

Effect of Fully Sulfated Glycosaminoglycans on Pulmonary Artery Smooth Muscle Cell Proliferation

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Received May 25, 1999, and in revised form August 30, 1999

Fully sulfated heparin and other glycosaminoglycans, namely heparan, chondroitin, and dermatan sulfates, and hyaluronan have been prepared by using sulfur trioxide under mild chemical conditions. All these derivatives were assayed for antiproliferative activity on cultured bovine pulmonary artery smooth muscle cells (BPASMCs). No appreciable difference was found between heparin and fully sulfated heparin. Chondroitin and dermatan sulfates actually stimulated BPASMCs growth but full sulfonation made them strongly antiproliferative. Native hyaluronan was not antiproliferative but became strongly so after sulfonation. Neither acharan sulfate nor *N*-sulfoacharan sulfate had any antiproliferative activity. This suggests that *O*-sulfonation of the polysaccharide is critical for antiproliferative activity, whereas *N*-sulfonation of glucosamine residues is not. © 1999 Academic Press

Key Words: chemical sulfonation; glycosaminoglycans; smooth muscle cell; proliferation.

Pulmonary hypertension due to chronic hypoxia is associated with increased quantities of smooth muscle cells (SMCs) in pulmonary arteries, due to thickening of the medial muscle layer in the proximal arteries and extension of the smooth muscle investment into peripheral, normally nonmuscular vessels (1–4). Heparin inhibits hypoxic pulmonary hypertension, possibly by an antiproliferative effect on SMCs (5–7). In earlier

studies, we have investigated the structure requirements for heparin's antiproliferative effect for pulmonary artery SMCs. These studies indicated that (a) the molecular size of the heparin does not effect its potency, (b) the protein core of heparin has no antiproliferative activity, (c) the heparin glycosaminoglycan chain is responsible for the antiproliferative activity, (d) the 3-*O*-sulfate on the internal glucosamine residue is not critical for natural heparin's antiproliferative activity, and (e) the charge density of heparin may affect the antiproliferative activity (8, 9).

The role of *N*- and *O*-linked sulfate groups on glucosamine and uronic acid sugar residues for the antiproliferative effect of heparin is not clear. Tozzio and coworkers have shown that 2-*O*-sulfonation of glucuronic acid in heparin is important for antiproliferative properties (10). On the other hand, Wright and coworkers found that 2-*O*-sulfonation in heparin is not essential for antiproliferative activity (11). The relationship between heparin *N*-sulfate groups and antiproliferative activity has not been found to be straightforward (10). To establish the role of *N*- and *O*-sulfonation in the heparin, we have chemically modified heparin (Fig. 1A), heparan (Fig. 1B), chondroitin (Fig. 1C), dermatan (Fig. 1D) sulfates, and hyaluronan (Fig. 1E) by *O*-sulfonation and acharan sulfate [a glycosaminoglycan of unusual structure found only in invertebrates (16) (Fig. 1F)], by *N*-deacetylation followed by *N*-sulfonation, and we have tested them for their antiproliferative activity on bovine pulmonary artery SMCs.

EXPERIMENTAL PROCEDURES

Reagents. Chondroitin sulfate (*M*, 15 K) from bovine tracheal cartilage, and dermatan sulfate (*M*, 30 K) from porcine skin were gifts from Shin-Nippon Yakugyo Co. (Tokyo, Japan). Hyaluronan

