Title
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Permalink
https://escholarship.org/uc/item/3cp321s9

Journal
The Journal of biological chemistry, 269(18)

ISSN
0021-9258

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Publication Date
1994-05-01

DOI
10.1016/s0021-9258(17)36804-7

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Peer reviewed
Fine Structure of Heparan Sulfate Regulates Syndecan-1 Function and Cell Behavior*

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Two myeloma cell lines, MPC-11 and P3X63Ag8.653 (P3), have almost identical amounts of syndecan-1 at their cell surface. The syndecan-1 molecules from both lines are similar in size, have indistinguishable core proteins, and have similarly sized heparan sulfate chains. Nevertheless, syndecan-1 on MPC-11 mediates cell adhesion to type I collagen, whereas P3 cells do not bind collagen. Affinity co-electrophoresis reveals that intact syndecan-1 isolated from P3 cells binds collagen poorly and that syndecan-1 heparan sulfate isolated from MPC-11 has a 20-fold higher affinity for collagen than syndecan-1 heparan sulfate from P3. Analysis of disaccharide composition and oligosaccharide mapping also reveals differences between MPC-11 and P3 heparan sulfate. Most notably, the level of N-sulfation and 2-O-sulfation is higher, and 6-O-sulfation lower, in syndecan-1 heparan sulfate from MPC-11 than from P3. Interestingly, levels of total sulfation of syndecan-1 heparan sulfate from MPC-11 and P3 are similar (75.6 and 72.6 sulfates/100 disaccharides, respectively), indicating that the difference in their affinity for collagen is not due to a difference in net charge. These data indicate that the fine structure of heparan sulfate can differ on identical proteoglycan core proteins, and these differences can control fundamental cellular properties such as cell-matrix adhesion.

Heparan sulfate proteoglycans are present on most cell surfaces where they participate in the regulation of cell behavior by binding structural proteins, enzymes, and growth factors (1, 2). Considerable diversity exists in the numbers and lengths of heparan sulfate chains that are found on cell surface proteoglycans, and considerable heterogeneity exists in the composition and fine structure of these chains.

The extent to which the molecular heterogeneity of heparan sulfate proteoglycans contributes to specificity in their biological activities is poorly understood. At least one heparan sulfate-binding protein, antithrombin III, binds specifically to a pentasaccharide sequence that is only found in some heparan sulfate chains (3, 4). Several other proteins bind with moderate preferences to subsets of the heparan sulfate-related glycosaminoglycan heparin, although the structural basis for this binding is not yet known (5). Studies indicate that bFGF1 is capable of interacting preferentially with certain heparan sulfate and heparin oligosaccharides of defined sequence (6–10). However, information is lacking on whether full-length naturally occurring heparan sulfates differ in their interactions with, and ability to mediate the biological activity of, bFGF, although one recent study suggests that this may be the case (11).

Indeed, the large size and complexity of intact proteoglycans has made it difficult to obtain clear correlations between proteoglycan structure and function. The most progress to date has been made with syndecan-1, a member of the syndecan family of proteoglycans (12). Syndecan-1 exhibits broad structural heterogeneity between cell and tissue types due to differences in the number and length of glycosaminoglycans, types of glycosaminoglycans (heparan sulfate versus chondroitin sulfate), and heparan sulfate fine structure (e.g., sulfate content, disaccharide composition, and distribution of heparinase cleavage sites) (13–16).

Syndecan-1 from various cell types has been shown to bind to interstitial collagens (17), fibronectin (18), thrombospondin (19), tenascin (16), and bFGF (20, 21). In some cases, cell type-specific differences in binding properties were seen. For example, solid phase assays indicate that a 250–300-kDa form of syndecan-1 isolated from tooth mesenchyme and having only heparan sulfate chains binds strongly to tenascin, whereas a 100–250-kDa form of syndecan-1 from normal murine mammary epithelial (NMuMG) cells and having both heparan sulfate and chondroitin sulfate chains binds weakly to tenascin (16).

