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Sulfated hyaluronan derivatives reduce the proliferation rate of primary rat calvarial osteoblasts

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Abstract Glycosaminoglycans (GAG) and proteoglycans, which are components of the extracellular bone matrix, are also localized in and at the membrane of osteoblasts and in the pericellular matrix. Due to their interaction with several growth factors, water and cations these molecules play an important role in regulating proliferation and differentiation of osteoblasts and bone development. The aim of this study was to assess *in vitro* the effects of two chemically sulfated hyaluronan (HyaS) derivatives on the proliferation of rat calvarial osteoblasts and to compare with those of native hyaluronan (Hya) and natural sulfated GAG such as chondroitin-4-sulfate (C4S), chondroitin-6-sulfate (C6S), dermatan sulfate (DS) and heparan sulfate (HS). Moderately and highly sulfated HyaS derivatives caused a time-dependent reduction of osteoblast proliferation. The anti-proliferative effect of HyaS was accompanied by a cell cycle arrest in the G1 phase, but was not associated with cell death. Whereas non-sulfated high molecular weight (HMW)- and low molecular weight (LMW)-Hya as well as C4S, C6S, DS and HS showed no effect on the cell proliferation.

Keywords Glycosaminoglycans · Sulfated hyaluronan · Osteoblast · Proliferation

Abbreviations

GAG	glycosaminoglycans
HyaS	sulfated hyaluronan
CS	chondroitin sulfate
DS	dermatan sulfate
HS	heparan sulfate
HMW	high molecular weight
LMW	low molecular weight

Introduction

The extracellular bone matrix (ECM) consists of inorganic components, mainly hydroxyapatite and organic components, predominantly type I collagen. Besides collagen and non-collagenous proteins, glycosaminoglycans (GAG) and proteoglycans (PG) are major constituents in the organic portion of the ECM [1]. GAG and PG are also intercalated into the cell membrane or associated with the cell membrane [2].

GAG are long unbranched polysaccharides consisting of a repeating disaccharide unit containing hexosamine and hexose or hexuronic acid. Moreover, the basic disaccharide unit is modified by sulfation, acetylation or epimerization. Thus, various GAG subclasses exist varying in composition and molecular fine structure of the disaccharide unit such as chondroitin sulfate (CS), dermatan sulfate (DS), heparin/heparan sulfate (HS), hyaluronan (Hya) and keratan sulfate

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(KS) [3]. Due to the presence of various sulfate and/or carboxylate groups, the GAG chains are highly negatively charged macromolecules. In the body, all GAG subclasses except of Hya are covalently linked to certain core protein molecules forming PG [4].

PG and GAG serve as a space-filling material necessary for the orientation, organisation and hydration of the ECM and act as potential binding sites for calcium ions in the bone ECM [4, 5]. However, an active participation of GAG and their PG backbone in cell-cell- and cell-matrix-communication was also demonstrated [4]. It is known that GAG are able to bind and present mediator proteins like growth factors and several cytokines. Sulfation degree and molecular fine structure of the carbohydrate chains are critical factors for the specific interaction with these small regulatory protein mediators [2, 6]. Therefore, GAG may influence proliferation and differentiation of cells by binding and presentation of several mediators or *per se* as demonstrated in numerous *in vitro* studies [2, 7–9]. It was shown that exogenously added GAG revealed inhibitory or stimulatory effects on the proliferation of cultured cells depending on cell type and degree of sulfation [1, 6, 10–13]. Moreover, native Hya was reported to exert both proliferative and anti-proliferative properties depending on its molecular mass [14]. Further on, it was demonstrated that in contrast to the natural Hya, which does not contain any sulfate group in the repeating unit, sulfated Hya (HyaS) derivatives caused an anti-proliferative effect on bovine pulmonary artery smooth muscle cells [15]. In rat calvarial osteoblasts, sulfated Hya derivatives enhanced the expression of cell adhesion proteins resulting in cell aggregate formation and induced alkaline phosphatase activity an early differentiation marker of osteoblasts [16]. An earlier report by Magnani *et al.* [17] showed that sulfated hyaluronan exerts anticoagulant and antithrombotic activities, which increased with increasing numbers of sulfate groups in the disaccharide unit. Similarly, sulfated Hya was demonstrated to inhibit in a sulfation degree-dependent manner HYAL-1 hyaluronidase activity, which is a strong promoter of bladder tumor growth and invasion [18].

