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Binding, Internalization, and Degradation of Antiproliferative Heparan Sulfate by Human Embryonic Lung Fibroblasts

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Abstract Binding, internalization, and degradation of ^{125}I-labeled, antiproliferative, or nonantiproliferative heparan sulfate by human embryonic lung fibroblasts was investigated. Both L-iduronate–rich, antiproliferative heparan sulfate species as well as L-iduronate–poor, inactive ones were bound to trypsin-releasable, cell-surface sites. Both heparan sulfate types were bound with approximately the same affinity to one high-affinity site (K_d approximately 10^{-8} M) and to one low-affinity site (K_d approximately 10^{-6} M), respectively. Results of Hill-plot analysis suggested that the two sites are independent. Competition experiments with unlabeled glycosaminoglycans indicated that the binding sites had a selective specificity for sulfated, L-iduronate–rich heparan sulfate. Dermatan sulfate, which is also antiproliferative, was weakly bound to the cells. The antiproliferative effects of heparan and dermatan sulfate appeared to be additive. Hence, the two glycosaminoglycans probably exert their effect through different mechanisms. At concentrations above 5 μg/ml (approximately 10^{-7} M), heparan sulfate was taken up by human embryonic lung fibroblasts, suggesting that the low-affinity site represents an endocytosis receptor. The antiproliferative effect of L-iduronate–rich heparan sulfate species was also exerted at the same concentrations. The antiproliferative species was taken up to a greater degree than the inactive one, suggesting a requirement for internalization. However, competition experiments with dextran sulfate suggested that both the high-affinity and the low-affinity sites are involved in mediating the antiproliferative effect. Structural analysis of the inactive and active heparan sulphate preparations indicated that although sulphated L-iduronate appears essential for antiproliferative activity, it is not absolutely required for binding to the cells. Degradation of internalized heparan sulfate was analyzed by polyacrylamide gel electrophoresis using a sensitive detection technique. The inactive species was partially degraded, whereas the antiproliferative one was only marginally affected. J. Cell. Biochem. 64:595–604.

Key words: glycosaminoglycans; binding; internalization; cell growth; degradation

Control of cellular proliferation involves an interplay between the cell and its environment, the peri- and extracellular matrix. The matrix contains fibrous proteins with structural (e.g., collagen) and adhesive (e.g., fibronectin) functions as well as multifunctional proteoglycans (PGs). The latter contain a variable number of glycosaminoglycans (GAGs) attached covalently to a protein core and are synthesized by most cell types [for extensive reviews of PG biochemistry and cell biology, see Kjellén and Lindahl, 1991; Bernfield et al., 1992]. PGs produced by fibroblasts usually contain two major classes of GAGs: dermatan sulfate (DS)/chondroitin sulfate (CS) and heparan sulfate (HS) [Schmidtchen et al., 1990].

The pericellular PGs seem to be involved in growth regulation both as stimulators and inhibitors. For example, it has been shown that cell-surface attached HS regulates the action of fibroblast growth factor (FGF). Binding of

Abbreviations used: CS, chondroitin sulfate; DexS, dextran sulfate; DS, dermatan sulfate; EGF, epidermal growth factor; GAG, glycosaminoglycan; Gdn-HCl, guanidine-hydrochloride; $GlcNSO_3$, N-sulphated glucosamine; GlcUA, D-glucuronic acid; HA, hyaluronan; HS, heparan sulfate; IdoUA, L-iduronic acid; MEM, minimal essential medium; PBS, phosphate-buffered saline; PG, proteoglycan.

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growth factor to HS is necessary for interaction with the signal-transducing receptor [Yayon et al., 1991; Rapraeger et al., 1991]. Exogenously added GAGs can also suppress cell growth. We have previously shown that human embryonic lung fibroblasts are growth-inhibited by certain L-iduronic acid (IdoUA)–rich GAGs, such as highly sulphated HS, heparin, and DS, whereas IdoUA-poor, low-sulphated HS and CS are much less active or inactive [Westergren-Thorsson et al., 1991]. Antiproliferative GAGs inhibit growth supported by endogenous growth factors, by exogenously added serum mitogens, and by epidermal (EGF), platelet-derived (PDGF), acidic (aFGF), or basic fibroblast growth factor (bFGF) [Westergren-thorsson et al., 1993].

The antiproliferative effect may be initiated by binding to receptors at the cell surface. This may result either in signal transduction via a second messenger or in endocytosis and subsequent action at an intracellular site. Cell-binding studies of exogenously supplied GAGs have been performed, but they have not been systematically correlated with the degree of antiproliferative activity of the bound GAG. Castellot et al. [1985] and Vannucchi et al. [1988] reported efficient binding and uptake of commercial heparin in smooth muscle and endothelial cells, respectively. Redini et al. [1989] found uptake of heparin, HS, and DS in weakly metastatic rhabdomyosarcoma cells. However, DS uptake is generally controlled via recognition of the parent PG core protein [Glössl et al., 1983; Hausser et al., 1992].