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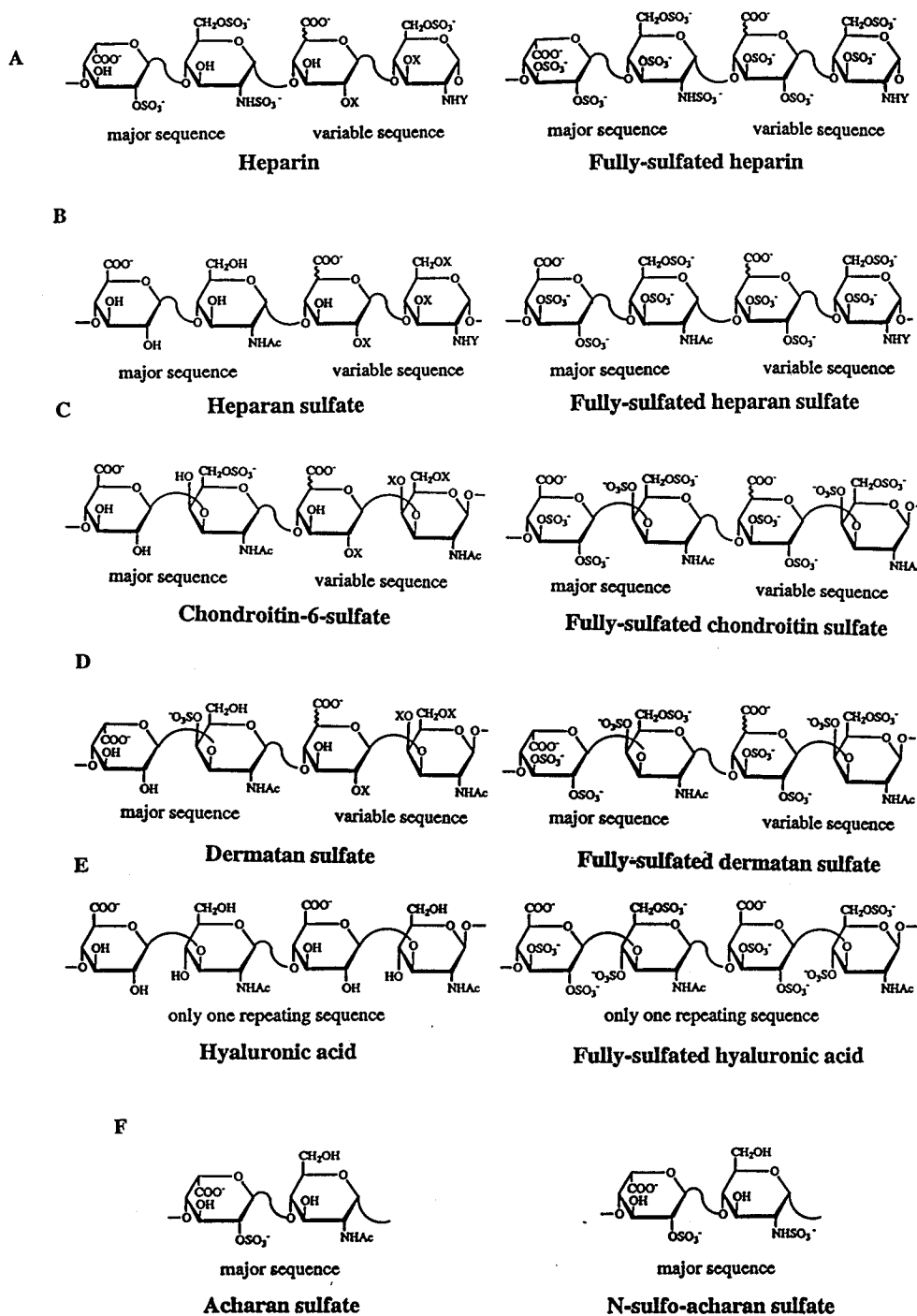


FIG. 1. Major and variable sequences of original and fully sulfated glycosaminoglycans: (A) heparin, (B) heparan sulfate, (C) chondroitin sulfate, (D) dermatan sulfate, (E) hyaluronan, (F) acharan sulfate and *N*-sulfoacharan sulfate, X = H or SO₃, Y = acetyl or SO₃.