In contrast, syndecan-1 from NMuMG epithelial cells binds with 4-fold higher affinity to type I collagen than does syndecan-1 from a murine myeloma cell line that expresses a form of syndecan-1 that is 70–150 kDa and has shorter and fewer heparan sulfate chains than does NMuMG syndecan-1 (22).

These data suggest that cell type-specific forms of syndecan-1 can have distinct ligand binding properties. However, two critical questions have yet to be resolved. First, what differences in syndecan-1 structure cause these differences in binding behavior? Conceivably, such differences could depend on

* This work was supported by National Institutes of Health Grants CA58729 (to R. D. S.) and NS20682 (to A. D. L.) and grants from the Cancer Research Campaign (United Kingdom) and by the Christie Hospital Endowment Fund (to J. T. G. and J. E. T.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: bFGF, basic fibroblast growth factor; P3, P3X63Ag8.653; NMuMG, normal murine mammary gland epithelial; RSA, bovine serum albumin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-(3-cholamidopropyl(dimethyl)ammonio)-1-propanesulfonate; MOPS, 4-(2-hydroxypropyl)-1-propanesulfonate; SAX, strong acid exchange; HPLC, high performance liquid chromatography; Gla, glucuronic acid; Idoa, iduronic acid; Idoa(2S), iduronic acid 2-sulfate; GlcNac, N-acetylgalactosamine; GlcNSO3, N-sulfated glucosamine; GlcNSO3(6S), N-sulfated glucosamine 6-sulfate; GlcA(2S), glucuronic acid 2-sulfate; UA, unsaturated uronic acid residue.

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aspects of large scale structure (e.g. number, length, or net charge of glycosaminoglycan chains), fine structure (specific oligosaccharide sequences with defined patterns of sulfation), or both. Second, are the differences that have been observed in syndecan-1 binding behavior sufficiently large to have a determining influence on cell behavior (i.e. does the structure of a cell's syndecan-1 control its behavior)?

To address these questions, we have been studying the role of syndecan-1 as the type I collagen receptor of myeloma cells. Previous work has demonstrated that syndecan-1 mediates the binding of murine and human myeloma cells to type I collagen (22, 23). In the present paper, we identify a murine myeloma cell line, P3, that possesses syndecan-1 that fails to mediate cell binding to collagen. These P3 cells contain the same amount of cell surface syndecan-1 as MPC-11 mouse myeloma cells (which do bind to collagen), but syndecan-1 from P3 cells possesses heparan sulfate chains that bind 20-fold less tightly to type I collagen than the heparan sulfate chains of syndecan-1 from MPC-11 cells. Furthermore, evidence is provided to show that P3 and MPC-11 syndecan-1 are similar in their gross properties, including core protein size and net charge, but substantially different in their fine structure of their heparan sulfate chains. Specifically, the heparan sulfate from MPC-11 syndecan-1 (which binds with high affinity to type I collagen) has higher levels of N-sulfation and 2-O-sulfation, lower levels of 6-O-sulfation, and a different arrangement of heparan sulfate cleavage sites than the heparan sulfate of P3 syndecan-1. These data suggest that a causal relationship may exist between heparan sulfate fine structure, proteoglycan binding properties, and at least some aspects of cell behavior.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—MPC-11 and P3 mouse myeloma cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.05 mM 2-mercaptoethanol, 100 units/ml penicillin, and 100 μg/ml streptomycin sulfate.