An intracellular site of action for endocytosed HS could be the nucleus, because HS has been found in preparations of nuclei from hepatocytes [Fedarko and Conrad, 1986]. Furthermore, heparin can suppress transcription of *c-fos*, *c-jun*, and *c-myc* when induced via a protein kinase C–dependent but not by an EGF-induced pathway [Wright et al., 1989; Herbert and Maffrand, 1991; Busch et al., 1992; Ottlinger et al., 1993]. However, nuclear association of HS could not be demonstrated when an improved method for isolation of nuclei was used in granulosa cells [Hiscock et al., 1994].

Despite much effort, it is thus still unclear how antiproliferative GAGs exert their effect. To be able to isolate relevant cell-surface receptors and to elucidate the fate of exogenously added GAGs, further primary characterization of binding parameters,uptake, and degradation are required. In the present study we have investigated relationships between the antiproliferative capacity of various GAGs and their structural features as well as their cell-surface binding, uptake, and degradation. We have used antiproliferative GAGs of different structure (HS and DS) as well as active and less active GAGs from the same family (HS/heparin).

TABLE I. Chemical Data for Sulphated Glycosaminoglycans*

Sample	$O\text{-}SO_3$ HexN (mole/ mole)	$N\text{-}SO_3$ HexN (mole/ mole)	IdoUA HexUA (mole/ mole)	$IdoUA\text{-}OSO_3$ HexUA (mole/ mole)	M_r (kDa)
HS2	0.30	0.26	0.30	0.10	20
HS6	0.91	0.72	0.65	0.60	20
DS	1.16	n.a.	0.90	0.20	30

*The preparations were obtained as described in Methods. Analyses were performed as described in Fransson et al. [1979, 1980a,b]. HexN, hexosamine; HexUA, hexuronic acid; n.a., not applicable; $N\text{-}SO_3$, N-sulphamate; $O\text{-}SO_3$, ester sulphate. Relative molecular mass (M_r) was determined by gel chromatography on Superose 6 calibrated with HS species of known molecular mass (determined by light scattering).

METHODS

Materials

The HS preparations (HS2 and HS6) were derived from beef lung and were prepared as described previously [Fransson et al., 1980a,b]. In brief, the starting material (heparin by-products) was dissolved in 2.1 M NaCl and sequentially precipitated as cetylpyridinium complexes by stepwise lowering of the NaCl concentration, yielding fractions of falling charge density. Thus, the low-charged preparation HS2 was obtained between 0.4 and 0.6 M NaCl and the high-charged HS6 between 1.2 and 2.1 M NaCl. The DS was obtained and characterized by methods described by Fransson and Malmström [1971] and by Fransson et al. [1979]. Chemical data are summarized in Table I. The hyaluronan (HA) and chondroitin sulphate (CS) were from rooster comb and bovine nasal cartilage, respectively, and were gifts from the Chicago standard collection [Rodén et al., 1972]. GAGs were released from their parent PGs (when applicable) by extensive proteolysis using papain. They were further purified by gel chromatography on Superose 6 under dissociative conditions before use for more details [see Westergren-Thorsson et al., 1991]. To remove possible contamination by HS in DS preparations, they were treated with a mixture of heparin lyase/heparinase I, HS lyase/hepari-

nase III (from Seikagaku Kogyo Co., Tokyo, Japan) and heparinase II (Sigma, St. Louis, MO) as described elsewhere [Schmidtchen and Fransson, 1992] or with nitrous acid [Shively and Contrad, 1976]. Recombinant human EGF was purchased from Genzyme (Cambridge, MA). Crystal violet came from Merck (Darmstadt, Germany) and glutaraldehyde and dextran sulfate (DexS) M_r 5,000) from Sigma. Eagle's minimal essential medium (MEM) and newborn calf serum were obtained from Nord Vacc (Stockholm, Sweden) and culture flasks and microtitre plates from Costar (Stockholm, Sweden).

Cell Culture

Human embryonic lung fibroblasts were established as described [Malmström et al., 1975] and grown in MEM with 10% newborn calf serum and split 1:2 every fourth day. Cells used for experiments were between passage 5 and 22 and screened for mycoplasma using GEN-PROBE Rapid Detection System (Skafte & Claesson AB, Mölndal, Sweden). Cells were counted with a Bürker chamber.

Proliferation Assay

The cells were seeded in 96-well microplates at a density of 3,000 cells/well and allowed to plate for 5 h in MEM with 10% calf serum. The medium was changed to MEM without serum, and the cells were serum-starved for 24 h. Cells were then grown in a defined medium consisting of Ham's F-12 fortified with insulin (10 µg/ml) and transferring (25 µg/ml) and with EGF (20 ng/ml) as mitogen. The cultures were incubated for 96 h at 37°C with or without GAGs solubilized in the medium, and the cell number was assayed by the crystal violet method [Gilles et al., 1986; Westergren-Thorsson et al., 1991].