(*M*, 100 K) was purchased from Kibun Food Chemipha Co. (Tokyo, Japan). Heparin (*M*, 16 K) and heparan sulfate (14.8 K) from porcine intestinal mucosa were purchased from Celsus (Cincinnati, Ohio). Fetal bovine serum and other reagents were obtained from Sigma (St. Louis, MO). Cell culture media, RPMI-1640 (Mediatech, Washington, DC), contained streptomycin (Lilly, Indianapolis, IN), penicillin (Pfizer, New York, NY), and amphotericin B (GIBCO, Grand Island, NY).

Sulfonation of heparin and other glycosaminoglycans. This procedure relied on mild sulfonation conditions similar to those described for the *O*-sulfonation of chondroitin sulfate (12). Briefly, the tributylammonium salt of each glycosaminoglycan was prepared from 100 mg of the sodium salt by strong cation-exchange chromatography and dried by lyophilization. The resulting salt was dissolved in 0.8 ml of *N,N*-dimethylformamide to which excess (15 mol/equivalent of available hydroxy group in glycosaminoglycan) pyridine-

sulfur trioxide complex had been added. After incubating 1 h (for chondroitin and dermatan sulfates) to 3 h (for heparin, heparan sulfate and hyaluronan) at 40°C, the reaction was quenched through the addition of 1.6 ml of water, and the crude product was precipitated with 3 vol of cold ethanol saturated with anhydrous sodium acetate and collected by centrifugation. The resulting fully *O*-sulfonated glycosaminoglycan was then dissolved in water, dialyzed to remove salts, and lyophilized to afford 120 mg of purified product. *N*-sulfonation of the oversulfated heparin and heparan sulfate was carried out under the conditions described earlier by Levy and Petracek (13). Briefly, 100 mg of *O*-sulfonated heparin was dissolved in 5 ml of water containing 180 mg sodium carbonate and incubated at 55°C and a fivefold excess sulfur trioxide-trimethylamine complex was added three times at 0, 5, and 10 h. After reaction at 55°C for 15 h, 5 ml of 16% NaCl and 45 ml of methanol were added, the mixture was kept on ice for 1 h, and the precipitated *O*-, *N*-sulfonated heparin was collected, dialyzed, and lyophilized to afford 92 mg of purified product. The molar ratio of sulfate to hexosamine for each fully sulfonated glycosaminoglycan, determined by high-performance liquid chromatography, was as follows: chondroitin sulfate, 3.96 ± 0.08 ; dermatan sulfate, 3.95 ± 0.04 ; hyaluronan, 3.79 ± 0.05 ; heparin, 4.88 ± 0.05 ; heparan sulfate, 4.22 ± 0.04 (SD was based on five independent syntheses). While the water solubility of each glycosaminoglycan was dramatically increased (>50 mg/ml) after chemical sulfonation (compared to that of intact glycosaminoglycan), the viscosity of each sulfonated glycosaminoglycan solution was reduced.

Disaccharide and compositional analysis. The determination of unsaturated disaccharides prepared from intact and modified glycosaminoglycans was performed following polysaccharide lyase digestion using high-performance liquid chromatography (14). Glycosaminoglycan samples were prepared for sulfate and hexosamine determination by exhaustive dialysis (MWCO 3500) against distilled water, lyophilization, and drying for 2 days over P_2O_5 . Determination of sulfate groups was performed by anion-exchange high-performance liquid chromatography after acid hydrolysis of the sample in 6 M HCl at 100°C for 2.5 h using conductivity detection (Tosoh Model CM-8, Tokyo, Japan). Hexosamine was analyzed by the postcolumn high-performance liquid chromatography derivatization method (15) following acid hydrolysis under identical conditions as described for sulfate analysis.

Preparation of acharan sulfate and *N*-sulfoacharan. Acharan sulfate and *N*-sulfoacharan sulfate were prepared as previously described (16, 17).