**Cell Binding Assays**—MPC-11 and P3 cell binding to type I collagen was assayed as described previously (17, 22). Polyvinyl 96-well U-bottom plates (Dynatech, Chantilly, VA) were incubated with type I collagen (rat tail, 1 mg/ml; Collaborative Biomedical Products, Bedford, MA) or BSA (Fraction V, 1 mg/ml, Sigma) overnight at 4 °C. Wells were washed with PBS, and remaining protein binding sites within the walls of the wells were saturated by incubation with 10 mg/ml BSA for 30 min. Plates were then washed three times with PBS prior to addition of cells. Cell cultures were established at a concentration of 2 x 10^6 cells/ml 24 h prior to performing cell binding assays. Cells were harvested by centrifugation at 4 °C, washed with ice-cold PBS, and resuspended in cold PBS at a concentration of 4 x 10^6 cells/ml. Cells were added to microtiter wells (200 μl/well), incubated for 30 min at room temperature, and the plate was centrifuged at 120 x g for 10 min in a Beckman TJ-6 centrifuge. Following centrifugation, 50 μl of 4% glutaraldehyde in PBS was added to each well and the plate placed at 4 °C overnight. After removal of the buffer, cells were stained with 4% trypan blue in PBS. In this assay, if cells do not bind to the protein-coated wells, a clearly visible pellet is formed in the bottom of the well following centrifugation. If cells bind to wells, they remain as a uniform coating over the well surface.

**Quantification of Syndecan-1**—Syndecan-1 on the surface of cells was quantified using 125I-labeled antibody 281.2, an antibody specific for an epitope within the extracellular domain of syndecan-1 (24). Cells growing at a concentration of 4 x 10^6 cells/ml were harvested by centrifugation and washed twice in ice cold complete cell culture media. Cells were then added to wells of a 96-well plate (6 x 10^5 cells/well) that had been preincubated with PBS containing 10 mg/ml BSA to prevent cells from binding to the wells. Cells in the wells were incubated with normal rat serum to prevent nonspecific binding of labeled antibody, followed by incubation for 30 min on ice with excess 125I-labeled monoclonal antibody 281.2, or with 125I-labeled antibody L3T4 (Pharmining, San Diego, CA), an isotype-matched control monoclonal antibody. Cells were then centrifuged, washed twice in cold complete media, resuspended, and bound antibody detected by γ counting.

**Purification and Analysis of Syndecan-1 and Syndecan-1 Heparan]].
by heating the sample at 100 °C for 2 min. Disaccharides were recovered by chromatography on a Bio-Gel P-2 column (1 x 120 cm) eluted with 500 mM NH₄HCO₃ at a flow rate of 4 ml/h. The disaccharide products (yields > 90%) were lyophilized and separated by SAX-HPLC on a ProPac PA1 analytical column (4 x 250 mm; Dionex, Surrey, United Kingdom). After equilibration in mobile phase (double-distilled water adjusted to pH 3.5 with HCl) at 1 ml/min, samples were injected and disaccharides eluted with a linear gradient of sodium chloride (0-1 M) in-line for 50 min. The eluant was monitored in-line for UV absorbance (A₂₅₄nm for unlabeled disaccharide standards) and ³¹P radioactivity (Radiomatic Flo-on/beta A-200 detector, Canberra Packard, Pangbourne, United Kingdom).

**Oligosaccharide Mapping by Gradient PAGE—Oligosaccharide mapping by gradient PAGE was carried out using methods described in detail previously (28, 30). Briefly, ³²P-labeled heparan sulfate samples depolymerized with heparitinase were electrophoresed (400 V for 4 h) on 25-30% gradient gels (24 cm x 16 cm x 0.75 mm) and electrotransferred to cationic nylon membranes (Biodyne B, Pall BioSupport Division, Portsmouth, United Kingdom). The fractionated oligosaccharides were detected by fluorography using EN3HANCE (DuPont NEN).

**RESULTS AND DISCUSSION**

**Identification of a Myeloma Cell Line That Expresses Syndecan-1 but Does Not Bind Collagen—** Previous studies have provided evidence that syndecan-1 mediates the binding of murine and human myeloma cells to type I collagen. For example, MPC-11 murine myeloma cells, which express syndecan-1 as their only major cell surface heparan sulfate proteoglycan, are inhibited from binding collagen by heparin, by pretreatment of cells with heparitinase or by growth of cells in an inhibitor of glycosaminoglycan sulfation (22). Moreover, isolated syndecan-1 from MPC-11 cells (which is virtually indistinguishable from the form of syndecan-1 found on normal plasma cells) exhibits demonstrable binding to collagen (22). Finally, when cells from several human myelomas were tested for binding to type I collagen, all of those that bound collagen expressed syndecan-1, whereas all of those that did not bind collagen lacked syndecan-1 (23).

