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Glycosaminoglycans Can Influence Fibroblast Growth Factor-2 Mitogenicity without Significant Growth Factor Binding

Huiming Wang,* Toshihiko Toida,† Yeong Shik Kim,‡ Ishan Capila,§ Ronald E. Hileman,§ Merton Bernfield,* and Robert J. Linhardt§

**Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115; †Faculty of Pharmaceutical Sciences, Chiba University, Ciba 263, Japan; ‡Department of Natural Products Research Institute, Seoul National University, Seoul 151-742, Korea; and §Division of Medicinal and Natural Products Chemistry and Department of Biochemical Engineering, University of Iowa, PHAR S328, Iowa City, Iowa 52242*

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Fibroblast growth factors are important heparin binding, mitogenic proteins. The binding site in heparin and heparan sulfate for fibroblast growth factor-2 (basic fibroblast growth factor) has been described as rich in glucosamine-2-sulfate 1→4 linked to iduronic acid-2-sulfate. The glucosamine residue in the heparin binding site is also 6-sulfated. A new glycosaminoglycan, acharan sulfate, has been chemically modified to prepare a polysaccharide, *N*-sulfoacharan sulfate, consisting of glucosamine-2-sulfate 1→4 linked to iduronic acid-2-sulfate. Acharan sulfate binds very weakly to fibroblast growth factor-2 while *N*-sulfoacharan sulfate binds with nearly the same affinity as heparin. Mitogenicity studies were performed using heparan sulfate-free cells stably transfected with fibroblast growth factor receptor-1. Acharan sulfate inhibits heparin's enhancement of fibroblast growth factor-2 mitogenic activity, without affecting cell viability, while *N*-sulfoacharan sulfate shows heparin-like activity but at a greatly reduced level. These results suggest additional mechanisms not requiring high affinity glycosaminoglycan binding to fibroblast growth factor-2 may be important in its mitogenic activity. © 1997 Academic Press

Fibroblast growth factor-2 (FGF-2 or bFGF) is an important mitogen controlling the growth and replication of a variety of cell types (1,2,3). The structure of the minimum sequence within heparan sulfate for mediating the mitogenic activity of FGF-2 (4,5,6) and the minimum sequence within heparin required for binding FGF-2 have been proposed (7). Both sequences contain a repeating unit structure containing →4)GlcNp2S(6S or OH) (1→4)IdoAp2S(1→ (where GlcNp, IdoAp and S are glucosamine, iduronic acid and sulfate, respectively). The structure of FGF-2-heparin oligosaccharide complex has been studied by x-ray crystallography (8) and high field NMR spectroscopy (9). These studies confirm the importance of the →4)GlcNpS(1→4) IdoAp2S(1→ sequence and suggest that a tetrasaccharide or hexasaccharide represents the minimum size requirement for binding a single FGF-2 molecule. The binding of two FGF-2 molecules, requiring a larger oligosaccharide (10), may facilitate the FGF-receptor dimerization required for FGF-2's mitogenic activity (1,2,3).

Recently, acharan sulfate, a novel glycosaminoglycan of the structure, composed primarily of the repeating unit, →4)GlcNpAc(1→4) IdoAp2S(1→ where Ac is acetate), was isolated from the giant African snail *Achatina fulica* (11). Chemical modification of this glycosaminoglycan converted it into a polysaccharide (*N*-sulfoacharan sulfate) with the predominant structure →4)GlcNpS(1→4)IdoAp2S(1→, corresponding to repeating units of the putative binding site for FGF-2. We now report the ability of these glycosaminoglycans to bind and influence the mitogenic activity of FGF-2.

MATERIALS AND METHODS

Glycosaminoglycans and their chemically modified derivatives. Heparin, low molecular weight heparin and heparan sulfate, sodium salts, were from porcine intestinal mucosa and were obtained from Celsus Laboratories (Cincinnati, OH). The syndecan-I ectodomain was from NMMG cells and was quantified based on its heparin sulfate content (12). Acharan sulfate, sodium salt (12) and homogenous fully sulfated heparin oligosaccharides (13) were prepared and purified as described previously. *N*-deacetylation and *N*-sulfation of acharan sulfate followed literature methods (14). The structure of acharan sulfate and *N*-sulfoacharan sulfate relied on enzymatic disaccharide analysis, NMR spectroscopy and gradient PAGE for molecular weight determination (13).

