Vol. 151, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1988 Pages 327-332

ISOLATION OF HEPARAN SULFATES WITH ANTITHROMBIN III AFFINITY AND ANTICOAGULANT POTENCY FROM BALB/C 3T3, B16.F10 MELANOMA, AND CUTANEOUS FIBROSARCOMA CELL LINES

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Received January 14, 1988

Summary: The heparan sulfates synthesized in vitro by three cell lines were isolated by proteolysis and preparative anion exchange chromatography and purified free of other glycosaminoglycans by selective enzymatic degradation. The isolates from the medium of BALB/c 3T3 fibroblasts, B16.F10 melanoma cells, and a cutaneous fibrosarcoma line, along with that from the detergent-extracted cell layer of the fibroblasts, were affinity-fractionated on columns of matrix-immobilized human antithrombin III. Each heparan sulfate contained subfractions with high affinity for the proteinase inhibitor, ranging from 3-34% of the starting material. The high affinity species possessed measurable anticoagulant activities by a clotting assay (6 to 30 units/mg). Since none of the lines were derived from cell types having any known biological role in vascular homeostasis, we suggest that anticoagulant activity of the glycosaminoglycan is a random property of its primary structure.

Heparan sulfate, a complex glycosaminoglycan (GAG), occurs in a wide variety of biological systems, but in spite of extensive investigative effort, relatively little is known about its precise functions. Recently, attention has been focused on the possibility that heparan sulfate on endothelial cell surfaces may contribute significantly to the antithrombogenic properties of vascular surfaces. This is an attractive proposal since heparin-like regions are present in heparan sulfates (1-4). A major component of the evidence in support of such a function has been the isolation of heparan sulfate with heparin-like activity from vascular tissue (5,6) and from cultured endothelial cells (7). Here, we have indirectly tested this hypothesis by searching for high affinity, and anticoagulantly-active, heparan sulfates within the GAGs synthesized in vitro by several mammalian cell lines derived from cell types that are irrelevant to vascular function.

MATERIAL AND METHODS

Materials: Chromatographic media were obtained from Pharmacia Inc. (Piscataway, NJ) and Bio-Rad Laboratories (Richmond, CA). AffiGel 15 was purchased from Bio-Rad. Human antithrombin III was isolated from blood bank platetet poor plasma according to Thaler & Schmer (8). The final product was homogeneous by SDS-PAGE. Tissue culture procedures were as described (9). GAG markers were heparan sulfate prepared from an Upjohn beef lung side fraction

0006-291X/88 $1.50

(2,10) and chondroitin 6-sulfate from Sigma Chemical Co. $Na_2[^{35}S]O_4$ (1 Ci/mMol) was a product of New England Nuclear Corporation (Boston, MA) and chondroitinase ABC of Miles Scientific (Naperville, IL).

Cell lines and tissue culture procedures: BALB/c 3T3 clone A31 (American Type Culture Collection, Rockville, MD), the murine (C57BL/6) melanoma line B16.F10 (Frederick Cancer Research Center, Frederick, MD), and a tumorigenic, ultraviolet light-induced, murine (C3H/HeN) cutaneous fibrosarcoma line, designated UV324 (9) were used in these studies.

Extraction of cell- and medium-derived glycosaminoglycans: The lines were expanded in 175 cm^2 flasks, and over 200 flasks per line were harvested for the present studies (approximately 1.3 x 10^8, 3.4 x 10^8, and 1.1 x 10^9 cells from the 3T3, B16.F10, and UV324 lines, respectively). The monolayers were extracted with 0.2% Triton X-100 in 25 mMol/L Tris HCl, pH 7.5, and proteinase inhibitors; most of the GAG so extracted we have found to be present in a cell surface location, based upon its susceptibility to heparinase digestion under nonlytic conditions prior to the detergent extraction (unpublished). The extracts were dialyzed against 10 volumes of water and lyophilized. The separately pooled medium preparations were precipitated by 10% cetylpyridinium chloride, and the precipitates were washed with NaCl-saturated 95% ethanol. Separately, two 175 cm^2 flasks of each line were labeled for 48 hr with 20 microCuries/ml [^{35}S]-sulfate, processed as above, and added to the bulk material as carrier in order to facilitate the analyses.

Analytical methodology: General methodology was as previously referenced (9). Preparations from the 3T3 and B16.F10 cells were treated with papain, diluted four times with water, added to BioRad Ag1x2 (Cl^-) columns (4x2.1 cm) and eluted sequentially with 25 ml of 0.5, 1.0, 1.25, 1.5, and 3.0 Mol/L NaCl, according to Linker and Hovingh (10). The cetyl pyridinium chloride-precipitate from the medium of the UV324 cultures was treated with 1% sodium dodecyl sulfate, and the solubilized material was eluted from Sepharose CL-4B (90 x 1 cm); residual undissolved material was treated with papain, applied to the Sepharose CL-4B column, and eluted with 1% sodium dodecyl sulfate in water. The anion exchange step was omitted for the UV324 medium preparations.