Radioactive Labeling of Glycosaminoglycans

The amino group of the peptide remnant at the reducing end of the glycan chains was coupled to p-hydroxyphenyl propionate as described [Fransson et al., 1990]. The p-hydroxyphenyl group was then radioiodinated using $Na^{125}I$ and chloramine T [Fransson et al., 1990]. Dextran sulphate (DexS) was radioiodinated after derivatization with p-aminobenzoic acid [Cheng et al., 1992, 1994]. Samples of HS were also radiolabeled in the same manner after cleavage of the xylose-to-serine bond by alkali [Cheng et al., 1992, 1994]. The radioactivity of the samples was measured in a 1272 Clini-Gamma counter. The specific radioactivities obtained for each GAG ranged from 64,000–24,000 cpm/µg for HS6, 54,000–270,000 cpm/µg for HS2, 180,000–192,000 cpm/µg for CS, 232,000–240,000 cpm/µg for DS, and 54,000–136,000 cpm/µg for DexS. The HS preparations (labeled either via the peptide remnant or via the reducing terminal xylose) were examined by enzymatic degradation and electrophoresis (see below, enzymatic degradations and polyacrylamide gel electrophoresis). Similar degradation patterns were obtained with preparations labeled according to either method.

Binding Assays

Experiments were performed at 4°C to avoid internalization of GAGs but also at 37°C for comparison. Fibroblasts were grown in 24-well microplates in 0.5 ml/cm^2 MEM with 10% newborn calf serum. The confluent cell monolayer was then incubated for various periods of time with 200 µl/cm^2 fresh, serum-free MEM containing radiolabeled GAGs. The medium was then removed, and the cell monolayer was rinsed twice with 250 µl/cm^2 fresh MEM. When binding was performed at 4°C, cells were extracted for 30 min with 0.5 ml/cm^2 of a buffer containing 4 M Gdn-HCl, 2% (v/v) Triton X-100, and 50 mM NaOAc, pH 5.8. The proteinase inhibitors, N-ethylmaleimide (10 mM) and diisopropylfluorophosphate (1 mM), were included in the buffer. GAGs remaining in the medium after incubation and rinsing were regarded as free, whereas those that were extracted from the cell monolayer were considered bound to the cell surface. Alternatively, when binding studies were performed at 37°C, cells were released from the monolayer by using 250 µl/cm^2 of 0.05% trypsin in PBS and incubating for 15–20 min at 37°C. The resulting cell suspension was centrifuged in a Biofuge A centrifuge (2,000 rpm for 3 min). GAGs released by trypsin were considered to be bound to the cell surface.

Uptake Assays

Experiments were performed at 37°C under sterile conditions. After incubation with 250 µl/cm^2 MEM containing radiolabeled GAGs for various periods of time, the medium was removed and the cell layer rinsed and subsequently subjected to trypsinisation and centrifugation. Radiolabeled GAGs present in the trypsin-liberated supernatant pool were regarded as surface-bound. Triton X-100 (250 µl/

cm^2) was added to the remaining cell pellet, and solubilized material was collected after a second centrifugation. Radiolabeled GAGs present in this supernatant was considered to represent GAGs taken up by the cells. Remaining detergent-insoluble material was solubilized in 4 M Gdn-HCl, and, after a third centrifugation, a Gdn-HCl extract was obtained.

Recovery of Radiolabeled HS From Cell Extracts

Extracts containing radiolabeled HS were passed over Q-Sepharose (0.2 ml gel in PBS) after 20x dilution with PBS to reduce ionic strength, if necessary. This ion exchanger binds also very low molecular weight HS oligosaccharides [Lindblom et al., 1991]. The columns were washed with 10 vols each of 6 M urea, 0.5 M NaOAc, pH 5.8, and 6 M urea, 10 mM Tris/HCl, pH 8.0, both containing 0.1% (v/v) Triton X-100 and 50 mM Tris/HCl, pH 7.5. Bound HS was eluted with 5 × 0.3 ml 2 M NH_4HCO_3 and 5 × 0.3 ml 4 M Gdn-HCl, 50 mM NaOAc, pH 5.8. HS2 was eluted with 2 M NH_4HCO_3 and recovered after freeze-drying. HS6 was eluted with 4 M Gdn-HCl and recovered by ethanol precipitation using 50 µg of dextran as carrier.

Enzymatic Degradations

Samples (100 µg) were dissolved in 50 µl 3 mM $Ca(OAc)_2$, 0.1% Triton X-100, 10 mM HEPES, pH 7.0, and digested with HS lyase (heparitinase) or heparin lyase (heparinase) using 20 mU/ml overnight at 37°C and 30°C, respectively.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed on linear gradient gels (20–30% T/0.5–3.6% C) as described by Fransson et al. [1990]. The buffers used were the same as described by Turnbull and Gallagher [1988]. Staining was performed with Azure A. For electroblotting we slightly modified the protocol of Kyhse-Andersen [1984]. ^{125}I-labeled material on the blotted membranes was visualized using a Fuji Bio-Imaging analyzer BAS 2000, Japan.

Statistics and Data Handling

Linear regression analysis was performed by Microsoft Excel. Mean values ± standard error of the mean was calculated.