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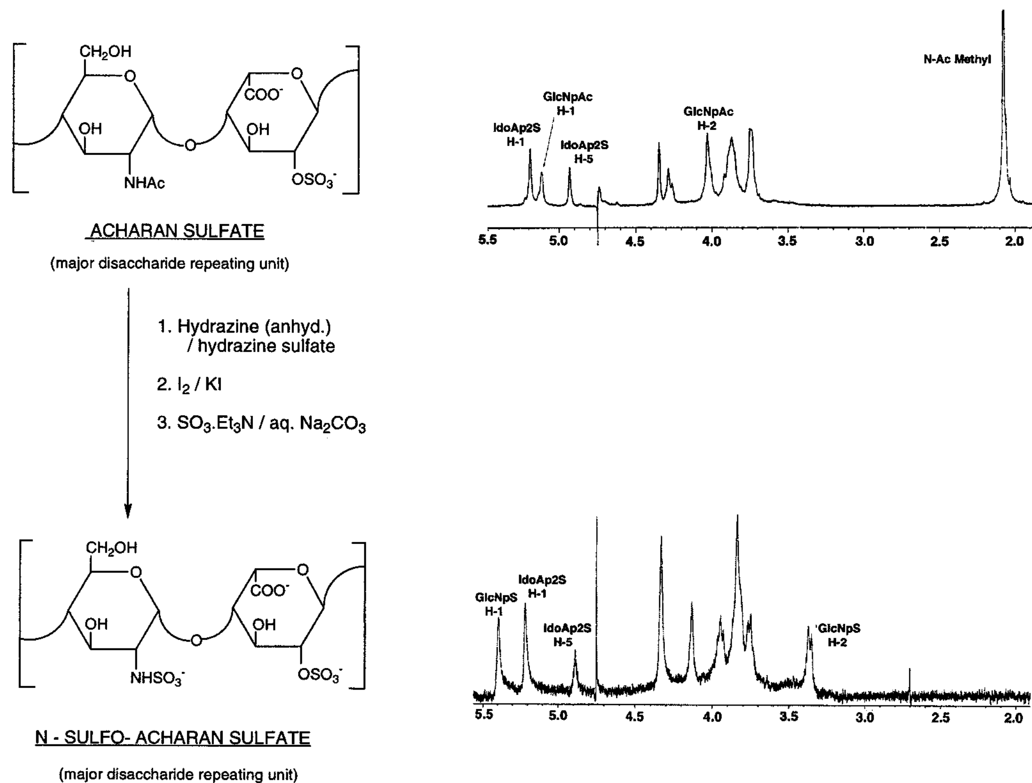

FIG. 1. Acharan sulfate was chemically converted to *N*-sulfoacharan sulfate by hydrazinolysis followed by *N*-sulfation. The 500 MHz ^{1}H-NMR of both are shown.

Interaction of glycosaminoglycans and their derivatives with FGF-2. Isothermal titration calorimetry was performed in 50 mM sodium phosphate buffer, pH 7.4, containing 100 mM sodium chloride at 25 °C as previously described (15).

Assay of FGF-2 mitogenic activity. FGF-2 mitogenicity assays were performed in F32 cells that express FGF-receptor 1 (FGF-R1) (16) but no detectable levels of heparan sulfate proteoglycans (17). Acharan sulfate and *N*-sulfoacharan sulfate were tested on F32 cells stimulated by 150 pM FGF-2 in the presence of 10 ng/ml of heparin (inhibition of heparin-mediated activity) or in the absence of heparin (stimulation of FGF-2 activity). Tested glycosaminoglycans were added in concentrations ranging from 1-5000 ng/ml. Proliferation was measured after 40 h incubation in 200 μl medium (RPMI 1640, 10% heat-inactivated newborn calf serum) in the presence of a 1 μCi/well pulse of [^{3}H]-thymidine during the final 6 h of the incubation. Radioactivity incorporated into DNA was quantified by scintillation counting. MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (Thiazole blue), used to determine cell viability, was from Sigma Chemical (St. Louis, MO).