In the course of these studies, however, one murine myeloma line, P3, was identified that did not attach to type I collagen (Fig. 1), yet P3 cells did express syndecan-1. This observation suggested that the syndecan-1 on P3 cells was either present in insufficient quantity or structurally different from the syndecan-1 on other myeloma cells. To address this question, experiments were undertaken to compare the abundance, structure, and binding properties of syndecan-1 isolated from P3 cells with syndecan-1 isolated from MPC-11 cells.

**MPC-11 and P3 Cells Express Syndecan-1 in Similar Amounts and of Similar Molecular Size—** To assess the basis for the difference in ability of these two cell lines to adhere to collagen, we first analyzed the amount and size of syndecan-1 on MPC-11 and P3 cell surfaces. Quantification of syndecan-1 using ¹²⁵I-labeled monoclonal antibody 281.2 indicates that nearly identical amounts of the proteoglycan are present on the surfaces of the two cell types (Table I).

To compare the size of syndecan-1 from the two cell types, purified syndecan-1 was analyzed by Sepharose CL-4B chromatography and its core proteins by Western blotting. The intact syndecan-1 from MPC-11 and P3 cells are similar in size (Fig. 2A), with the P3 being only slightly smaller, and the core proteins are identical in size (Fig. 2B). Because the core proteins are the same size, and there is no evidence for alternative splicing of syndecan-1 mRNA (31, 32), it is likely that the core proteins from the two cell lines are identical in structure. Analysis of isolated heparan sulfate chains from the syndecans by Sepharose CL-6B chromatography shows that a large proportion of the MPC-11 and P3 heparan sulfate peaks overlap, with the overall average size of the MPC-11 heparan sulfate chains being slightly larger than the P3 chains (Fig. 2C). These results
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FIG. 3. Analysis of binding of syndecan-1 and syndecan-1 heparan sulfate to type I collagen by affinity co-electrophoresis. A, labeled syndecan-1 was prepared from NMuMG mouse mammary epithelial cells and MPC-11 and P3 cells by trypsinization of cells cultured in [35S]sulfate and purified by DEAE chromatography and immunoaffinity chromatography using antibody 281.2. Collagen concentrations are shown at the bottom. Progressive retardation of syndecan-1 mobility from low to high collagen concentration demonstrates binding (collagen-free zones between each lane permit the mobility at each collagen concentration to be compared with the mobility of free syndecan-1 molecules). The presence of smears in some lanes, rather than tight bands, is indicative of binding heterogeneity within each sample, i.e. some syndecan-1 molecules bind collagen more tightly than others. Arrows illustrate the presence of high-affinity subpopulations in the MPC-11 and NMuMG samples that are not seen in the P3 sample. B, heparan sulfate chains were prepared from the samples used in A and subjected to affinity co-electrophoresis. Collagen binding isotherms derived from the data in B. For each lane of the autoradiograms, an average retardation coefficient ($R$) was determined after numerically determining (by integrating pixel intensities) the median mobility ($\nu$). $R$ values vary from 0 (no retardation) to 1 (complete arrest of mobility) and are plotted as a function of collagen concentration. Curves were fit to the equation $R = R_{\text{max}}/[1 + (K_d/\nu)^2]$, where $R_{\text{max}} = 0.97$ ($22$) and yield apparent values of $K_d$ of $5.8 \times 10^{-7}$ M (NMuMG), $6.4 \times 10^{-7}$ M (MPC-11), and $1.1 \times 10^{-5}$ M (P3).

Determine that the amount of syndecan-1 and the molecular size of syndecan-1 and its heparan sulfate chains are similar on MPC-11 and P3 cells and therefore are unlikely to account for the difference in the ability of these cells to bind to type I collagen.

