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Published in:
Cell Biology International Reports

DOI:
[10.1016/0309-1651\(89\)90061-1](https://doi.org/10.1016/0309-1651(89)90061-1)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1989

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

BRAND, HS., VANKAMPEN, GPJ., VANDESTADT, RJ., Kuijer, R., & VANDERKORST, JK. (1989). Effect of sulfate concentration on glycosaminoglycan synthesis in explant cultures of bovine articular cartilage. *Cell Biology International Reports*, 13(2), 153-162. [https://doi.org/10.1016/0309-1651\(89\)90061-1](https://doi.org/10.1016/0309-1651(89)90061-1)

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EFFECT OF SULFATE CONCENTRATION ON GLYCOSAMINOGLYCAN SYNTHESIS IN
EXPLANT CULTURES OF BOVINE ARTICULAR CARTILAGE

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ABSTRACT

The effects of the sulfate- and FCS concentration on the rate of synthesis and the biochemical properties of glycosaminoglycans, synthesized in bovine articular cartilage in vitro, were studied. 20 % FCS in the culture medium stimulated the rate of synthesis. In media without FCS, the rate of synthesis decends from day 0 on. The differences in incorporation rates of [35 S]-sodium sulfate and 1,6- [3 H]-glucosamine-HCl into glycosaminoglycans in serum free media containing 9 μ M and 900 μ M sulfate were used to discuss the inorganic sulfate concentration in cartilage. In 9 μ M sulfate medium, the newly synthesized glycosaminoglycans contain higher levels of unsulfated disaccharides than the endogenous glycosaminoglycans. In each culture medium, the ratio 6-sulfated disaccharides to 4-sulfated disaccharides of the newly synthesized glycosaminoglycans becomes higher after 3 days in culture. The glycosaminoglycan synthesis is underestimated, when chondrocytes are cultured in media containing less than 200 μ M sulfate.

INTRODUCTION

Articular cartilage consists of chondrocytes, sparsely distributed in an abundant extracellular matrix mainly composed of collagen and proteoglycans. The chondrocytes are actively involved in both synthesis and degradation of the extracellular matrix. In explants of bovine articular cartilage, the turnover of the proteoglycans can be affected by the addition of modulators to the culture medium, e.g. bovine fetal or adult serum, insulin, bacterial lipopolysaccharides or retinoic acid (Campbell et al., 1984; Campbell and Handley, 1987; Hascall et al., 1983; McQuillan et al., 1986; Morales et al., 1984).

The highly hydrophilic proteoglycans are composed of glycosaminoglycans (GAGs), chains of repeating disaccharide units, covalently attached to a protein core. SO_4^{2-} can be attached by two different sulfotransferases to the 4 or 6 position of the N-acetylgalactosamine^{*} residue (Habuchi and Miyashita, 1982; Heinegard and Paulsson, 1984).

Intraperitoneal administration of sodium salicylate, a widely used nonsteroidal antiinflammatory drug, to mice leads to a marked fall in the inorganic sulfate concentration of the serum (de Vries et al., 1985). The sulfate concentration of the culture medium

affects the biochemical composition of newly synthesized proteoglycans in mouse patellae and chick embryo cartilage in vitro (Ito et al., 1982; Sobue et al., 1978; van der Kraan et al., 1988). Undersulfation of GAGs in articular cartilage of dogs with spondyloepiphyseal dysplasia, leads to altered biomechanical properties (Bingel et al., 1986).

In this study, the effect of high and low sulfate concentrations on the rate of synthesis and the biochemical composition of GAGs, synthesized in explant cultures of bovine articular cartilage, was studied.

MATERIALS AND METHODS

[^{35}S]-Sodium sulfate (22.126 GBq/mmol) and 1,6-[^3H]-glucosamine-HCl (1572.5 GBq/mmol) were purchased from New England Nuclear. Nutrient mixture F-12 (HAM), Gey's Balanced Salt Solution (GBSS), Fetal Calf Serum (FCS) and penicillin/streptomycin were purchased from Gibco Europe, Protease K type XI from Sigma, Chondroitinase ABC (EC 4.2.2.4) and chondroitinase AC II (EC 4.2.2.5) from Miles Scientific. Instagel II was obtained from Packard. All other chemicals were obtained from Merck or BDH (analytical or suprapur grade). Bacteriological petridishes, diameter 6 cm, were purchased from NUNC. HPLC equipment from Waters was used, with a Polyol Si300 column (Serva) and a Radial-PAK $\mu\text{BONDAPAK NH}_2$ column (Waters).