In the present study, the effects of HyaS derivatives on the proliferation of primary rat osteoblasts were evaluated and compared to the effects of native non-sulfated low molecular weight (LMW)- and high molecular weight (HMW)-Hya as well as natural sulfated GAG including chondrotin-4-sulfate (C4S), chondroitin-6-sulfate (C6S), DS and HS.

Materials and methods

Materials

Cell culture plastic material was obtained from Greiner (Frickenhausen, Germany). 24 well plates were supplied from Nunc (Wiesbaden, Germany). Collagenase P (from *C. histolyticum*) was from Roche Diagnostics (Mannheim, Germany). Bovine pancreatic trypsin, penicillin/streptomycin antibiotics, Dulbecco`s minimum essential medium (DMEM) and phosphate buffered saline (PBS) were purchased from Biochrom (Berlin, Germany), and the fetal calf serum (FCS) was from BioWest (Germany). HMW-Hya (from *Streptococcus*, molecular weight see Table 1) was obtained from Aqua Biochem (Dessau, Germany). Mertiolate, sulfur trioxide/dimethylformamide complex (SO_3-DMF, purum, ≥ 97%, active SO_3 ≥ 48%) and sulfur trioxide/pyridine complex (SO_3-pyridine, pract.; ≥45% SO_3) were from Fluka (Switzerland). C4S from bovine trachea, C6S from shark cartilage, DS from porcine intestinal mucosa and HS from bovine kidney were obtained from Sigma-Aldrich (Steinheim, Germany). Methyl-[^{3}H]thymidine (1 mCi/ml) was from GE Healthcare Life Sciences (Germany). Ascorbic acid, β-glycerophosphate and aprotinin were purchased from Sigma. Cell lysis buffer was from New England Biolabs (Frankfurt, Germany), ribonuclease (RNase) A was from Applichem (Darmstadt, Germany) and propidium iodide was bought from Calbiochem (Darmstadt, Germany). Cytotoxicity Detection Kit (LDH) was obtained from Roche. For determination of protein concentration the RotiQuant assay from Roth (Karlsruhe, Germany) was used. Quant-iT PicoGreen dsDNA Assay Kit was bought from Invitrogen (Karlsruhe, Germany). Fluorimetric Kit for detection of caspase-3 activity was purchased from Sigma.

LMW-Hya

LMW-Hya was prepared by a controlled thermal degradation of the native HMW-Hya in analogy to a described procedure [19]. 700 ml of a solution of 1% (w/v) of HMW-Hya in distilled water were heated in a steam autoclave (Hirayama Manufacturing Corp., Japan) at 130°C for 90 min. After cooling to room temperature, the solution is purified by dialysis against distilled water followed by lyophilization of the solution and drying of the resulting polymer in vacuum. IR and NMR spectra of the obtained

Table 1 Molecular weight (Mw) and molecular weight distribution (PD) values of HMW-Hya, LMW-Hya and HyaS derivatives determined by gel permeation chromatography