Determination of molecular weight. Gradient polyacrylamide gel electrophoresis analysis (18) together with gel permeation high-performance liquid chromatography (19) were used to estimate the weight average molecular weight of each sample. The relative molecular weight of each glycosaminoglycan was based on both their migration and elution positions (18, 19).

Anti-factor IIa activity. Anti-factor IIa activity was determined by incubating 50 μ l of glucosaminoglycan-containing sample with 30 μ l of normal human plasma and 20 μ l of human thrombin (1.2 NIH units/ml) at 37°C for 30 s. Chromogenic thrombin substrate (ethylmalonyl-PrO-Arg-p-nitroaniline hydrochloride) was added and amidolytic thrombin activity was measured on an ACL 300 plus instrument (Lexington, MA) (12). Activity in units per milligram was calculated in comparison to USP heparin reference standard K-3 (The United States Pharmacía, 1990, 1457).

Cultured pulmonary artery smooth cells proliferation assay. Smooth muscle cell proliferation assays were performed as previously described (8, 9). Briefly, isolated bovine pulmonary artery smooth muscle cells (BPASMC) were seeded at 1.5×10^4 cells/well into six-well tissue culture plates, grown for 2 days, and then growth arrested for 48 h by reducing the serum concentration of the medium from 10 to 0.1%. Media were then changed for experimental samples to contain either standard medium (RPMI-1640 with 10% FBS),

growth arrest medium (0.1% FBS), or standard medium containing heparin or other GAG derivative (10 μ g/ml). All media contained streptomycin (10 μ g/ml), penicillin (100 U/ml), and amphotericin B (1.25 μ g/ml). After 4–5 days, the BPASMCs present in the cell culture wells were rinsed with Hank's balanced salt solution to remove the remaining cell culture medium and unattached dead cells. No dead cells (trypan blue exclusion) either in control or treated with GAGs (both sulfated or nonsulfated) were observed. After detachment of BPASMCs with trypsin/EDTA, the cell number was counted by Coulter counter.

The glycosaminoglycans, including the fully sulfated ones (1 mg) were each dissolved (1 mg/ml) in distilled sterile water. This solution (140 μ l) was added to culture medium (13.86 ml) and 2 ml of the resulting medium was added to each well. The fully sulfated glycosaminoglycans were completely soluble, as the culture medium was clear (no turbidity) after addition of the sulfated glycosaminoglycans.

The effective dose of heparin has been previously found to be identified as 1 to 10 μ g/ml (20). In the present study 10 μ g/ml was used for each of the glycosaminoglycans.

The percentage growth was calculated as

$$\% \text{ growth} = \frac{\text{net cell growth in treated medium}}{\text{net cell growth in standard medium}} \times 100,$$

where net cell growth equals cell growth in standard or treated medium minus cell growth in growth arrest medium.

Statistics. Results are presented in each case as the mean \pm standard error of the mean. Comparisons among groups were made with a factorial analysis of variance (ANOVA), using the Stateview software package (BrianPower, Inc., Calabassas, CA) for Macintosh computers. If ANOVA were significant, multiple comparisons were made using Fisher protected least significant difference (PSLD) test. In all cases, significance was set as $P < 0.05$.

RESULTS AND DISCUSSION

It is well established that heparin inhibits the proliferation of arterial smooth muscle cells *in vitro* and *in vivo* (21). Heparin and other glycosaminoglycans including hyaluronan are linear polysaccharides; the backbone of these polysaccharides has the formula $(A-B)_n$, where A is a uronic acid residue and B is a hexosamine residue, as described in Table I (22).

Acharan sulfate is also a linear polysaccharide consisting primarily of 2-sulfo-iduronic acid α -(1 \rightarrow 4) and *N*-acetylglucosamine α -(1 \rightarrow 4) (16). Heparin and heparan sulfate are composed of different proportions of the same building blocks. In heparin the content of sulfamido groups greatly exceeds the content of acetamido groups, and the number of *O*-sulfo groups exceeds that of *N*-sulfo groups (23).