RESULTS

Preparation and structural characterization of glycosaminoglycan derivatives. Acharan sulfate was *N*-deacetylated and *N*-sulfated to obtain *N*-sulfoacharan sulfate (Fig. 1). The structure of acharan sulfate and *N*-sulfoacharan sulfate were assigned based on one and two dimensional ^{1}H NMR spectroscopy (Table 1). The average molecular weight of acharan sulfate was 29,000 while the molecular weight of the *N*-sulfated acharan sulfate derivative was reduced to ~8,000 as determined by gradient PAGE and confirmed by the sharpening of its NMR signals (see Fig. 1). Treatment of acharan sulfate and *N*-sulfoacharan sulfate with heparin lyases I, II and III afforded the major (>90%) disaccharide products, ΔUAp2S(1→4)GlcNpAc (where ΔUAp is 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid) and ΔUAp2S(1→4)GlcNpS, respectively, as determined by capillary electrophoresis (11).

Determination of the FGF-2 binding affinity of glycosaminoglycans and their derivatives. Isothermal titration calorimetry was performed with heparin oligosaccharides, heparin, acharan sulfate and *N*-sulfoacharan sulfate. The heat of interaction (ΔH) is directly measured in this experiment. From this heat of interaction the Kd, and n (stoichiometry) of interaction can be deduced. The minimum FGF-2 binding site is a tetra- or hexasaccharide. An n of 1.8 was observed for a dodecasaccharide suggesting a tetradecasaccharide is required to bind two molecules of FGF-2 (Fig. 2). Heparin and *N*-sulfoacharan sulfate bind FGF-2 most tightly, heparin oligosaccharides bind less well and acharan

TABLE 1

Assignment of ^{1}H-NMR Signals for Glycosaminoglycan Samples[a]

	Acharan sulfate[b]		*N*-sulfoacharan sulfate	
Proton	L-IdoAp2S	D-GlcNpAc	L-IdoAp2S	D-GlcNpS
H-1	5.189	5.114	5.216	5.392
H-2	4.345	4.020	4.331	3.374
H-3	4.284	3.74	4.331	3.749
H-4	4.027	3.47	4.130	3.838
H-5	4.930	3.867	4.889	3.946
H-6	—	3.87, 3.90	—	3.84, 3.84
N-Ac Methyl	—	2.083	—	—

[a] ^{1}H-NMR was performed at 500 MHz in ^2H_2O. Chemical shifts were determined relative to the internal standard, 3-(trimethylsilyl) propionic acid-1, sodim salt.
[b] S is used to designate sulfate and Ac to designate acetate.

sulfate binds very poorly. The n value is consistent with the molecular weight of each ligand (Table 2).

Influence of glycosaminoglycans and their derivatives on FGF-2 mitogenic activity. The direct influence of acharan sulfate and its derivative on FGF-2 mitogenic activity was measured in the absence of added heparin. *N*-sulfoacharan sulfate, while binding FGF-2 with similar affinity as heparin, was about 150-fold less active than heparin on a weight basis, having about the same activity as the syndecan-1 ectodomain (Fig. 3A). In the presence of 10 ng/ml heparin (where [^{3}H]-thymidine incorporation would increase with increased heparin concentration), up to 1.5 μg/ml of *N*-sulfoacharan failed to further stimulate FGF-2 mitogenicity (Fig. 3B). In contrast, acharan sulfate markedly decreased the mitogenic activity in a concentration dependent manner, beginning at concentrations greater than that of the heparin. The effect of acharan sulfate on cell viability was assessed by measuring the formation of a chromogen from MTT, a tetrazolium dye that is converted solely by living cells (18). The assay showed no reduction in the number of living cells during 40 h incubation with the same acharan sulfate concentrations as used in the mitogenicity assays. Thus, the inhibition of mitogenicity is not due to toxicity leading to reduced cell number.