Sample	HMW-Hya	LMW-Hya	HyaS06	HyaS13
Degree of sulfation	0.0	0.0	1.0	2.8
Mw [gmol^{-1}]	1.15×10^6	8.41×10^4	3.32×10^4	7.40×10^4
PD	13.45	4.94	2.08	1.69

product were identical with those of starting HMW-Hya [20]. Molecular weight determination was performed by gel permeation chromatography analysis (Jasco PU 980 pump, Postnova Analytics PN 3000 (15°) laser light scattering (LLS) detector, Jasco RID-1531 refraction (RI) detector, and Suprema-Gel 10 μm−100 Å, 10 μm−1,000 Å and 20 μm−30,000 Å columns). The derivatives were eluted with PBS at 0.5 and 0.8 ml/min, respectively. Concerning the refraction index detection, the system was calibrated with standard pullulan (PSS, Mainz, Germany) samples. LLS detection of analytes was used for the determination of molecular weight values (Mw) and RI detection for the estimation of the polydispersity indices (PD). Data are summarized in Table 1.

Synthesis and characterization of HyaS derivatives

Sulfated hyaluronan (HyaS) derivatives were prepared as described previously [16, 17, 21] with some modifications. Sulfation reaction was performed in DMF at room temperature using the tetrabutylammonium salt of HMW-Hya as a starting material. Sulfation reaction with SO_3-pyridine complex in a molar Hya:SO_3 ratio of 1:7 resulted in sulfated hyaluronan containing 6% sulfur (HyaS06, degree of sulfation=1.0). By the reaction of Hya with SO_3-DMF complex in a molar Hya:SO_3 ratio of 1:20 a HyaS derivative with 13% sulfur content (HyaS13, degree of sulfation=2.8) was formed. The HyaS derivatives were isolated as sodium salts by precipitation with acetone and subsequent neutralisation with NaOH in ethanol. The precipitates were separated, washed several times with acetone and purified by dialysis against distilled water. Finally, after lyophilization the HyaS derivatives were dried in vacuum to completely remove remaining water. Yields for HyaS and LMW-Hya were 65–70% and 90%, respectively.

The degree of sulfation (Table 1), which indicates the average number of sulfate ester groups per disaccharide repeating unit of HyaS, was calculated on the basis of the sulfur content of prepared HyaS samples. An automatic elemental analyzer (CHNS-932 Leco, Mönchengladbach Germany) was used for the sulfur determination. FT-IR and ^{13}C-NMR spectra of the prepared HyaS were in accordance with literature data [17, 20].

NMR analyses of C4S, C6S and DS

C4S, C6S and DS samples were characterized by ^{1}H- and ^{13}C-NMR spectroscopy. The samples were dissolved in 50 mM phosphate buffer (pH 7.4) to give 70–100 mg/ml solutions. Mertiolate was added in a concentration of 200 μg/ml to prevent the growth of microorganisms. Spectra were recorded on a Bruker Avance 600 MHz NMR-spectrometer. Both, ^{13}C- and ^{1}H-NMR spectra were in accordance with previous reports and confirmed the structure of the glycosaminoglycans [22, 23]. The ^{1}H-NMR spectra also confirmed the absence of common impurities often present in biopolymers isolated from biological tissues (*e.g.* solvents such as ethanol or acetone and precipitation reagents). There were only minimal amounts of acetate and formate indicating that the intact polymers were used and previous degradation processes did not play a major role.

Cell isolation and culture

NIH guidelines for the care and use of laboratory animals were applied for the study. Rat calvarial-derived osteoblasts were isolated according to Geissler *et al*. [24]. Briefly, cells were obtained from the calvariae of newborn Wistar rats by sequential digestion with collagenase P (3.65 U/ml) and trypsin (15 U/ml) in PBS and subcultured in DMEM containing 10% FCS [25]. The osteoblast phenotype was confirmed by determination of alkaline phosphatase activity, collagen type I synthesis, osteocalcin mRNA, and formation of calcium phosphate deposits after addition of osteogenic supplements (10 mM β-glycerophosphate, 300 μM ascorbic acid) to the medium beginning at day 4 of culture.

12,500 cells/cm^2 were placed in 24 well plates and covered with 1 ml of DMEM containing 10% FCS. GAG (100 μg/ml) were first added 24 h after plating and then with medium change at day 4. The cells were cultured for maximum 8 days.

