Structure and Properties of an Under-sulfated Heparan Sulfate Proteoglycan Synthesized by a Rat Hepatoma Cell Line

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ABSTRACT A rat hepatoma cell line was shown to synthesize heparan sulfate and chondroitin sulfate proteoglycans. Unlike cultured hepatocytes, the hepatoma cells did not deposit these proteoglycans into an extracellular matrix, and most of the newly synthesized heparan sulfate proteoglycans were secreted into the culture medium. Heparan sulfate proteoglycans were also found associated with the cell surface. These proteoglycans could be solubilized by mild trypsin or detergent treatment of the cells but could not be displaced from the cells by incubation with heparin. The detergent-solubilized heparan sulfate proteoglycan had a hydrophobic segment that enabled it to bind to octyl-Sepharose. This segment could conceivably anchor the molecule in the lipid interior of the plasma membrane. The size of the hepatoma heparan sulfate proteoglycans was similar to that of proteoglycans isolated from rat liver microsomes or from primary cultures of rat hepatocytes. Ion-exchange chromatography on DEAE-Sephacel indicated that the hepatoma heparan sulfate proteoglycans had a lower average charge density than the rat liver heparan sulfate proteoglycans. The lower charge density of the hepatoma heparan sulfate can be largely attributed to a reduced number of *N*-sulfated glucosamine units in the polysaccharide chain compared with that of rat liver heparan sulfate. Hepatoma heparan sulfate proteoglycans purified from the culture medium had a considerably lower affinity for fibronectin-Sepharose compared with that of rat liver heparan sulfate proteoglycans. Furthermore, the hepatoma proteoglycan did not bind to the neoplastic cells, whereas heparan sulfate from normal rat liver bound to the hepatoma cells in a time-dependent reaction. The possible consequences of the reduced sulfation of the heparan sulfate proteoglycan produced by the hepatoma cells are discussed in terms of the postulated roles of heparan sulfate in the regulation of cell growth and extracellular matrix formation.

Heparan sulfate proteoglycans are present in a large variety of vertebrate tissues and appear to be preferentially located at the surface of cells, either directly associated with the cell membrane (1) or in close contact with cells, as in basement membranes (2) and in the pericellular matrix of cultured cells (3, 4).

Previous studies in our laboratory have indicated that heparan sulfate proteoglycans are associated with the plasma membrane of rat liver cells by two independent mechanisms (5). The proteoglycan may be bound via its polysaccharide portion to cell surface receptors, or the core protein may be inserted into the lipid interior of the membrane (6).

The mechanisms responsible for the anchorage of heparan sulfate in the basement membrane or in extracellular matrices are not clear. Immunofluorescent studies have shown a co-distribution between heparan sulfate proteoglycans and fibronectin in the extracellular matrix (7, 8) and a direct binding of heparan sulfate chains to fibronectin has been demonstrated (7, 9).

Transformed cells usually do not assemble an extracellular matrix (10–12), although exceptions to this rule have been observed (12). Their inability to form a matrix does not appear to be caused by failure of the transformed cells to synthesize any of the identified matrix components (13). It is possible,

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therefore, that successful matrix formation by cells depends on structure as well as on the quantity of the synthesized matrix components. In several systems, transformed cells have been shown to produce a heparan sulfate that has a reduced sulfate content compared with the proteoglycan synthesized by the normal counterpart (14–16).

The pericellular location of the heparan sulfate proteoglycans enables them to interact directly with cells and may allow these molecules to affect the behavior of cells. Recent interest has focused on the possibility that heparan sulfate may regulate cell proliferation (17–20). In particular, mucopolysaccharides isolated from normal rat liver were found to reduce the rate of proliferation of cultured hepatoma cells (21).

The current study was undertaken to investigate the hypothesis that structural differences in the heparan sulfate proteoglycan produced by transformed cells result in an impaired function of the proteoglycan in terms of its interaction with other molecules.