RESULTS

Characterization of GAGs

Chemical data for the HS and DS preparations used are shown in Table I. HS has the common core structure (-4GlcUAβ1-4GlcNAcα-1-)$_n$ modified to various degrees by exchange of N-acetyl for N-sulphate, by 5-epimerization of D-glucuronic acid (GlcUA) to L-IdoUA, and by additional O-sulphation on both sugars. The HS2 preparation has approximately one-third IdoUA, most of which is nonsulphated. As the contents of N-sulphate and nonsulphated IdoUA are almost equal, most of the estersulphate should be associated with the N-sulphated glucosamine ($GlcNSO_3$) at C-6. In HS6 there is more IdoUA, and most of these residues are 2-O-sulphated. As the total ester-sulphate content is not excessively high, several $GlcNSO_3$ residues must lack 6-O-sulphation. DS/CS has the common core structure (-4GlcUAβ1-3GalNAcβ1-)$_n$ also modified to various degrees by 5-epimerization and O-sulphation. The DS preparation used is IdoUA-rich but not excessively oversulphated.

Sensitivity to enzymatic degradation of the HS2 and HS6 preparations was performed by electrophoresis (results not shown). HS2 was minimally degraded by heparin lyase, confirming that IdoUA is generally unsulphated in this species. Treatment of HS2 with heparan sulphate lyase, which cleaves bonds betwen GlcNAc/$GlcNSO_3$ and GlcUA, generated a series of oligosaccharides centered on decasaccharide. These should contain IdoUA-$GlcNSO_3$ as the major disaccharide repeat. HS6 was mostly sensitive to heparin lyase generating a wide spectrum of fragments from disaccharide to over 20 mers, in keeping with a high content of 2-O-sulphated IdoUA, present as continuous runs of disaccharides as well as in solitary positions. HS6 was only minimally degraded by heparan sulphate lyase.

Binding Sites

Cells were incubated for 2 h at 4°C with different concentrations of antiproliferative IdoUA-rich HS (HS6), inactive IdoUA-poor HS (HS2), antiproliferative DS (IdoUA-rich), or inactive CS (IdoUA-deficient). After incubation, GAGs that remained free in the medium were collected, and those that were bound to the cell layer were released by treatment with Gdn-HCl and detergent. The binding data are presented as Scatchard plots (Fig. 1). The curved shape of the plots for the two HS species indicate either cooperativity between dependent sites of the same type or the existence of two separate, independent binding sites. Depen-