Tritiated-thymidine incorporation assay

Cellular proliferation rate was monitored by quantifying DNA synthesis via incorporation of methyl-[^{3}H]thymidine. At indicated time points, medium was supplemented with methyl-[^{3}H]thymidine (1 mCi/ml) to obtain 1 μCi/ml medium, and cells were incubated for 24 h. Then, the culture medium was removed, and cells were washed with PBS and treated twice with 10% trichloroacetic acid at 4°C for 10 min to precipitate the DNA. After washing with ethanol cells were lysed in a solution containing 100 mM NaOH, 22 mM Na_2CO_3 and 1% SDS followed by scintillation counting in a Trilux 1450 Microbeta counter (Wallac, Turku, Finland).

Cellular DNA content

After washing with PBS, cells were lysed in lysis buffer containing 1% (v/v) aprotinin and 1 mM PMSF. The content of cellular DNA of the lysate was measured using the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) following the manufacturer's instructions. The cellular DNA concentration (μg/ml cell lysate) was calculated from a standard curve made with λ-phage-derived DNA.

Cell cycle analysis

For cell cycle analysis, cells were washed with PBS, and subsequently trypsinized for 5 min at 37°C. After centrifugation (5 min, 1,400 rpm), 1 ml of ice-cold 70% ethanol/PBS was added drop-by-drop followed by incubation on ice for minimum 45 min. After addition of 1 ml of PBS, the cells were centrifuged (10 min, 1,000 rpm, room temperature). The cell pellet was resuspended in 0.5 ml 1% BSA/PBS and 0.5 ml of a buffer solution (pH 7.8) containing 0.1 M citrate and 0.2 M Na_2HPO_4, and incubated at room temperature for 10 min. Subsequent to further centrifugation (10 min, 1,000 rpm, room temperature), cells were incubated in 50 µl of a staining solution containing 60 µg propidium iodide and 200 µg RNase A/ml for 30 min at room temperature. DNA content was measured at least with 25,000 cells using the fluorescence-activated cell sorter FACScan (BD Biosciences Pharmingen, Heidelberg, Germany). The distribution of cells in different cell-cycle stages (G_1/S/G_2 + M) was determined according to their DNA content using the WinMDI software. Thus, resting cells (G_0/G_1 phase) contain diploid DNA (2n DNA amount), dividing cells contain between 2n and 4n DNA amount and G_2/M phase cells have 4n DNA amount revealing the twofold fluorescence intensity of the 2n cells. The sum of all cells in the cell-cycle analysis was set to 100% and the distribution into G_1, S, and G_2/M was determined.

Protein content

For determination of protein content, cells were lysed as described above. Afterwards, protein content of the lysate was determined using the RotiQuant assay (Roth). The protein concentration (µg/µl cell lysate) was calculated from a standard curve made with BSA.

Lactate dehydrogenase activity

Lactate dehydrogenase is a constitutive cytoplasmic enzyme that is present in all cells. The cellular and extracellular LDH activity was monitored by Cytotoxicity Detection Kit (Roche). For determination of cellular (c)LDH, cells were lysed as described above. For determination of extracellular, (ec)LDH conditioned medium was used. Further procedure followed the manufacturer's instructions. The viability was calculated by the ratio of cLDH to whole LDH (= cLDH + ecLDH).

Caspase-3 activity

Apoptosis of cells was monitored by analysis of caspase-3 activity (Caspase-3 Assay Kit, Sigma). Therefore, cells were placed in 96 well plates and covered with 0.2 ml of medium per well. At indicated time points, cells were washed with PBS. Further procedure followed the manufacturer's instructions. Briefly, cells were incubated with 25 µl of lysis buffer on ice for 20 min. Then 200 µl of caspase-3 substrate (17 µM Ac-DEVD-AMC) solution were added. The reaction performed in presence or absence of a caspase-3 inhibitor (200 µM Ac-DEVD-CHO), 200 µl of this reaction solution were transferred to a fluorimeter multiwell plate. The plate was incubated at 37°C and the fluorescence (excitation 360 nm, emission 460 nm) were measured after 60 min. The caspase-3 activity (nmole of AMC released per ml of cell lysate) was calculated from an AMC standard curve.