MATERIALS AND METHODS

Materials: The hepatoma cells were obtained from Dr. Eve Briles, University of Alabama, Birmingham. [^{35}S]Sulfate (carrier-free) was purchased from the Radiochemical Centre, Amersham, England and 6-[^{3}H]glucosamine (20.2 Ci/mmol) from New England Nuclear, Boston, MA. Heparin and bovine liver heparan sulfate were prepared as previously described (5) and chemically labeled with tritium by acetylation of free amino groups with [^{3}H]acetic anhydride (22). Heparan [^{35}S]sulfate proteoglycans isolated from cultured rat hepatocytes as described (5) were a gift from Dr. L. Kjellén, Uppsala, Sweden. Trypsin (pancreatic, type II), soybean trypsin inhibitor, DNase I (DN-25), papain (twice crystallized, type III), and BSA were obtained from Sigma Chemical Co., St. Louis, MO. Chondroitinase ABC was purchased from Miles Laboratories, Inc., Elkhart, IN. DEAE-Sephacel, Sepharose CL-4B, Sephadex G-50 and G-200, octyl-Sepharose, Percoll, and dextran sulfate were from Pharmacia Fine Chemicals, Uppsala, Sweden. Fibronectin was purified from human plasma using the procedure of Vuento and Vaheri (23) and coupled to CNBr-activated Sepharose (Pharmacia Fine Chemicals) according to the manufacturer's directions. Eagle's α-modified minimal essential medium (MEM) (catalogue number 12-313), L-glutamine, and fetal calf serum were purchased from Flow Laboratories, Inc., McLean, VA. Culture flasks, Petri dishes, and 16-mm well dishes were obtained from Costar Data Packaging, Cambridge, MA. The rabbit antiserum to basement membrane heparan sulfate proteoglycan (BMI) was a kind gift from Dr. John Hassell, National Institutes of Health, Bethesda, MD (24). The rabbit antiserum raised against rat fibronectin has been described (25). Goat anti-rabbit IgG antiserum conjugated with fluorescein isothiocyanate (Miles Laboratories, Inc.) was diluted 1:50 before use.

Indirect Immunofluorescence: Hepatoma cells were grown on coverslips and fixed with 3.5% paraformaldehyde. Unspecific binding sites were blocked by incubating the cells with 1% BSA in PBS. After a thorough wash, the cells were incubated at 37°C for 45 min with rabbit antibodies raised against either basement membrane heparan sulfate proteoglycan or against rat plasma fibronectin. These antibodies were then visualized by incubation of the cells with a second antibody, goat anti-rabbit gamma globulin conjugated with fluorescein isothiocyanate. No staining was observed in controls where cells were stained with the second antibody only.

Isolation of Radiolabeled Proteoglycans Synthesized by Cultured Hepatoma Cells: Hepatoma cells were seeded in 50.0-ml culture flasks in 5.0 ml of α-modified MEM supplemented with 10% fetal calf serum and 0.03 mg/ml L-glutamine. Radioisotope was added at the time of seeding (50 μCi/ml of [^{35}S]sulfate or 20 μCi/ml of [^{3}H]glucosamine), and the flasks were incubated at 37°C for 48 h.

After the incubation, the culture media were collected and centrifuged to remove detached cells. The proteoglycans that were subsequently isolated from the supernatant (see below) were denoted medium proteoglycans. The cell layer was washed three times with PBS and then solubilized by treatment first with 2% Empigen BB in 0.1 mM HEPES, pH 7.4, containing protease inhibitors (the protease inhibitors 5 mM benzamidine HCl, 0.1 mM phenylmethylsulfonyl fluoride, and 10 mM EDTA were used in all buffers) for 20 min at room temperature, followed by the addition of an equal volume of 8.0 M GuHCl/2% Triton X-100 in 0.1 M HEPES buffer, pH 7.4. The cell extract was incubated at 4°C on an end-over-end mixer for 30 min and then dialyzed against 0.05 M Tris-HCl, pH 8.0, containing 0.1% Triton X-100. The proteoglycans isolated from the dialyzed cell fraction were designated cell-associated proteoglycans.

The samples were applied to columns (1 × 3 cm) of DEAE-Sephacel at 4°C. The columns were washed with 0.35 M NaCl in 0.05 M Tris-HCl, pH 8.0, and eluted by a linear gradient from 0.35 to 1.50 M NaCl in 0.05 M acetate, pH 4.0. The buffers used in processing the cell-associated proteoglycans were supplemented with 0.1% Triton X-100.