Each anion exchange salt fraction was dialyzed, lyophilized, and analyzed for uronic acid content by the meta hydroxy-diphenyl assay. The preparations were then treated with chondroitinase ABC, precipitated with cetylpyridinium chloride, washed with NaCl-saturated 95% ethanol, dissolved in water and lyophilized. The samples were analyzed for radioactivity and uronic acid content. Heparan sulfate was conclusively identified by at least three of four criteria: 1) resistance to chondroitinase ABC; 2) elution at 1.0-1.25 Mol/L NaCl from Ag1x2 anion exchange columns, coordinately with the authentic heparan sulfate standard but clearly distinct from elution of heparin standards at 1.5-3.0 Mol/L NaCl; 3) characteristic electrophoretic migration on cellulose acetate in 0.1 M calcium acetate; and 4) susceptibility to nitrous acid degradation at low pH.

Antithrombin III affinity chromatography and anticoagulation assay: Antithrombin III, complexed with pig mucosal heparin to block the heparin binding site(s), was coupled to AffiGel 15, using the general instructions of the manufacturer. Before use, the heparin bound to the matrix was eluted with extensive high-salt buffer washes. The fractionations were essentially as described (11).

Each high and low affinity heparan sulfate was assayed for its anticoagulant potency by the activated partial thromboplastin time with commercial reagents (American Dade, Miami, FL). The reference standards were prepared from pig mucosal heparin (Sigma) assumed to have the anticoagulant activity specified by the manufacturer (158.9 units/mg by the U.S.P. standard).

RESULTS AND DISCUSSION

GAG yields from the cell layers and respective medium preparations are listed in Table I along with [^{35}S]-sulfate incorporation and calculated specific activities using the uronic acid determinations. As revealed by the effect of chondroitinase ABC, the majority of the sulfated GAGs within each

Table I

Glycosaminoglycan Yields from Cell Lines[1]

Cell Line/Preparation	Dowex fraction (NaCl molarity)	HS yield(ug) before/after ABCase	[^{35}S]-dpm/ug HS
3T3/medium	1.0	2180/423	208
	1.25	2480/130	338
	1.5	3480/103	686
	3.0	1560/125	625
3T3/0.2% Triton	1.0	560/43	374
	1.25	870/250	1266
	1.5	560/58	1507
	3.0	ND/15	1020
B16.F10/medium	1.0	2063/1023	72
	1.25	580/85	299
	1.5	328/20	245
	3.0	75/0	-
B16.F10/0.2% Triton	1.0	ND/28	339
	1.25	ND/55	289
	1.5	ND/33	294
	3.0	ND/28	193
UV324/Medium SDS extract	NA	1298/60	4
papain digest	NA	2850/835	3

[1]abbreviations: HS, heparan sulfate; ND, not done; ABCase, chondroitinase ABC; SDS, sodium dodecyl sulfate; NA, not applicable.

Dowex fraction is represented by the chondroitin sulfates. The elution of the heparan sulfates over a broad range in salt concentrations from the Dowex columns reflects its basic structural heterogeneity.

Heparan sulfates in the 1.25 and 1.5 M NaCl Dowex fractions from the 3T3 medium, the 1.0 M fraction from the B16.F10 medium, the 1.0 and 1.5 Mol/L NaCl fractions from the 3T3 cell layer extracted with 0.2% Triton, and the UV324 medium preparation (SDS extract) were analyzed for their antithrombin III affinities and anticoagulant potencies (Table II). The fractionations were controlled by the chromatography of 1.0 mg beef lung heparan sulfate standard on columns of similar size as that used for the cell-derived meterial. This was done twice, each time with one of two separately-prepared batches of the matrix, and showed 6 versus 16% of the recovered material to be present in the high salt fraction; respective anticoagulant activities of the high and low affinity isolates were 29.7/36.2 and 1.6/3.3 units/mg. Such variability reflects in part the different efficiencies of separate batches of affinity matrix; furthermore, we have found that varying the starting material weight/matrix volume ratio has an effect on the apparent proportional content of high affinity material (11). In view of these variables, we found that values for fractional content of high affinity material within the cell layer and medium samples ranged from 3-34%. For the high affinity fractions, the anticoagulant activities were from ~6-~30 units/mg. Little correlation existed between the high affinity content of a heparan sulfate and the anticoagulant

Table II

Antithrombin III Affinity and Anticoagulant Potencies of Heparan Sulfates[1]

Preparation	Dowex fraction	Wt(ug)	ATIII fraction	Recovery (ug)	% in each fraction	Anticoagulant activity(U/mg)
3T3/medium	1.25 +1.5	233				
			LA	140	68%	1.3
			HA	66	32%	13.2
3T3/0.2% Triton[2]	1.0	43				
			LA	31	88%	NA
			HA	4	12%	NA
	1.5	58				
			LA	33	66%	NA
			HA	17	34%	NA
B16.F10/ Medium	1.0	1023				
			LA	708	97%	0.4
			HA	23	3%	6.2
UV324/medium SDS extract	-	60				
			LA	35	67%	3.3
			HA	17	33%	28.2
Ox lung standard[3]	1.0	-				
			LA	-	94/84%	1.6/3.3
			HA	-	6/16%	36.2/29.7