Statistical analysis

All presented data were derived from three independent experiments (3 different litter of neonatal rat pups) performed in triplicate. The results are presented as mean ± standard deviation (SD). One-way or two-way ANOVA including Bonferroni posttest was applied to determine statistical significance.

Results

Synthesis and characterization of Hya derivatives

HMW-Hya was used for synthesis of HyaS06 and HyaS13 derivatives. As indicated by NMR spectra, the sulfate groups are located at the C6-position of the *N*-acetylglucosamine (HyaS06) and in the C6-, C4- and partially C2-position (HyaS13), respectively (data not shown). Sulfation of Hya resulted in a considerable decrease in the molecular weight (Mw) of the formed products as shown in Table 1. Starting from HMW-Hya (Mw ~1 Mill g/mol) HyaS with Mw ranging between 30,000 and 70,000 g/mol were obtained. The decrease in Mw was only slightly influenced by the used type of sulfation agent. The degradation might be due to the known susceptibility of the glycosidic bond in Hya to hydrolytic cleavage caused by intermediately formed highly acidic -OSO_3H groups. Compared to the Hya starting material, both HyaS derivatives showed a narrower Mw distribution. Thus, a low molecular weight Hya (LMW-Hya) was prepared by controlled thermal degradation of native HMW-Hya to rule out that effects of HyaS on the proliferation were due to the lower Mw.

Effect of sulfated hyaluronan on the proliferation of primary rat osteoblasts

Proliferation rate and cellular DNA content of rat calvarial osteoblasts were determined from day 2 to day 8 of culture.

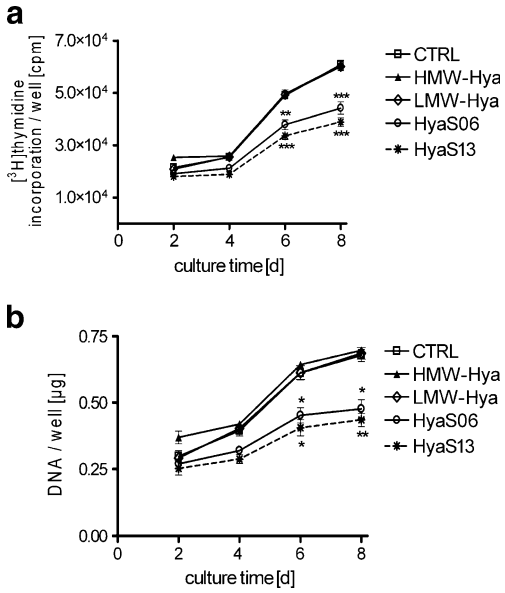

Fig. 1 Proliferation of primary rat osteoblasts. Cells were plated on polystyrene (TCPS) and incubated with either 100 µg HMW-Hya, LMW-Hya, HyaS06 or HyaS13/ml 24 h after seeding. Untreated cells are used as a control (CTRL). At day 1, 3, 5, and 7 of culture cells were incubated with 1 µCi [^{3}H]thymidine/ml. After 24 h the reaction was stopped and the incorporated radioactivity was determined by liquid scintillation counting **a**. At day 2, 4, 6, and 8 of culture cell lysates were analysed for the cellular DNA content using the fluorescent DNA-binding PicoGreen dye and λ-phage-derived DNA for calibration **b**. The results are presented as mean ± SD. Significant differences of GAG treatment *versus* control are indicated with * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$). $N=3$