Heparan Sulfate Chains of Syndecan-1 from P3 Cells Have a Low Affinity for Type I Collagen—To more closely analyze the molecular basis for the difference in cell binding to collagen, we purified syndecan-1 from MPC-11 and P3 cells and, using the technique of affinity co-electrophoresis (27), compared their affinities for type I collagen (Fig. 3). As a control, we also analyzed intact syndecan-1 from normal murine mammary gland epithelial (NMuMG) cells which is known to have a high affinity for type I collagen (17, 22). All of the samples tested show broad heterogeneity in binding affinities; however, syndecan-1 from both MPC-11 and NMuMG cells has a distinct high affinity fraction that is absent from the syndecan-1 from P3 cells (Fig. 3A, arrows). Analysis of heparan sulfate chains isolated from purified syndecan-1 gave similar results although these showed less heterogeneity than did the intact syndecan-1 (Fig. 3B). Calculated values of $K_d$ of the heparan sulfate chains for type I collagen were $5.8 \times 10^{-7}$ M, $6.4 \times 10^{-7}$ M, and $1.1 \times 10^{-5}$ M, for NMuMG, MPC-11, and P3, respectively (Fig. 3C). Thus, the heparan sulfate chains of syndecan-1 from MPC-11 cells have an approximately 20-fold higher affinity for type I collagen than do the heparan sulfate chains of syndecan-1 from P3 cells. This difference provides a likely explanation for the observed inability of P3 cells to attach to collagen (Fig. 1).

It is noteworthy that although the heparan sulfate chains of MPC-11 syndecan-1 are significantly smaller than those of NMuMG syndecan-1 (17 versus 36 kDa, respectively) (22), they have very similar affinities for collagen. This indicates that at least in this instance, heparan sulfate fine structure may be more important than chain length in determining affinity of heparan sulfate for collagen. This notion is further supported by the observation that P3 heparan sulfate, which is only slightly smaller than MPC-11 heparan sulfate (Fig. 2), has a much lower affinity for collagen than does MPC-11 heparan sulfate.

Heparan Sulfate of Syndecan-1 from MPC-11 and P3 Cells Differ in Their Fine Structure—Because the heparan sulfate chains from MPC-11 and P3 cells are similar in size (Fig. 2C),
their difference in affinity for collagen is likely due to differences in their fine structure. Consistent with this, data on disaccharide composition (Fig. 4 and Table II) and oligosaccharide mapping profiles (Figs. 5 and 6) demonstrate that syndecan-1 heparan sulfate from MPC-11 and P3 cells are structurally distinct. In the disaccharide composition it is particularly noticeable that the P3 heparan sulfate has reduced levels of both N-sulfate and 2-O-sulfate groups relative to MPC-11 heparan sulfate (Table II). Indeed, the disaccharide \( \text{UA(2S)}-(\text{GlcNSO})_3(6S) \) is entirely absent from the P3 heparan sulfate. However, the level of 6-O-sulfate is higher in P3 than in MPC-11, and thus the overall level of sulfation of the heparan sulfate species from the two cell lines is very similar (72.6 and 75.6% respectively, Table II). Therefore, the difference in the affinity of P3 and MPC-11 syndecan-1 heparan sulfate for type I collagen are not simply due to differences in net charge. Although the overall level of sulfation of syndecan-1 from normal B cells is unknown, the levels seen here for P3 and MPC-11 cells are broadly within the same range as several normal cell types (33), despite the relatively low N-sulfate content. This indicates that these transformed cells may not differ significantly from their normal counterparts in regard to their average level of sulfation.

The data in Table II suggest that heparan sulfate affinity for collagen correlates strongly with the presence of N- and 2-O-sulfate groups. However, compositional data per se are not necessarily predictive of the nature of the collagen binding sequence.