Metacarpophalangeal joints from six months old calves were obtained from the local slaughterhouse, within 2 hours after the animal was killed. The joint was opened under aseptical conditions, the full depth cartilage was excised, collected in GBSS and cut to pieces of approximately 3 x 3 x 1 mm. Explants (150-200 mg wet weight) were transferred to a bacteriological culture dish, containing 5 ml culture medium. The culture media consisted of Ham F-12 with 0.1 % penicillin/streptomycin with/without 20 % FCS (vol/vol). An adjusted amount of sodiumsulfate was added to the culture media to give a final sulfate concentration of 9 or 900 μM .

The incubation was carried out at 37° C (5 % CO_2 in air) and the culture media were changed daily. Quadruple cultures were used for each determination.

After 0, 3, 7 or 10 days incubation, the culture medium was replaced by 5 ml of the corresponding culture medium to which 185 kBq ^{35}S -sulfate/ml and 148 kBq ^3H -glucosamine/ml was added and incubated for 17 hours. Thereupon the culture medium was replaced with cold GBSS (4° C). The explants were washed 3 times in cold GBSS (Maroudas and Evans, 1974), lyophilized and dry weight was determined. The culture media were dialyzed exhaustively against water and counted for radioactivity.

The lyophilized explants were digested with protease K type XI (2.5 U in 1 ml 0.05 M Tris-HCl, 0.001 M CaCl_2 , pH 7.9) and a reduction was performed (0.1 M NaOH, 1 M NaBH_4 , 48 hours at 45° C). The GAGs were precipitated with 4 volumes of ethanol (-20° C) and counted for radioactivity (Hascall et al., 1983). The amount of sulfate, incorporated into GAGs (nmol), was calculated for each

culture from the concentration of sulfate in the culture medium, the specific activity of the radiolabel in the culture medium and the amount of radiolabel, incorporated into GAGs.

The hydrodynamic volumes of the GAGs were determined on a Polyol-Si300 gelpermeation column (van de Stadt et al., 1986). Fractions were monitored for radioactivity and uronic acid (Bitter and Muir, 1962).

The GAGs were digested with chondroitinase AC II (0.05 IU/mg GAG). The undigested material was precipitated with ethanol/sodium acetate, washed twice with ethanol and digested with chondroitinase ABC (Gurr et al., 1985). The unsulfated (Δ Di-OS), 6-sulfated (Δ Di-6S) and the 4-sulfated (Δ Di-4S) disaccharides were separated, collected and counted for radioactivity (Hjerpe et al., 1979).

Inorganic sulfate was determined using the benzimidazole method (de Vries et al., 1987)

RESULTS

Initially, the incorporation of glucosamine into GAGs of explants cultured in both media with 0 % FCS is less compared to medium with 20 % FCS 900 μ M sulfate. Prolonged incubation of articular cartilage explants, in media without FCS, leads to a significant decrease in incorporation of glucosamine after 3 days (0 % FCS 9 μ M sulfate -26 %, 0 % FCS 900 μ M sulfate -37 % compared to day 0). After 7 days incubation a constant level is reached (fig. 1). In medium with 20 % FCS 900 μ M sulfate, the incorporation of glucosamine into GAGs is stimulated on day 3 and 7 (+ 32 %, + 27 % compared to day 0, respectively). On day 10, this effect of FCS has disappeared.

The calculated amount of sulfate, incorporated into GAGs in explants, cultured in medium with 0 % FCS 9 μ M sulfate on day 0, is very low compared to the media with 900 μ M sulfate (< 10 %). Prolonged incubation of articular cartilage explants in media without FCS leads to a progressive decline in incorporation of sulfate into GAGs upto day 7, after which a constant level of incorporation was achieved. In medium with 20 % FCS 900 μ M sulfate, the incorporation of sulfate is stimulated significantly on day 3 and 7 (+ 35 % and + 34 % respectively, fig 2).

Irrespective of the culture medium or days in culture, less than 7 % of the radiolabel incorporated into GAGs, was recovered from the media. These fractions were not analysed further.

The endogenous and the newly synthesized, radiolabeled GAGs had identical hydrodynamic volumes, K_{av} 0.30 - 0.33, irrespective of the presence of FCS, the sulfate concentration in the culture medium or number of days in culture (results not shown).

75 $\pm$ 3 % of the newly synthesized GAGs are chondroitinase AC II sensitive. The relative amounts of the composing disaccharides are presented in table I. The endogenous GAGs contained 15 - 26 % Δ Di-OS. The amount of Δ Di-OS in newly synthesized GAGs in explants

FIG 1.