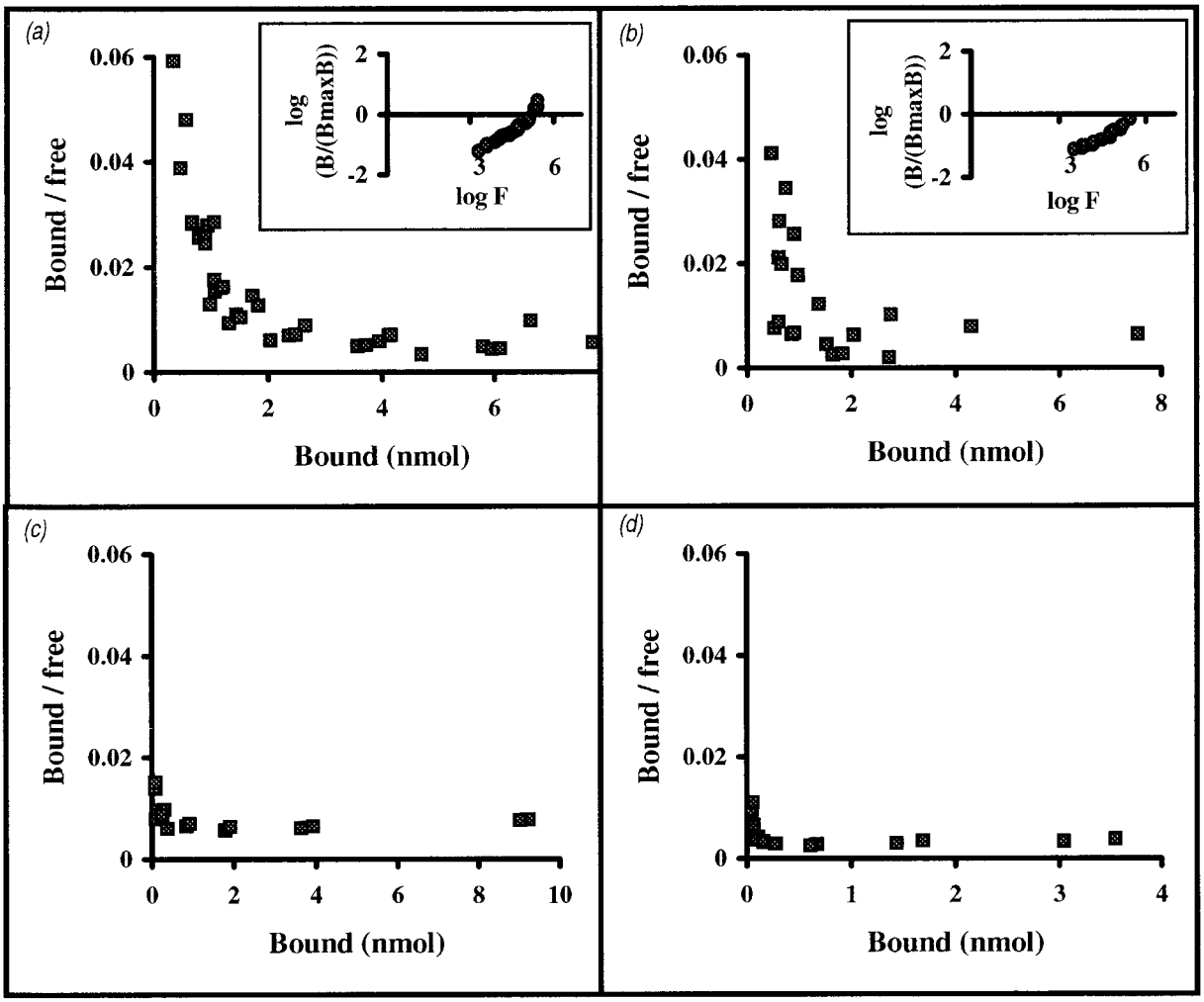

Fig. 1. Scatchard plots representing binding at 4°C to human fibroblasts of IdoUA-rich HS6 (***a***), IdoUA-poor HS2 (***b***), CS, (***c***) and DS (***d***). The concentration of GAGs ranged from 0.05–10 µg/ml in *a* and from 0.1–10 µg/ml in *b–d*. The curved shape of the plots for both HS species (*a, b*) is consistent with one high-affinity site (approximate $K_d = 10^{-8}$ M and 10^6 sites/cell) and one low-affinity site (approximate $K_d = 10^{-6}$ M and 10^7 sites/cell). **Inserts:** Hill plots of the binding of IdoUA-rich HS6 (*a*) and IdoUA-poor HS2 (*b*), indicating, in both cases, the presence of two independent high- or low-affinity sites on the cells. B is the amount of GAG bound to the fibroblasts and F the amount that remains unbound and free in the medium.

dent sites of the same type can display either positive or negative coperativity. Data are also presented as Hill plots (inserts, in Fig. 1*a*, *b*) to determine if binding is cooperative. Both plots had slopes close to unity (0.91 for HS6 and 0.94 for HS2). Therefore, the fibroblasts probably contain two independent high- and low-affinity sites for the binding of HS. Both the dissociation constant (K_d) and the number of sites/cell were similar for the high-affinity binding of HS6 ($K_d = 6.0 \times 10^{-8}$ M; 6.1×10^6 sites/cell) and HS2 ($K_d = 6.9 \times 10^{-8}$ M; 5.0×10^6 sites/cell). Also, low-affinity binding of HS6 ($K_d = 4.7 \times 10^{-6}$ M; 7.9×10^7 sites/cell) and HS2 ($K_d = 2.9 \times 10^{-6}$ M; 5.7×10^7 sites/cell) yielded similar parameters. The individual K_d values were not affected by the incubation time (2 h or 4 h). CS and DS showed very little binding to fibroblasts (Fig. 1*c*, *d*). In both cases, binding data in the low-affinity range gave no slope in the Scatchard plot, and no K_d could be obtained.

Binding of GAGs to fibroblasts was also studied at 37°C. Incubation of cells with various concentrations of HS6 for different periods of time indicated that a saturation level was usually reached between 6 and 24 h of incubation. At a concentration of 100 µg/ml of HS6, on an average 3 µg/well were bound (Fig. 2). A comparison between different GAGs showed that HS6 displayed the greatest binding, followed

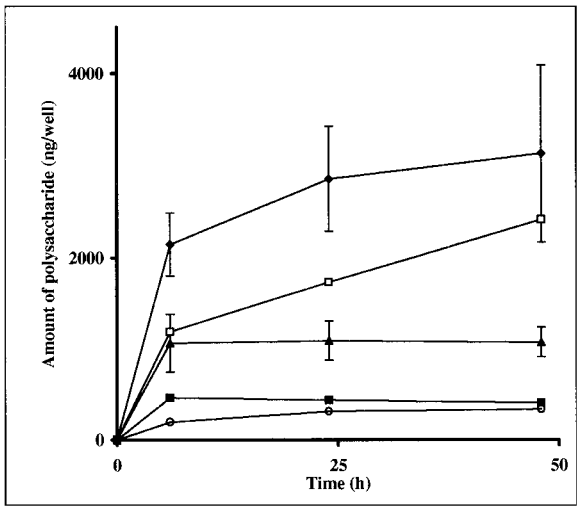

Fig. 2. Binding at 37°C of IdoUA-rich HS6 (◆), Dex S (□), IdoUA-poor HS2 (▲), DS (■), and CS (○) to fibroblasts. The concentration of the GAGs was 100 µg/ml. $x \pm$ S.E.M. (n = 4) is shown for HS2 and HS6.