Chemical *O*-sulfonation, to obtain fully sulfated heparin, heparan sulfate, dermatan sulfate, chondroitin sulfate, and hyaluronan, was performed with adducts of sulfur trioxide in aprotic solvents (24). Sulfate analysis and NMR spectroscopy (25) showed that the *O*-sulfonated glycosaminoglycans had no free hydroxyl groups. No breakdown of the polysaccharide backbone was detected, under the mild sulfonation conditions that were used, based on the observed increase in molecular weight expected from the additional sulfate

TABLE I
General Composition of Different Glycosaminoglycans (A-B)_n

Glycosaminoglycan	A	Linkage	B	Linkage	NAc	SO ₃ ⁻
Hyaluronan	GlcA	β-(1 → 3)	GlcN	β-(1 → 4)	+	0
Chondroitin sulfate	GlcA	β-(1 → 3)	GalN	β-(1 → 4)	+	+
Dermatan sulfate	IdoA (GlcA)	α-(1 → 3) β-(1 → 3)	GalN	β-(1 → 4)	+	+
Heparan sulfate	IdoA (GlcA)	α-(1 → 4) β-(1 → 4)	GlcN	α-(1 → 4)	(+)	+
Heparin	IdoA (GlcA)	α-(1 → 4) β-(1 → 4)	GlcN	α-(1 → 4)	(+)	+++

Note. GlcA, glucuronic acid, residue shown in parenthesis is minor component; IdoA, iduronic acid; GlcN, glucosamine; GalN, galactosamine; (+) indicates partially *N*-acetylated glucosamine residues; ++ and +++ indicate more than one sulfate group in repeating disaccharide units are present.

groups. The *O*-sulfonation of heparin and heparan sulfate resulted in partial de-*N*-sulfonation of the glucosamine residue. Thus, these derivatives were re-*N*-sulfonated (13). *N*-sulfonated acharan sulfate was prepared by *N*-deacetylation and *N*-sulfonation of acharan sulfate following the known method (16, 17). Properties of fully sulfated derivatives and anticoagulant activity are listed in Table II. These sulfonation reactions were performed five times and repeatedly afforded uniformly fully sulfated glycosaminoglycan products.

Effects of fully sulfated derivatives and starting glycosaminoglycans at the concentration of 10 μg/ml on the growth of bovine pulmonary artery smooth muscle cells are given in Fig. 2 in broken and solid columns, respectively. BPASMC growth in heparin-free media was defined as 100%. When heparin was added to cultured BPASMCs, there was a significant inhibition of cell growth as compared to standard media (36.4 ±

2.5% growth, $P < 0.05$). Fully sulfated heparin showed a similar degree of growth inhibition at 38.8 ± 3.9%. Growth was significantly reduced from standard media ($P < 0.05$) but was not different from the original heparin. Native heparan sulfate did not effect BPASMC growth (85.3 ± 5.2% growth). However, fully sulfated heparan sulfate containing either *N*-acetyl or *N*-sulfate group on glucosamine residues (Fig. 2) suppressed the growth of BPASMCs in comparison to native heparan sulfate and standard media (59.4 ± 4.6% growth, $P < 0.05$). Chondroitin and dermatan sulfates were actually proliferative to BPASMCs (116.8 ± 5.8 and 158.1 ± 20.9% growth, respectively, $P < 0.05$ as compared to standard media). Fully sulfated chondroitin (50.7 ± 3.9% growth) and dermatan sulfate (48.7 ± 3.2% growth) inhibited the growth of BPASMCs in comparison to native polysaccharides ($P < 0.05$ for both sulfated polysaccharides). Native

TABLE II
Physical-Chemical Properties Anticoagulant Activity of Heparin and Other Glycosaminoglycans, Acharan Sulfate, and Their Derivatives

