of bone ECM, dependent on the bone type, localization and mammalian species, is in general less than 1% of bone dry weight [9–11]. GAGs/proteoglycans in the bone ECM are very dynamic and reveal great diversity in chain length, sulfation pattern, and fine structure [12]. During in vivo and in vitro osteogenic differentiation the pattern of proteoglycans changes continuously, as the amounts of chondroitin sulfate- and dermatan sulfate-containing proteoglycans decrease, whereas the amount of heparan sulfate proteoglycans increases [13,14]. Hyaluronan is of importance for the recruitment of osteoblast precursor cells from the bone marrow and plays a role in directing stem cells towards osteogenesis [15,16].

In vitro osteogenic differentiation of hMSC involves three phases, (i) proliferation; (ii) matrix synthesis and maturation/differentiation (decreased proliferation rate, elevated endogenous ECM synthesis, induction of osteoblast-specific genes), and (iii) mineralization (controlled deposition of calcium phosphate, formation of mineralized nodules), and requires the osteogenic supplements dexamethasone (Dex), ascorbic acid and β-glycerophosphate [17–20]. It is known that ascorbic acid is an essential coenzyme for Col synthesis and that β-glycerophosphate is a donor of organic phosphate. Dex is known to induce the expression of several osteogenic genes such as *tnap*, *bsp II* and *osteoadherin*; however, Dex has also been reported to suppress osteogenic parameters such as osteocalcin expression and to induce adipogenic markers such as peroxisome proliferator-activator receptor-γ [21]. The importance of GAG for osteoblast differentiation was evaluated in several in vitro studies with partially contradictionary results, probably caused by using different species, cells or GAG sources [14,22–28]. Natural oversulfated chondroitin sulfate (chondroitin sulfate E) influenced bone remodeling by both enhancing osteoblast differentiation (positive effect on collagen deposition, activity of tissue non-specific alkaline phosphatase (TNAP), and mineral accumulation) and inhibiting osteoclastogenesis [26,29]. Several tissue engineering approaches based on the usage of GAG have been reported. Heparan sulfate-containing polycaprolactone polymers stimulated proliferation of hMSC [30]. A porous collagen/chondroitin 6-sulfate scaffold material supported osteogenic differentiation of rat bone marrow cells [31]. Collagen/chondroitin 4-sulfate (C4S) matrices were used as a substrate for osteoblasts to study the adhesion and proliferation and bone healing processes of mini-pig maxilla and rat tibia [32–35]. A synthetic substratum prepared from heparin-binding peptides was found to support the proliferation and to facilitate the long-term culture of human pluripotent stem cells [36].

Synthetically sulfated hyaluronan derivatives (HyaS) were at first characterized and described as anti-thrombogenic substances [37–40] and inhibitors of tumor necrosis factor α [41]. An osteogenic effect of such hyaluronan sulfates on rat calvarial cells was described by Nagahata et al. [42], demonstrating that treatment with hyaluronan sulfate in solution resulted in the formation of cell aggregates with increased TNAP activity. HyaS derivatives are known to induce the *N*-cadherin, connexin-43 and TNAP activity of rat osteoblasts [42]. Kunze et al. [23] reported a dose-dependent inhibitory effect of low and high sulfation hyaluronan derivatives on the proliferation of rat osteoblasts. Low sulfation hyaluronan was used to coat tissue culture polystyrene (TCPS) and was found to induce the differentiation of keratinocytes [43]. The authors hypothesized that the low sulfation hyaluronan might bind and stabilize growth factors, in particular bFGF. Hintze et al. [44] demonstrated that low and high sulfation hyaluronan derivatives had a high affinity interaction with the bone anabolic mediator BMP-4 with binding constants of 20 nM and 13 pM, respectively. Recently artificial extracellular matrices (aECMs) containing col and HyaS derivatives or chemically over-sulfated chondroitin sulfate derivatives were shown to promote the adhesion and proliferation of human dermal fibroblasts, whereas collagen synthesis by fibroblasts and hMSC was reduced [45,46]. Taking all the data together, natural GAGs and synthetically sulfated hyaluronan derivatives seem to be potent molecules for regulating cellular differentiation processes. However, the heterogeneity of natural GAGs makes it difficult to assign specific effects to a particular structure or a specific GAG/protein interaction [24]. Thus synthetically sulfated GAGs with a characterized defined molecular structure could contribute to a better understanding of these molecular interactions.

In the present study the influence of aECMs containing Col and defined HyaS derivatives or the natural GAG hyaluronan and C4S on the osteogenic differentiation of hMSC was investigated. In parallel, osteogenic differentiation of hMSC was induced by the established osteogenic supplements Dex, ascorbate and β-glycerophosphate. Osteogenic differentiation of hMSC was determined by analyzing osteoblast-specific gene expression, TNAP activity and deposition of calcium phosphate.

2. Materials and methods

Cell culture plastic material was obtained from Greiner (Frickenhausen, Germany) and Nunc (Wiesbaden, Germany). Dulbecco's minimum essential medium (DMEM), trypsin, the antibiotics penicillin and streptomycin and phosphate-buffered saline (PBS) were purchased from Biochrom (Berlin, Germany). Fetal calf serum was from BioWest (via Th.Geyer, Hamburg, Germany). Biochemical reagents were from Sigma (Taufkirchen, Germany). The RotiQuant assay from Roth (Karlsruhe, Germany) was used to determine the protein concentration. Rat tail collagen I was from BD Bioscience (Heidelberg, Germany). C4S (CSA, from bovine trachea) was obtained from Sigma (see Table 1 for analytical data) and hyaluronan (Hya, from *Streptococcus* sp.) from Aqua Biochem (Dessau, Germany) (see Table 1 for analytical data).

2.1. Synthesis and characterization of the Hya derivatives

The low sulfation hyaluronan derivative (HyaS1, DS_S ~1) and high sulfation hyaluronan derivative (HyaS3, DS_S ~3) were synthesized as described in Kunze et al. [23] and Hintze et al. [44] (see Table 1 for analytical data). To prepare the medium sulfation hyaluronan derivative (HyaS2, DS_S ~2) the procedure for the preparation of HyaS1 was used with the modification that the SO_3–pyridine complex was added to a suspension of Hya (tetrabutylammonium salt) in dimethylsulfoxide at a Hya:SO_3 ratio of 1:8, followed by stirring the reaction mixture for 20 min at room temperature. The reaction yield was 75% relative to Hya.

The average degree of sulfation per disaccharide repeating unit of the hyaluronan sulfates was determined by estimation of the sulfur content using an automatic elemental analyzer (CHNS-932, Leco, Mönchengladbach, Germany). Molecular weight determination was performed by gel permeation chromatography (GPC) using a Jasco PU 980 pump and a combination of three SupremaGel columns 8 mm in diameter × 300 mm long with grain and pore sizes of 10 μm/100 Å, 10 μm/1000 Å and 20 μm/30,000 Å, respectively. The samples were eluted with PBS at a flow rate of 0.8 ml min^{-1}. A double detection system consisting of a Postnova Analytics PN 3000 (15°) laser light scattering (LLS) detector and a Jasco RID-1531 refraction index (RI) detector was used. Absolute values of weight-average (M_w) molecular weights were determined using the LLS detection system. Calculation of polydispersity PD (PD = M_w/M_n) was performed on the basis of M_n and M_w values obtained by RI detection. In the latter case calibration of the detection system was performed with commercially available

ID	Title	Pages
383	Sulfated hyaluronan/collagen I matrices enhance the osteogenic differentiation of human mesenchymal stromal cells in vitro even in the absence of dexamethasone	9