After a lag phase between day 2 and 4, the cells started to proliferate moderately up to day 8. The [³H]thymidine incorporation rate increased within 6 days of culture by about threefold (Fig. 1a). A similar effect was observed by determination of DNA content in the cell lysates (Fig. 1b). As seen in Fig. 1, the addition of HyaS led to a significant decrease of the osteoblastic proliferation rate within the whole culture period. The effect was mostly pronounced at day 8 for both high and moderately sulfated derivative (27±3% for HyaS06 and 36±1% for HyaS13, respectively) (Fig. 1a). These results were confirmed by measurement of cellular DNA with a maximum at day 8 (30±6% for HyaS06 and 36±3% for HyaS13, respectively) (Fig. 1b). However, in the presence of HMW-Hya or LMW-Hya (which has a similar Mw as the HyaS derivatives, Table 1) both, the proliferation rate and DNA content were not affected within the whole culture period (Fig. 1a, b). In preliminary experiments, the reduction of cellular DNA content by both HyaS06 and HyaS13 was shown to be dose-dependent between 10 and 500 µg/ml with a half-maximal cell effect at 100 µg GAG/ml (data not shown).

To verify whether the decrease of the [³H]thymidine incorporation and cellular DNA content induced by HyaS derivatives was caused by a specific block in the cell cycle, FACS analysis of propidium iodide-stained cells was performed at day 2 of culture. Propidium iodide is a DNA-intercalating fluorescent molecule, which allows the distinction of cells by their DNA content. Resting cells (G_0/G_1 phase) contain diploid DNA (2n DNA amount), dividing cells (S phase) contain between 2n and 4n DNA amount and G_2/M phase cells have 4n DNA amount revealing the twofold fluorescence intensity of the 2n cells. As shown in Fig. 2, 60% of the untreated cells were in G1 phase, 11% in S phase and 29% in G2/M phase. For cells grown in the presence of HMW-Hya no significant alteration of the number of cells in the G1 (57% to 60%), S (10% to 11%) and G2/M (33% to 29%) phase were found. In contrast, HyaS13 caused a significant increase in the percentage of cells in G1 phase (74% to 60%) and an accordant decrease in the percentage of cells in the S phase (8% to 11%) and G2/M phase (18% to 29%) compared to the control (Fig. 2).

As seen in Fig. 3, total cellular protein content (Fig. 3a) and cellular LDH activity (Fig. 3b) strongly correlated with the [³H]thymidine incorporation and DNA content of primary rat osteoblasts. Addition of HyaS derivatives to the cells induced a time-dependent decrease of protein content with a maximum effect at day 8 (33±4% for HyaS06 and 48±5% for HyaS13, respectively) (Fig. 3a). Further, HyaS derivatives caused a time-dependent decrease of LDH activity with a maximal effect at day 8 (44±2% for HyaS06 and 52±4% for HyaS13, respectively) (Fig. 3b).

To exclude whether the HyaS-induced decrease in [³H] thymidine incorporation, LDH activity, DNA and protein content was due to reduced viability or apoptosis, the viability of the cells was examined by measuring the LDH release. Additionally, caspase-3 activity was measured as an apoptosis marker. Treatment of the cells with HMW-Hya, LMW-Hya or HyaS derivatives did not change the cell

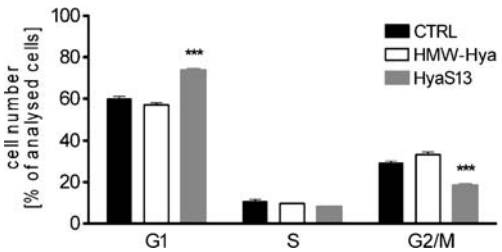

Fig. 2 Cell cycle analysis of primary rat osteoblasts. Cells were plated on TCPS and incubated with either 100 µg HMW-Hya or HyaS13/ml 24 h after seeding. Untreated cells are used as a control (CTRL). At day 2 after seeding, cells were trypsinized, fixed and stained with propidium iodide. Analysis of the different cell cycle phases ($G_1/S/G_2$ + M) was performed by FACS. The quantitation of the results was performed with WinMDI software. The total number of cells assessed by FACS was set to 100%. The results are presented as mean ± SD. Significant differences of GAG treatment *versus* control are indicated with *** ($p<0.001$). $N=3$