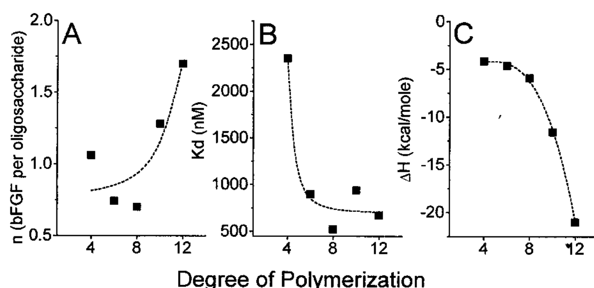

FIG. 2. Calorimetric determination of heparin oligosaccharide size requirements for bFGF binding and dimerization. Shown are (A) binding stoichiometry (n), (B) K_d, (C) ΔH of interaction, as function of oligosaccharide size (*i.e.*, 4 = tetrasaccharide, 6 = hexasaccharide, etc.) These oligosaccharides were of >95% purity and have the structure ΔUAp2S(1→[4)GlcNp2S6S(1→4)IdoAp2S(→]m=1-5).

DISCUSSION

The mitogenic activity of the FGF family of growth factors has been under extensive investigation (1,2,3). Heparin is known to enhance the activity of FGF-2 through its binding to this growth factor. The heparan sulfate chains of syndecan or glypican proteoglycans, while believed to be the endogenous molecules responsible for this activity, have considerably less mitogenic activity than heparin (6,19). Oligosaccharides prepared from heparin and heparan sulfate glycosaminoglycans bind FGF-2. The minimum FGF-2 binding site in heparin is a tetrasaccharide or hexasaccharide but neither enhances its mitogenic activity. It has been suggested that a larger heparin oligosaccharide that can promote dimerization of FGF-2 is required for mitogenic activity (20). The current study clearly demonstrates that a tetradecasaccharide is required for binding two FGF-2 molecules (Fig. 2). A tetradecasaccharide fraction has been prepared from heparan sulfate both binds FGF-2 (6).

The sequences of the oligosaccharides derived from heparin and from heparan sulfate that bind FGF-2 share a common feature. The FGF-2 binding heparin oligosaccharides contain the repeating sequence →4)GlcNp2S6S(1→4)IdoAp2S(1→ (7,8) while the heparan sulfate oligosaccharides contain the repeating sequence →4)GlcNp2S(1→4)IdoAp2S(1→ (4,5,6). The binding contribution of the 6-sulfate groups, commonly found in the glucosamine residues of heparin, and in the FGF-2 binding heparin oligosaccharides is unclear (21).

TABLE 2

Binding of Glycosaminoglycans and Their Derivatives to FGF-2

Sample	ΔH (kcal/mole)	K_d (μM)	MW[a]	n[b]	Avg binding site size[c]
Low molecular weight heparin	−13.4	0.036	4,800	3.6	tetrasaccharide
N-Sulfoacharan sulfate	−15.4	0.090	7,800	4.4	hexasaccharide
Acharan sulfate	−15.1	4.5	29,000	13.5	octasaccharide

[a] MWavg as determined by gradient polyacrylamide gel electrophoresis (11) based on a repeating disaccharide unit (sodium salt) of mass 665, 563 and 504 for heparin, *N*-sulfoacharan sulfate and acharan sulfate, respectively.
[b] n is the average number of FGF-2 molecules occupying a single glycosaminoglycan chain.
[c] The size of oligosaccharide occupied by a single FGF-2 molecule is 2 (MW/disaccharide unit mass)/n.

Recently, acharan sulfate, a novel glycosaminoglycan of the structure →4)GlcNpAc(1→4)IdoAp2S(1→, was isolated. Unlike the more structurally complex heparin or heparan sulfate, acharan sulfate contains a major (>90%) repeating disaccharide unit, making it a relatively simple structure (11). Acharan sulfate's simple but unusual structure was chemically converted to a new derivative, *N*-sulfoacharan sulfate, containing the repeating saccharide present in heparan sulfate that binds FGF-2. The structure of *N*-sulfoacharan sulfate, →4)GlcNp2S(1→4) IdoAp2S(1→, was established using NMR spectroscopy (Fig. 1). Although its average molecular weight was somewhat reduced, it gave a single disaccharide of the structure ΔUA2S(1→4)GlcNS on treatment with heparin lyase I, consistent with its structure.