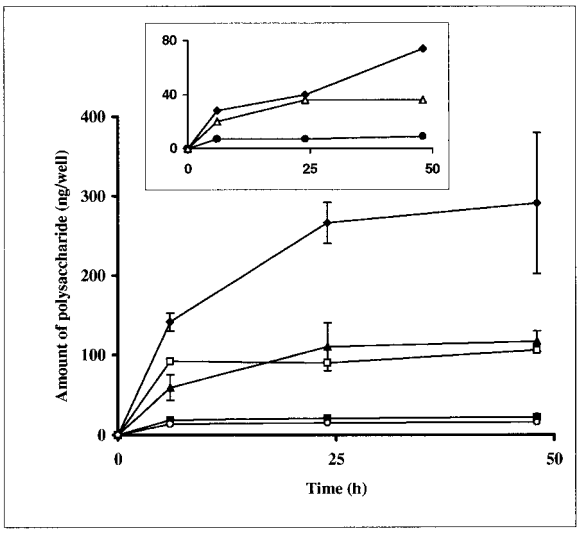

Fig. 3. Internalization at 37°C of IdoUA-rich HS6 (◆), DexS (□), IdoUA-poor HS2 (▲), DS (■), and CS (○) by human fibroblasts. The concentration of the various GAGs was 100 µg/ml. **Insert:** Inhibition by DexS of internalization of IdoUA-rich HS6 at 37°C. Cells were incubated with 10 µg/ml of radiolabeled HS6 alone (◆) or with a combination of 10 µg/ml of radiolabeled HS6 and either 1 µg/ml (△) or 100 µg/ml unlabelled DexS (●). x ± S.E.M. (n = 4) is shown for HS2 and HS6.

by DexS and HS2. Very little DS and CS were bound. The K_d values for HS6 and HS2 obtained at 37°C were similar to values obtained at 4°C.

Selectivity of Binding

To determine if the binding sites were selective for HS, a 100-fold excess of various unlabeled GAGs or of the sulfated polysaccharide DexS was added to radiolabeled HS6 at 1 or 10 µg/ml and at 4°C. A 100-fold excess of HS6 or heparin inhibited binding of radiolabeled HS6 by 80 ± 3% and 73 ± 4%, respectively, at 1 µg/ml HS6 and by 67 ± 4% and 59 ± 4%, respectively, at 10 µg/ml (n = 3 in all cases). In contrast, HS2 and DS were less efficient competitors, and CS and HA were essentially inactive. However, a 100-fold excess of DexS competed strongly with HS6 (at 1 µg/ml) for binding to the cells (88 ± 4%).

Uptake of HS

Radiolabeled HS was added to cells at 37°C, and after various time periods the cell layer was trypsinized and released cells collected by centrifugation and lysed with detergent. Insignificant amounts of HS6 were recovered from the cell lysate after incubation with 0.5 µg/ml, but significant uptake was observed after 6 h with 10 µg/ml and more with 100 µg/ml. The IdoUA-rich, antiproliferative HS6 was taken up to a greater extent than the IdoUA-poor, inactive HS2 (Fig. 3). A small amount of DexS was initially taken up, but it did not appear to increase with time. No significant uptake of either DS or CS was observed. As shown in the insert of Figure 3, a tenfold excess of DexS almost abolished uptake of HS6 (at 10 µg/ml and at 37°C). Also, binding of HS6 under the same conditions was inhibited by a tenfold excess of DexS (88%).

Degradation of HS

The HS6 and HS2 bound or taken up by the cells were recovered on Q-Sepharose and quantified. After 24 h of incubation, 20% of cell layer–associated HS6 and 12% of HS2, were recovered from the cell lysate. Hence, the major portion of cell layer–attached HS was at the cell surface (trypsin-releasable). Only minor amounts were attached to the extracellular matrix remaining in the plates after trypsinization.

To ascertain if degradation of HS had taken place, radiolabeled HS species that had been incubated with cells for various periods of time were subsequently recovered from the trypsinate or the cell lysate or extracted from the plates with 4 M Gdn-HCl and analyzed by poly-

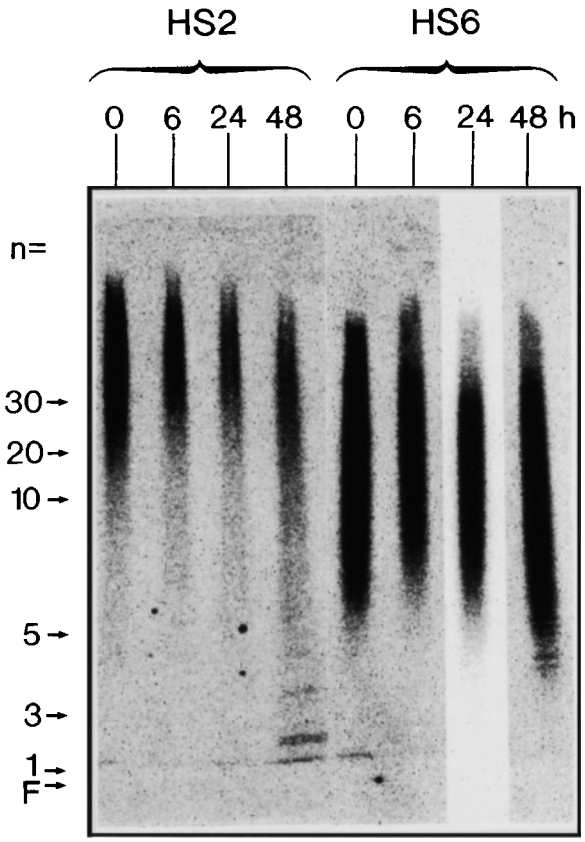

Fig. 4. Polyacrylamide gel electrophoresis of radiolabeled HS2 and HS6 recovered from the internalized and detergent-solubilized fraction. Cells were incubated with 100 µ/ml of radiolabeled HS2 or HS6 for the indicated periods of time. The cell monolayers were then trypsinized, and radiolabeled HS was isolated from the detergent lysate of the recovered cells and subjected to electrophoresis as described in Methods. Similar amounts of HS were applied. The migration distance of standard oligosaccharides is indicated (n = 1, 2, 3, . . . is di-, tetra-, hexasaccharide and so on). F, front.