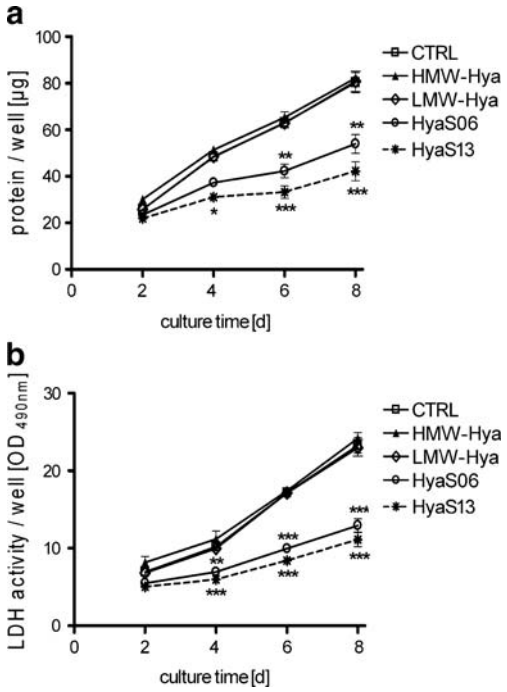

Fig. 3 Cellular protein content and LDH activity. Cells were plated on polystyrene and incubated with either 100 µg HMW-Hya, LMW-Hya, HyaS06 or HyaS13/ml 24 h after seeding. Untreated cells are used as a control (CTRL). At day 2, 4, 6, and 8 of culture cells were lysed. The lysate was used for total protein determination by Bradford method **a** and for spectrophotometrically determination of LDH activity **b**. The results are presented as mean ± SD. Significant differences of GAG treatment *versus* control are indicated with * ($p<0.05$), ** ($p<0.01$) or *** ($p<0.001$). $N=3$

viability and the caspase-3 activity (data not shown) indicating that both HyaS derivatives did not cause a cytotoxic or apoptotic effect.

Effect of native sulfated GAG on the proliferation of primary rat osteoblasts

Primary rat osteoblasts were treated with C4S, C6S, DS and HS. The sulfation degree of these commercially available substances was given by the manufacturer to about 5 -7%, which is in the range of the sulfation degree of the HyaS06 derivative. The results of [^{3}H]thymidine incorporation, LDH activity, DNA and protein content are summarized in Table 2. With the exception of C4S, none of the GAG induced a significant change of the investigated parameters. C4S induced a reduction of [^{3}H]thymidine incorporation at day 2 (32±5%), and at day 8 (35±1%) compared to untreated control. However, this effect could not be confirmed by measuring cellular DNA, protein content and cellular LDH activity (Table 2) indicating that C4S might interfere with the uptake or incorporation of [^{3}H] thymidine but did not affect the proliferation of rat osteoblasts.

Discussion

GAG play an important role in bone development. They are potent mediators of osteoblastic proliferation and differentiation. For example, bone-derived HS chains were shown to affect both proliferation and differentiation of primary murine and human osteoblasts [1, 10]. Moreover, HS was reported to promote the differentiation of MC3T3-E1 preosteoblasts by increasing the expression of runt domain transcription factor 2 (Runx2), alkaline phosphatase (ALP) and osteopontin (OPN) [26]. However, the high heterogeneity/variability of the molecules including the fine structure of disaccharide units, number and position of the sulfate residues, presence of *N*-acetyl and/or *N*-sulfate groups and relative molecular mass makes it difficult to link cellular effects to molecular structures [6]. Thus, the aim of the present study was to investigate the effect of defined chemically sulfated Hya in comparison to native non-sulfated Hya as well as native sulfated GAG including C4S, C6S, DS and HS on the proliferation of rat calvarial osteoblasts.