Compound	Disaccharide unit		Average molecular weight (kDa)	Optical rotation [α] _D	Anti-IIa activity
	mol O-SO ₃ ⁻	mol N-SO ₃ ⁻			
Heparin (HP)	2.0	0.88	16.0	+46.5	327
Fully sulfated HP	4.0	0.68	20.0	+22.3	74
Heparan sulfate (HS)	1.0	0.22	14.8	+70.0	3
Fully sulfated HS	4.0	0.22	24.0	+31.5	38
Chondroitin sulfate (CS)	1.0	0	15.0	-30.0	4
Fully sulfated CS	4.0	0	23.8	-8.0	39
Dermatan sulfate (DS)	1.0	0	30.0	-41.5	1
Fully sulfated DS	4.0	0	47.5	-10.5	62
Hyaluronan (HA)	0	0	100	-32.5	0
Fully sulfated HA	4.0	0	198	-25.0	65
Acharan Sulfate (AS)	1.0	0	29	nd	0
<i>N</i> -sulfo AS	1.0	1.0	8	nd	21

Note. nd, not determined; Anti-IIa activity is described as units per mg.

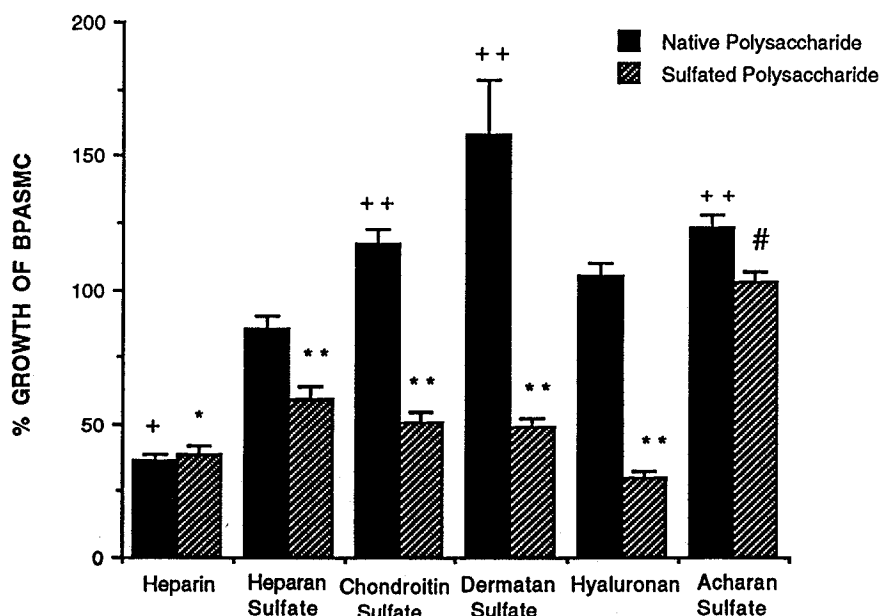


FIG. 2. Effect of various polysaccharides on bovine pulmonary artery smooth muscle cells grown in media containing 10% fetal bovine serum plus either original (solid bars) or fully sulfated glycosaminoglycan (striped bars) or acharan sulfate (solid bar) or *N*-sulfoacharan sulfate (striped bar). Samples are standardized to growth in media containing 10% serum without polysaccharide. +, Significant reduction in cell growth as compared to the standard ($P < 0.05$); ++, significant increase in cell growth as compared to the standard ($P < 0.05$); *, significant reduction in cell growth as compared to the standard ($P < 0.05$), but no difference as compared to the native polysaccharide; **, significant reduction in cell growth as compared to both the standard and the native polysaccharide ($P < 0.05$); #, significant reduction in cell growth as compared to native acharan sulfate ($P < 0.05$), but not to the standard.

hyaluronan had no significant effect on BPAMSC proliferation but became strikingly antiproliferative with sulfonation ($29.7 \pm 2.6\%$ growth, $P < 0.05$). Acharan sulfate had a significant proliferative effect on BPAMSCs ($123 \pm 4.2\%$ growth, $P < 0.05$ as compared to standard media). *N*-sulfoacharan sulfate significantly decreased BPAMSC growth ($P < 0.05$ as compared to native acharan sulfate) but failed to demonstrate a significant antiproliferative effect as compared to standard media.