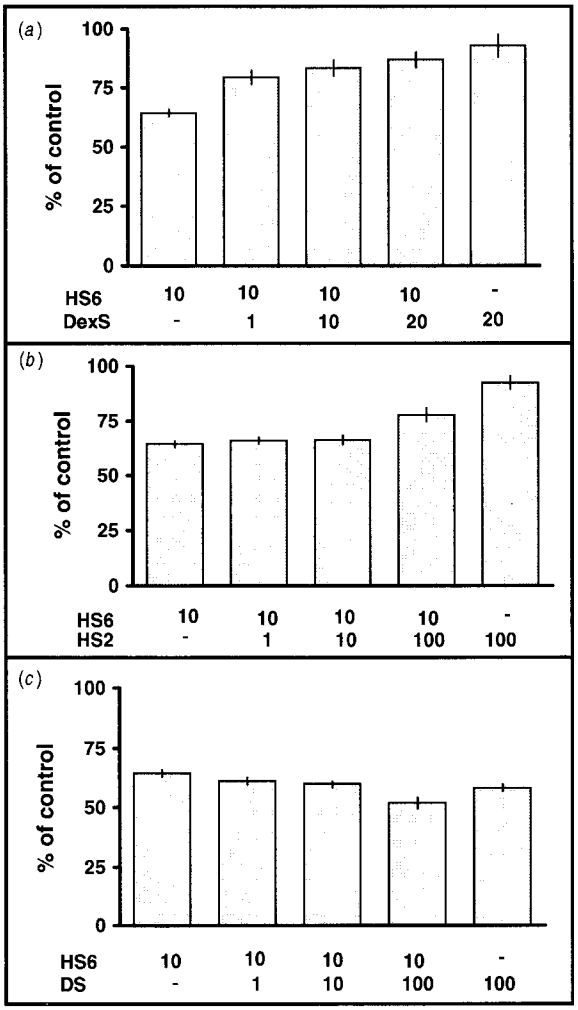

Fig. 5. Competitive effects of various sulphated glycans on the growth-inhibitory capacity of HS6. Fibroblasts were pretreated as described in Methods and plated at a density of 5,000/well. Growing cells were incubated with HS6 alone (10 µg/ml throughout) or together with the indicated concentrations of DexS (***a***), HS2 (***b***) or DS (***c***). On each plate a set of control incubations was made. Cell number was estimated by measuring the amount of crystal violet adsorbed. The values represent the mean ± S.E.M. from n = 15, 10, and 15, respectively in *a*, *b*, and *c*.

acrylamide gel electrophoresis (Fig. 4). As the HS preparations used were labeled in the reducing end, only fragments extending from the reducing end to the point of cleavage can be observed. HS2 that was recovered from the cell lysate showed signs of partial degradation after 48 h of incubation. Not all chains had been cut, but degradation products migrating like small oligosaccharides (n = 1–5) were seen. Hence, cleavage sites were situated near the linkage region. Only minor degradation of HS6 appeared to take place in the same compartment. Both HS2 and HS6 that were recovered from the trypsinate remained intact (results not shown). Also, material bound to the extracellular matrix remaining in the plates appeared undegraded (results not shown).

Blocking the Antiproliferative Effect of HS

As shown previously [Westergren-Thorsson et al., 1991], HS6 but not DexS can inhibit growth of human embryonic fibroblasts. In the present study, it was found that DexS is a potent competitor of the binding of HS6 to the same cells. Therefore, it was of interest to examine if DexS could bind to the cell surface in place of HS6 and thereby block its antiproliferative activity. As shown in Figure 5*a*, HS6 alone at 10 µg/ml caused approximately 35% inhibi-

tion of growth, whereas DexS at double the concentration inhibited less than 15%. However, 1 μg/ml of DexS could reduce the antiproliferative activity of 10 μg/ml of HS6 by 50%.

HS2, which is a weak inhibitor of cell growth [Westergren-Thorsson et al., 1991, 1993], was also tested for its ability to affect the antiproliferative activity of HS6 (Fig. 5*b*). It is seen that a tenfold excess of HS2 was required to reduce the activity of HS6 by approximately 50%.

DS is also antiproliferative [Westergren-Thorsson et al., 1991] despite no specific binding to the cells (see Fig. 1*d*). To ascertain if HS and DS operate via different mechanisms, they were tested separately or in combination (Fig. 5*c*). Whereas 10 μg/ml of HS6 alone caused approximately 35% inhibition of growth and 100 μg/ml DS alone approximately 40% inhibition, HS6 and DS together gave approximately 50% reduction. As approximately 65% inhibition is the greatest effect observed so far with any GAG fraction at high concentration [Belting et al., 1996], the cumulative results suggest that the antiproliferative effects of HS and DS are additive.

In separate experiments possible competitive effects of DexS on the growth-inhibitory capacity of DS were also tested. Whereas DexS interfered with HS6 (see Fig. 5*a*), DexS did not interfere with the antiproliferative effect of DS (results not shown).