The incorporation of $[^3\text{H}]$ -glucosamine in GAGs per mg dry weight of explants cultured in medium with 0% FCS 9 μM sulfate (●---●), 0% FCS 900 μM sulfate (○---○) or 20% FCS 900 μM sulfate (□---□). Each point represents the average of quadruple determinations, the vertical bar represents the standard deviation. Groups sharing a common character do not differ significantly (Student T-test, $p < 0.01$).

FIG 2.

The incorporation of sulfate in GAGs (nmol) per mg dry weight of explants cultured in medium with 0% FCS 9 μM sulfate (●---●), 0% FCS 900 μM sulfate (○---○) or 20% FCS 900 μM sulfate (□---□). Each point represents the average of quadruple determinations, the vertical bar represents the standard deviation. Groups sharing a common character do not differ significantly (Student T-test, $p < 0.01$).

TABLE I

The disaccharide composition of chondroitinase AC II sensitive glycosaminoglycans of explants cultured in media with or without 20 % FCS and with low or high sulfate concentrations.

- A. The percentage unsulfated (Δ Di-0S) disaccharides of endogenous (end.) and newly synthesized, ^3H -labeled glycosaminoglycans (^3H).
 B. Ratio of 6-sulfated: 4-sulfated disaccharides (Δ Di-6S : Δ Di-4S) of endogenous and newly synthesized glycosaminoglycans.

		DAYS IN CULTURE:			
		0	3	7	10
A. Percentage Δ Di-0S					
0 % FCS 9 μM sulfate	end:	26	21	24	25
	^3H :	40	54	54	51
0 % FCS 900 μM sulfate	end:	20	19	19	16
	^3H :	16	23	24	24
20 % FCS 900 μM sulfate	end:	17	16	17	15
	^3H :	16	18	19	18
B. Ratio Δ Di-6S : Δ Di-4S					
0 % FCS 9 μM sulfate	^3H :	0.83	1.14	1.11	1.11
0 % FCS 900 μM sulfate	^3H :	0.66	0.96	0.81	0.79
20 % FCS 900 μM sulfate	^3H :	0.68	1.05	0.98	0.98
Endogenous		0.53 - 0.65			

cultured in both media with 900 μM sulfate, did not differ from the amount in endogenous GAGs. However, in 9 μM sulfate medium explants produced ^3H -labeled GAGs, which contained significantly higher levels of $\Delta\text{Di-OS}$ (Table IA).

The ratio $\Delta\text{Di-6S} : \Delta\text{Di-4S}$ in the endogenous GAGs was 0.53-0.65. Irrespective of the number of days in culture, the ratios $\Delta\text{Di-6S} : \Delta\text{Di-4S}$ of newly synthesized GAGs in explants cultured in 0 % FCS 9 μM sulfate medium were always higher (0.83 - 1.14) than the corresponding ratios of the endogenous GAGs. In explants cultured in media with 900 μM sulfate, the ratios $\Delta\text{Di-6S} : \Delta\text{Di-4S}$ of the newly synthesized GAGs were, on day 0, 0.66 - 0.68, which is comparable with the corresponding ratios of the endogenous GAGs. After 3 days incubation, however, the ratio in the newly synthesized GAGs was higher. This phenomenon was more pronounced in explants cultured in 20 % FCS 900 μM sulfate medium, than in those cultured in 0 % 900 μM sulfate medium (1.05 and 0.96, respectively)(Table IB).

In all culture conditions, 10 % of the endogenous GAGs were chondroitinase AC II resistant, but chondroitinase ABC sensitive. These GAGs contained 2 - 4 % $\Delta\text{Di-OS}$ and were predominantly 4-sulfated (table II). The newly synthesized chondroitinase ABC sensitive GAGs, produced by explants cultured in both media with 900 μM sulfate on day 0 contained 14 % $\Delta\text{Di-OS}$ and in medium with 0 % FCS 9 μM sulfate 23 % $\Delta\text{Di-OS}$. From day 3 up to 10, newly synthesized GAGs contained a constant amount of $\Delta\text{Di-OS}$: In 0 % FCS 9 μM sulfate and 0 % FCS 900 μM sulfate medium 28 % $\Delta\text{Di-OS}$, in 20 % FCS 900 μM sulfate medium 24 %.

TABLE II

The disaccharide composition of chondroitinase AC II resistant, but chondroitinase ABC sensitive glycosaminoglycans cultured in media with or without 20 % FCS and with high or low sulfate concentrations. Ratio of 6-sulfated : 4-sulfated disaccharides of endogenous and newly synthesized ^3H -labeled glycosaminoglycans.