Isothermal titration calorimetry has been used to measure the binding of heparin and heparin oligosaccharides to FGF-2 (8,10,15). Similar analysis showed that while *N*-sulfoacharan sulfate bound (K_d of 0.09 μM) with nearly the same affinity as heparin (K_d of 0.036 μM), acharan sulfate bound with much lower affinity (K_d > 4 μM) (Table 2). In addition, *N*-sulfoacharan sulfate tightly bound multiple FGF-2 molecules suggesting that it is capable of dimerizing FGF-2. These data confirm that the presence of 6-sulfate groups have little effect on the binding avidity of glycosaminoglycans to FGF-2 (6,21).

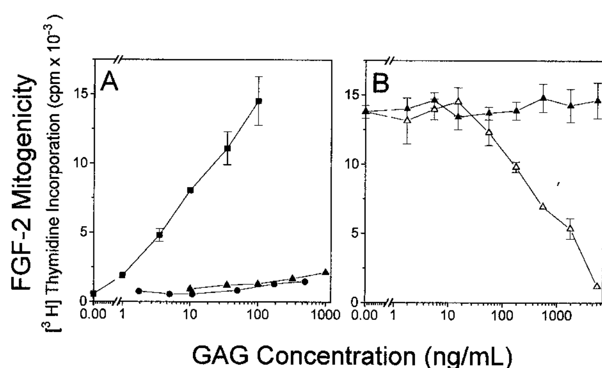

FIG. 3. Mitogenic activity of FGF-2 in the absence (A) and presence (B) of added heparin. Heparin (B), *N*-sulfoacharan sulfate (H), syndecan-1 (J) and acharan sulfate (C)were used at increasing concentrations and the incorporation of [^{3}H]-thymidine into DNA by F32 cells was measured. The heparin (alone) and FGF-2 (alone) controls gave ~750 cpm.

Acharan sulfate and *N*-sulfoacharan sulfate have distinctly different effects on the mitogencity of FGF-2 for F32 cells, a B-cell-derived cell line stably transfected with FGFR-1. Despite its high binding affinity for FGF-2, *N*-sulfoacharan sulfate had minimal mitogenic activity compared with that of heparin. This is in apparent contrast to both the high binding affinity and high mitogenic activty previously reported for the heparan sulfate-derived tetradecasaccharide fraction having a similar repeating structure (6). However the presence of minor levels of 6-sulfate groups in this fraction may account for the observed difference (6,21). The 6-sulfate groups in the tetradecasaccharide fraction (or in heparin) may be important for enhancing its mitogenic activity presumably through their interaction with FGFR-1 (22). *N*-sulfoacharan sulfate had no discernible effect on FGF-2 mitogenicity induced by heparin. In contrast, acharan sulfate inhibited FGF-2 mitogenicity in the presence of heparin. This inhibition was seen at low GAG concentrations (IC_{50} of ~400 ng/ml in the presence of 10 ng/ml heparin). The inhibition was not due to either direct binding of FGF-2, because the growth factor-acharan sulfate interaction is very weak, or to toxicity that alters cell viability. Because acharan sulfate is a large anionic polysaccharide, it is not likely to exert its inhibitory effect by entering cells. However, acharan sulfate might bind to FGF-2 dimers, thought to be the growth factor's mitogenically active form (1-3,8,9,20). The dimer shows a second glycosaminoglycan binding surface when stabilized by heparin (8,9,22). Interaction of acharan sulfate with this surface could account both for its low affinity for FGF-2 monomers (Fig. 2) and its inhibition of heparin-mediated FGF-2 mitogenicity (Fig. 3B).

Several inhibitors of FGF-2 mitogenic activity have

been described, including the synthetic polymers sulfated β-cyclodextrins, sulfated malto-oligosaccharides and phophorothioate oligodeoxynucleotides as well as the drug suramin (22,23). While these inhibitors have been proposed to be anti-angiogenic agents, potentially useful in cancer chemotherapy and in preventing restenosis following vascular injury, their use as FGF-2 antagonists has been limited because of their anticoagulant potency or *in vivo* toxicity. Acharan sulfate differs from these agents in being a natural product with minimal anticoagulant activity. Further studies will assess its utility as a pharmacologic agent.

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