GAGs and Plating Efficiency

As DS could inhibit growth of fibroblasts in the absence of strong binding to the cells, it was considered important to test whether GAGs could interfere with growth in other ways, such as by blocking the initial plating and cell attachment to the substratum. Cells were therefore pretreated as described above and seeded at densities of 5,000 or 20,000/well, using regular medium containing 10% serum, and with or without 100 μg/ml of GAG (HS6, HS2, DS, or CS). After incubation for 2, 4, or 6 h, the number of cells was measured. In no case did the presence of GAG affect the number of cells that were plated.

DISCUSSION

Highly sulphated, heparin-like HS preparations (like HS6) and IdoUA-rich DS inhibit growth of normal fibroblasts. Cells in monolayer culture require an appropriate substratum for attachment and growth. Addition of exogenous GAGs may therefore interfere with cell-substratum interaction. Cell plating under the growth conditions used here was not affected by exogenous GAG (HS, CS, or DS). Also, other studies have shown that adhesion of fibroblasts to plates coated with intact fibronectin is not affected by added DS/CS-containing PGs [Bidanset et al., 1992]. However, San Antonio et al. [1992] reported that heparin and DexS inhibit attachment of 3T3 fibroblasts to type I and V collagen films.

The present study also showed that antiproliferate HS and DS have different binding characteristics when tested with human embryonic lung fibroblasts. Whereas HS6 binds well to the cell layer, DS binds very little. A low-sulphated heparan sulphate preparation (HS2) does not suppress cell growth, but, like the antiproliferative HS6, it is bound to the cells. Structural analysis of the two HS preparations support the notion that sulphated IdoUA is essential for the antiproliferative activity but not for binding to the cells. For the two HS forms we have found one high-affinity ($6–7 \times 10^{-8}$ M) and one low-affinity ($3–5 \times 10^{-6}$ M) site that appear to be independent of one another. The high-affinity site becomes saturated already at 1 μg/ml (i.e., 5×10^{-8} M), but the antiproliferative effect of HS6 is noticeable first at ten times higher concentration [Westergren-Thorsson et al, 1991]. Therefore, binding to the low-affinity site is probably necessary to elicite the growth-arresting effect. However, as the inactive HS2 shows the same binding characteristics as the antiproliferative HS6, contact with the cell surface may not be sufficient.

We therefore studied possible uptake of the two HS species by the fibroblasts. GAGs recovered from a detergent lysate of the trypsin-released cells were considered to represent material taken up by the cells. The results showed that the antiproliferative species HS6 was taken up to a greater extent than the inactive one (HS2). Uptake was significant at concentrations of 5 μg/ml or higher, suggesting that the low-affinity site could represent an endocytosis receptor and that uptake is necessary for induction of the antiproliferative effect. However, there were no signs of degradation of the active species.

To characterize the two binding sites further, we used DexS, which is a nonnatural polysulfated glycan. DexS is not antiproliferative to lung fibroblasts per se [Westergren-Thorsson et

al., 1991], but, as shown in this study, it interferes with binding, uptake, and growth effects of HS6. Binding of HS6 at 10 μg/ml, which should involve the low-affinity site, was greatly inhibited by a tenfold excess of DexS. Also, uptake of HS6 was essentially abolished by the same excess of DexS. In contrast, the antiproliferative effect of 10 μg/ml of HS6 was reduced 50% by as little as 1 μg/ml of DexS. At this level, DexS had very little effect on the uptake of HS6 (see insert in Fig. 3). Thus, the effect of DexS on the antiproliferative ability of HS6 cannot be mediated via interference with uptake of HS. These findings strongly suggest that HS6 exerts its growth-inhibiting effect by interacting both with the high-affinity and the low-affinity sites.

HS represents a family of GAGs that includes a vast number of structural variants with multiple functions [Fransson, 1987]. Also, preparations HS2 and HS6 used in this study are heterogenous [Fransson et al., 1980a], and, in affinity-labeling experiments, the HS6 preparation binds several cell surface–derived proteins [Westergren-Thorsson, Fransson, and Malmström, unpublished results]. Hence, the ligand is both polymeric and contains a number of potential binding sequences. The present results also suggest that more than one cell-surface receptor is involved in mediating the antiproliferative effect, which constitutes valuable information for future studies. Hence, various HS fragments generated via different forms of controlled degradation must be tested for growth effects and binding characteristics before attempts to isolate individual HS receptors can be initiated.

The mechanism of growth inhibition by DS appears to be different from that of HS, as DS has no specific binding sites on fibroblasts and as their effects appear to be additive. DS may instead interact with cell-derived soluble factors essential for growth. Polyamines (mainly spermine and spermidine), produced intracellularly by all cells, are essential for growth. Although they are believed to have an intracellular site of action, they are also exchanged with the external environment via carrier systems [see Bradbury and Parish, 1989]. Recently, Belting and Fransson [1993] showed that spermine can be bound to certain IdoUA-rich regions in DS and that the same fragments, in oligosaccharide form, are antiproliferative. Thus, DS may inhibit cell growth by blocking uptake of polyamines.

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