	DAYS IN CULTURE:			
	0	3	7	10
0 % FCS 9 μM sulfate	$^3\text{H} : 0.52$	0.65	0.71	0.68
0 % FCS 900 μM sulfate	$^3\text{H} : 0.44$	0.68	0.76	0.84
20 % FCS 900 μM sulfate	$^3\text{H} : 0.46$	0.78	0.79	0.67
Endogenous	0.27 - 0.34			

On day 0, in each culture medium, the ratio $\Delta\text{Di-6S} : \Delta\text{Di-4S}$ of the newly synthesized chondroitinase ABC sensitive GAGs (0.44-0.52) was higher than in the endogenous ones. After 3 days incubation, the ratios $\Delta\text{Di-6S} : \Delta\text{Di-4S}$ of the newly synthesized GAGs reached a constant level (0.65-0.84).

DISCUSSION

Bovine articular cartilage can be maintained in explant culture for several weeks. 20 % FCS has a stimulatory effect to the rate of GAG synthesis. After 7 days incubation, the ratio of GAG synthesis in medium with FCS to medium without FCS is 4. This value is not different from explants cultured in DMEM (Hascall et al., 1983; Handley et al., 1986).

The incorporation of sulfate into GAGs in explants, cultured in media with 900 μM sulfate, correlates well with the incorporation of glucosamine. However, in 0 % FCS 9 μM sulfate medium, the sulfate incorporation is extremely low, compared to the glucosamine incorporation (fig. 2). The ^3H -glucosamine incorporation rates in both media without FCS and the hydrodynamic volumes of the newly synthesized GAGs are identical. An increase in the specific activity of the endogenous glucosamine or UDP-sugars pools is improbable (Kim and Conrad, 1976; Sweeney et al., 1988), which leads to the conclusion that the specific activity of the inorganic sulfate concentration in the culture media must be decreased by an amount of inorganic sulfate from the cartilage. The synovial fluid of the metacarpophalangeal joint contained 0.87 mM inorganic sulphate. In mammals, the molar distribution coefficient between cartilage and synovial fluid is 0.37 - 0.45 (Maroudas, 1975), which means that the explants contain 64 - 78 nmol inorganic sulphate per 200 mg wet weight. This is 1.4 - 1.7 times the amount of sulphate, available in 5 ml 9 μM 0 % FCS culture medium. Another, minute, source of sulphate is turnover of cysteine (Esko et al., 1986).

The ratio $\Delta\text{Di-6S} : \Delta\text{Di-4S}$ disaccharides of newly synthesized chondroitinase AC II sensitive GAGs in explants became higher. In 0 % FCS 900 μM sulfate medium this increase was less pronounced, compared with 0 % FCS 9 μM or 20 % FCS 900 μM sulfate medium (Table IB). A change in the ratio $\Delta\text{Di-6S} : \Delta\text{Di-4S}$ disaccharides was also reported for epiphyseal chick cartilage, cultured in media with sulfate concentrations, ranging from 0.03 up to 300 μM (Ito et al., 1982). The activity of chondroitin 4-sulfotransferase and chondroitin 6-sulfotransferase in chick embryo cartilage, was also affected by the culture media used (Habuchi and Miyashita, 1982).

The endogenous GAGs that are chondroitinase AC II resistant, but chondroitinase ABC sensitive are dermatansulfate, which is consistent with the low amount of $\Delta\text{Di-OS}$ and the low $\Delta\text{Di-6S} : \Delta\text{Di-4S}$ ratio in these GAGs (Swann et al., 1983).

We conclude that the sulfate concentration of the culture medium, in which chondrocytes are cultured, is important for the sulfation degree of the newly synthesized GAGs. For the culture of bovine

articular cartilage explants, the culture medium should contain at least 200 μM sulfate. In culture media with less than 200 μM sulfate, the contribution of the inorganic sulfate in cartilage to the total pool of sulfate available will be considerable, depending on the amount of cartilage and the volume of the culture medium. Secondly, at extreme low environmental sulfate concentrations chondrocytes will synthesize chondroitinsulfate chains that are less sulfated, compared to the endogenous ones. When the incorporated amount of ^{35}S -sodiumsulfate in GAGs by explants, cultured in media containing less than 200 μM sulfate, is used to measure the synthesis of proteoglycans, both effects will lead to an underestimation of the GAG synthesis. However, even explants, cultured in media that contain enough sulfate, synthesize GAGs that, judged by the $\Delta\text{Di-6S} : \Delta\text{Di-4S}$ ratio, differ from the endogenous ones.

ACKNOWLEDGEMENT

The authors thank E. Kiljan and M.H.M.T. de Koning for their expert technical assistance. Bovine metacarpophalangeal joints were obtained from the Amsterdam slaughterhouse by courtesy of G. Gruijthuijsen. This work was supported by a grant of the Dutch League against Rheumatism.

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Received 25.9.88

Accepted 27.11.88