Gradient PAGE oligosaccharide mapping using the enzyme heparitinase which acts specifically on the hexosamine-glucuronic acid linkage (34) demonstrates qualitatively that the MPC-11 and P3 \( \text{3H}\) labeled heparan sulfates give rise to distinct banding patterns (Fig. 5). Differences in the presence and relative content of specific oligosaccharide bands were evident, clearly indicating that the fine structures of these two heparan sulfate species are markedly different. This was confirmed by quantitative oligosaccharide mapping by SAX-HPLC using heparitinase which also revealed distinct elution profiles (Fig. 6).

The greater susceptibility of the P3-derived heparan sulfate (note the high content of small \( \text{3H}\) fragments eluting between 15 and 20 min) suggests a relative enrichment of N- and O-sulfate groups in the vicinity of the heparitinase cleavage sites. In contrast, the MPC-11 heparan sulfate contains an increased proportion of larger more highly sulfated saccharides composed of heparitinase-resistant GlcNSO\(_2\)-IdoA repeats, with variable O-sulfation (28, 29), which elute between 30 and 80 min. Because the overall level of sulfation of syndecan-1 heparan sulfate is similar between MPC-11 and P3 cells (Table II), the mapping data suggest that in the MPC-11 polysaccharide the sulfated residues are tightly clustered in relatively large blocks or domains, whereas in the P3 counterpart, the blocks of sulfated disaccharides are more commonly interspersed with GlcA-containing disaccharides that can be cleaved by heparitinase.

It seems likely that critical differences in the distribution of sulfated disaccharides such as those described above are responsible for the observed differences in collagen binding properties of syndecan-1 heparan sulfate. It is becoming increasingly clear that heparan sulfate binds biologically relevant protein ligands through specific sugar sequences. For example, high affinity binding of heparan sulfate to bFGF is mediated by a heparitinase-resistant fragment seven disaccharides in length with an internal repeat of five IdoA(2S)-GlcNSO\(_2\) units (6–8), although it is not clear whether all the sulfate groups within sequences of this type are required for the bFGF interaction (9). Sequences of the same basic size and structure have been shown to mediate activation of bFGF in a mitogenic assay.
employing 3T3 cells rendered heparan sulfate-deficient by chlorate treatment (35). By contrast, the anti-thrombin III binding site in heparan sulfate (and heparin) is characterized by 3-O- and 6-O-sulfated glucosamines and a glucuronic acid residue (3, 4). Within the context of the present study, it will be essential to identify the structure of the collagen binding site in the syndecan-1 heparan sulfate as this sequence will have a major role to play in regulating cell adhesion and migration in the interstitial matrix.

Syndecan-1 Heparan Sulfate and Cell Behavior—Overall from these data we conclude that: (i) the syndecan-1 core protein can bear structurally different heparan sulfate chains in different, yet related, cells, and (ii) such structural variation in syndecan-1 heparan sulfate can give rise to substantial differences in the proteoglycan's binding properties. Furthermore, the data strongly suggest that such differences in proteoglycan binding properties can play a determining role in an important cell behavior, cell-matrix adhesion.

These results have several important implications regarding the regulation of proteoglycan function. First, they support the view that, at least for syndecan-1, the fine structure of heparan sulfate is not determined by information encoded in the core protein. Second, they argue that structural variations in heparan sulfates serve to encode functionally important information and do not merely reflect redundant biosynthetic variability. This is in accord with the demonstration of specific binding sequences for protein ligands such as anti-thrombin III (3, 4) and bFGF (6–8). Third, they demonstrate that an extracellular matrix molecule, type I collagen, can recognize information encoded in heparan sulfate, by a means other than simple detection of net polymer charge.

Taken together, these findings indicate that heparan sulfate fine structure is a critical determinant of proteoglycan function. It is clear that more needs to be learned about the range of heparan sulfate structural variation that occurs in vivo, as well as the range of heparan sulfate-binding proteins that are sensitive to such variation. Until then, caution should be exercised in drawing conclusions about the in vivo functions of any heparan sulfate proteoglycan based solely on the distribution of its core protein.

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