We demonstrated that both moderately and highly sulfated Hya derivatives reduced the proliferation of primary rat osteoblasts. In the presence of HyaS derivatives a higher percentage of cells remained in G1 phase of cell cycle with a concomitant decrease of the amount of cells in the S and G2/M phase. Our data are in accordance with recent findings showing that natural Hya has no significant effect on the proliferation of bovine pulmonary artery

Table 2 Effect of native sulfated GAG on [^{3}H]thymidine incorporation, cellular LDH activity, cellular DNA and protein content of primary rat osteoblasts

	Effect of GAG treatment [% of untreated control]							
	Day 2				Day 8			
	[^{3}H]-thymidine	DNA	Protein	LDH	[^{3}H]-thymidine	DNA	Protein	LDH
C4S	68±3*[a]	105±3	98±3	98±3	65±1*	98±2	100±1	94±4
C6S	97±1	106±1	96±2	97±4	102±3	100±2	100±1	93±3
DS	98±3	99±3	89±3	96±6	100±1	91±3	88±4	88±3
HS	102±2	109±2	92±3	105±8	97±9	89±1	89±4	103±2

[a] Data are presented as mean ± SD of three independent experiments (each in triplicate); significant differences are indicated with *($p<0.01$). $N=3$

smooth muscle cells but becomes highly anti-proliferative with sulfation [15]. Similarly, highly sulfated bone-derived HS was shown to induce a reversible arrest of adult human osteoblasts in the G2/M phase by down-regulation of the cell cycle related proteins Retinoblastoma (Rb), CDK1 and Cyclin A. These effects of osteoblast-derived HS were assigned to the interaction of HS with the bone-related growth factor TGF-β1 [10].

In contrast, native HMW- and LMW-Hya did not affect the osteoblastic proliferation between day 2 and 8 of culture. Huang *et al*. [27] demonstrated that short-term application of Hya with a wide molecular mass range between 60–2,300 kDa stimulated cell growth of rat calvarial-derived mesenchymal cells. In contrast, reports by Cuff *et al*. [14] and Kothapalli *et al*. [28, 29] revealed that the binding of HMW-Hya to CD44 antagonized the mitogen-induced cell cycle progression, whereas the binding of LMW-Hya to CD44 stimulates cell cycle progression in vascular smooth muscle cells and fibroblasts.

We could show that the natural sulfated GAG C4S, C6S, DS and HS have no effect on the cell proliferation of rat calvarial osteoblasts. The inhibitory effect of C4S on the tritiated-thymidine incorporation seems to be due to an interference of C4S with the assay because the inhibitory effect could not be confirmed by measuring cellular DNA, protein content and cellular LDH activity. This assumption is in line with an earlier report by Pietilä and Nikkari [30] showing that C4S diminishes the incorporation of tritiated-thymidine into DNA in rabbit aortic smooth muscle cells without affecting the growth rate, determined by cell number and cellular protein content. Thus, the results indicate that the presence of sulfate residues in the polysaccharide backbone alone is not sufficient for GAG to exert anti-proliferative properties. Rather a disaccharide unit composed of glucuronic acid and *N*-acetylglucosamine supplemented with sulfate residues in at least the C6-position of the N-acetylglucosamine are required for the anti-proliferative effect on primary rat osteoblasts.

In conclusion, our data show that sulfated Hya derivatives reduce the proliferation of primary rat osteoblasts probably by inducing an arrest of the cells in the G1 phase. In contrast, naturally non-sulfated HMW- and LMW-Hya do not affect the osteoblastic proliferation. By comparison, other native sulfated carbohydrates such as chondroitin sulfate, heparan sulfate and dermatan sulfate have no effect on the proliferation of osteoblasts. The inhibitory effect of C4S on the tritiated-thymidine incorporation assay seems to be due to an interference of C4S with the assay system.

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