The relationship of anticoagulant activity to antiproliferative activity on BPAMSC growth was weak. Full sulfonation of native heparin reduced its anticoagulant activity as measured by anti-factor IIa activity by 77% (Table II), while this had no effect on antiproliferative activity (Fig. 2). Full sulfonation of the other glycosaminoglycans in most cases produced marked increase in antiproliferative activity but only a very modest increase in anticoagulant activity (Fig. 2, Table II). As previously reported (26), these data suggest that the structural determinants for the two biological activities, namely the anticoagulant and antiproliferative, are different.

This study was designed to examine the role of the degree of sulfation, linkage of acidic and basic sugar residues, significance of *N*-sulfate groups, and importance of glucosamine residues of heparin, in determining the antiproliferative activity of BPAMSCs. No

change in the growth of BPAMSCs by fully sulfated heparin suggests that native heparin contains optimal sulfate groups necessary for antiproliferative activity. However, heparan sulfate does not appear to have sufficient *O*-sulfo groups to have full potency and therefore an increase in sulfonation of heparan sulfate decreases the growth of BPAMSCs. Our data on the effect of full sulfonation of chondroitin and dermatan sulfates on the growth of BPAMSCs also suggest that *O*-sulfo groups in both the sugar residues of the repeating disaccharide unit are very important for antiproliferative activity. Further, the galactosamine sugar residues in chondroitin and dermatan sulfate are all *N*-acetylated (Table I). These results are supportive of the earlier findings on heparin that the *N*-sulfate groups on glucosamine residues could be replaced with *N*-acetyl groups without significant loss of activity (27, 28). However, these results are at variance with an earlier report that 2-*O*-sulfonation and a negative charge at the *N*-position of the basic residues present in heparin are required for antiproliferative activity (10). Our results for fully sulfated chondroitin and dermatan sulfates suggest that basic sugar residues of glucosamine in heparin could be replaced by galactosamine without significant loss of activity. A remarkable increase in the antiproliferative activity of fully sulfated hyaluronan in comparison to heparin suggests that the anomeric linkage between acidic and basic

sugar residues [Fig. 1A, heparin, α -(1 \rightarrow 4); Fig. 1E, hyaluronan, β -(1 \rightarrow 3)] is not critical for antiproliferative activity. These results also further confirm that *N*-sulfonation is not critical for antiproliferative activity as all the glucosamine residues in hyaluronan are *N*-acetylated and the potency of fully sulfated hyaluronan is similar to heparin.

Oversulfated glycosaminoglycans have been previously reported (29–31). Chondroitin sulfates D and E, for example, have two sulfate groups in each disaccharide repeating unit (29, 30), compared to the single sulfate group commonly observed in chondroitin sulfates A and C (CS, Table II). An even more highly sulfated dermatan that contains three sulfate groups in each disaccharide unit has also been reported (31). While these oversulfated chondroitin/dermatan sulfates were first isolated from shark, squid, and hagfish cartilage, they have been recently observed in higher animals such as the mammalian mast cells (32, 33). Endogenous oversulfated chondroitin sulfates present in these tissue might be responsible for various biological activities through their effect on cell proliferation (34).

In conclusion, we have demonstrated that: (1) a certain number of *O*-sulfo groups of heparin is essential for antiproliferative activity, (2) the *N*-sulfo group on basic sugar residues is not critical for antiproliferative activity, (3) the basic sugar residues of glucosamine are replaceable with galactosamine residues, and (4) anomeric linkages of acidic and basic sugar residues are not essential for antiproliferative activity.

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