[1]Abbreviations: LA, low affinity; HA, high affinity; NA, not available. [2]Anticoagulant activities not available due to heparin contamination; percentage values in the fractions are based upon the measured specific activities of the [^{35}S]-sulfate labeled products. [3]Results from two separate fractionations using separate batches of affinity matrix.

potency of the fractionated material. The activities of the low affinity preparations varied somewhat as well, from <1 to ~3 units/mg.

The foregoing data represent analyses for which authenticity of the cellular source of the material was established. A substantial problem, however, was heparin contamination of the initial high affinity fractions. Its source was determined to be the affinity matrix, sporadically occurring despite the repeated high salt washings after preparation. The heparin was identified by its characteristic cellulose acetate electrophoretic mobility and susceptibility to nitrous acid degradation. A cellular source was ruled out since its presence was sporadic, it was never radioactive, it was never found in any preparation prior to affinity fractionation, and furthermore heparin would not likely elute in 1.0-1.25 M NaCl from the Dowex columns. Within the affinity matrix the heparin contaminant has two potential origins, namely from its use as an immobilized affinity matrix upon which the antithrombin III was originally isolated, and from its use to block heparin binding sites during complexing of the protein to Affigel (matrix prepared without this step we have found to be less efficient in resolving high affinity species). Since slow leaching of small quantities of residual material from the matrix was apparently occurring, we obviated the problem by a high salt wash immediately prior to chromatography of the cellular isolates. In view of this inherent

problem, we must be critical of the high affinity "heparin-like" heparan sulfate of vascular origin shown in Figure 2A of Marcum & Rosenberg (6) to comigrate with a heparin standard. This material may actually be heparin.

GAG remaining after the affinity fractionations was further analyzed to confirm its chemical composition. Material recovered from the cellulose acetate in the heparan sulfate bands and that left from the anticoagulant assays were subjected to deamination at low pH. Their degradation by this treatment supported identification of the GAGs as authentic heparan sulfate in each instance.

It has been proposed that endothelial cell heparan sulfate proteoglycan may contribute to the antithrombogenic properties of vascular surfaces (5). The mechanism for this would involve the interaction of heparin-like regions of heparan sulfate chains with circulating antithrombin III, thereby catalyzing reactions between the serine proteinase inhibitor and activated clotting factors. Among the initial supportive evidence was the isolation from vascular tissue of heparan sulfate which contained molecules with high affinity for antithrombin III and which possessed estimated anticoagulant activity (6); precise values for the activity on a per mg basis remain to be reported (7), probably because of insufficient quantitative yields from the cultures. It should also be noted that in the foregoing studies the proportion of starting material eluting in high affinity fractions is quite low. Different experimental approaches have included analyzing the effects of vascular segments or of endothelial cell monolayers on thrombin neutralization, in the presence and absence of antithrombin III; such functional studies have yielded conflicting conclusions (12-17). Recently, detailed structural studies of heparan sulfate proteoglycan derived from endothelial cells have shown the presence of heparin-like sequences within the polymer (3).

Hovingh & Linker (1) analyzed the products resulting from Flavobacterial heparinase and heparitinase digestion of beef lung heparan sulfate and described the presence of heparin-like trisulfated disaccharide repeating-units, clustered near the nonreducing terminus of the polymer (2). Analysis of heparan sulfates derived from vertebrate lung and aortic tissues demonstrated that up to 5% of the starting materials possessed high affinity for antithrombin III and that the high affinity fractions varied from ~2-30 units/mg in anticoagulant potency; however, the data suggested a random variation between activity and source of the GAG (11). The present work increased the scale of isolation from several cell lines in vitro, utilizing lines established from cell types having no known relevance to vascular homeostasis. It was reasoned that if substantial content of high affinity material showing anticoagulant potency was found, then it could be concluded that these properties are coincidental to the primary structure and have no implications for vascular function. Our work, which has underscored the heterogeneous nature of cellular heparan sulfates, enabled direct calculations of anticoagulant potencies based upon quantitative recoveries of GAG, thereby

showing heparan sulfate subfractions with high affinity for antithrombin III and with anticoagulant activities to be present in all samples examined. Accordingly, the data support the argument that possession of such activity by heparan sulfate derived from endothelial cells does not necessarily reflect participation of this substance in the antithrombogenicity of vascular surfaces.

ACKNOWLEDGEMENTS

This work was supported in part by PHS grant number CA 41591, from the National Cancer Institute, DHHS. The authors acknowledge helpful discussions with Dr. Alfred Linker.

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