To remove chondroitin sulfate proteoglycans, the proteoglycan mixture was dialyzed for 24 h against 0.05 M Tris-HCl buffer, pH 8.0, and then digested with chondroitinase ABC according to the procedure described previously (26). The digest mixtures were applied to a column (107 × 1.2 cm) of Sephadex G-50 equilibrated with 0.5 M NaCl. The chondroitinase ABC degradation products were eluted close to the total volume of the column. The material eluting in the void volume was heparan sulfate, as indicated by its susceptibility to nitrous acid treatment at pH 1.5 (27).

Purification of Heparan Sulfate Proteoglycans from Rat Liver Microsomes: Heparan sulfate proteoglycans labeled in vivo with [^{35}S]-sulfate or [^{3}H]glucosamine were purified from rat liver microsomes using a procedure previously described (28) with the following additional steps. The microsomal preparation was suspended in 0.35 M NaCl in 0.05 M Tris-HCl buffer, pH 8.0, containing 0.1 mg/ml of dextran sulfate. The suspension was incubated at 4°C in an end-over-end mixer for 30 min and then centrifuged at 35,000 *g* for 30 min. This procedure released the heparan sulfate proteoglycans bound to the plasma membranes via polysaccharide-receptor interactions (5). Radioactively labeled heparan sulfate proteoglycans were isolated from the supernatant by ion-exchange chromatography on DEAE-Sephacel as described above.

Estimation of Molecular Size: The molecular sizes of heparan sulfate proteoglycans were estimated by gel chromatography on a column (98 × 1.2 cm) of Sepharose CL-4B eluted with 4 M GuHCl in 0.05 M Tris-HCl, pH 8.0. The column was calibrated with reduced and alkylated proteins of known molecular weights (phosphorylase *b*, 94,000 mol wt; α-chains of fibrinogen, 70,000 mol wt; heavy chains of IgG, 53,000 mol wt). Previous studies have shown that this method is reliable for determining the molecular weight of heparan sulfate proteoglycans isolated from rat livers (28).

The sizes of the polysaccharide chains cleaved from the proteoglycan core protein by alkaline/borohydride treatment (0.05 M NaOH, 1.0 M $NaBH_4$ at 45°C for 24 h) were determined by gel chromatography. The neutralized reaction mixtures were applied to a column (105 × 1.2 cm) of Sephadex G-200 equilibrated and eluted with 0.2 M NaCl at a flow rate of 4 ml/h. The relative molecular weights of the polysaccharide chains were estimated from their K_{av} values using the calibration curve of Wasteson (29) obtained from chondroitin sulfates.

Nitrous Acid Deamination: Two methods of nitrous acid deamination were used. One involves treatment of the polysaccharides at pH 1.5 and results in the cleavage of *N*-sulfated glucosamine residues, whereas *N*-unsubstituted and *N*-acetylated hexosamine units are left intact (27). This procedure was used to quantitate the proportion of heparan sulfate in a mixture of glycosaminoglycans. In the other method, the pH 1.5 procedure is followed by a second treatment at pH 3.9 (30). This method resulted in the cleavage of *N*-sulfated and *N*-unsubstituted glucosamine residues but not of *N*-acetylated hexosamine units. The extent of degradation of the heparan sulfate chains was estimated after fractionation of the deamination mixtures on a column (62 × 0.6 cm) of Sephadex G-50.

Release of Cell Surface-associated Heparan Sulfate by Trypsin or Heparin: The ^{35}S-labeled hepatoma cells were incubated with 100 μg heparin/ml of medium for 30 min at 37°C, followed by an incubation with 100 μg trypsin/ml of PBS for 2–5 min at 37°C. The reaction was stopped by the addition of 250 μg/ml of soybean trypsin inhibitor. The incubation mixtures were centrifuged to remove any detached cells, and trypsin-released proteoglycans were isolated from the supernatants. For quantitation of ^{35}S-labeled glycosaminoglycans released by heparin or trypsin, samples were digested with papain (26) and radiolabeled glycosaminoglycans were precipitated onto filter papers with cetylpyridinium chloride (31).

Hydrophobic Chromatography of Proteoglycans: Solutions of ^{3}H-labeled heparan sulfate proteoglycans purified from (*a*) the medium of cultured hepatoma cells, (*b*) the cell layer after solubilization with detergent and (*c*) the trypsin-released material were made 2% cholate in 0.02 M Tris-HCl, pH 7.3, by a procedure previously described (7). The samples were then applied to a column (3 × 1 cm) of octyl-Sepharose and eluted in a stepwise manner with 25-ml aliquots of 0.02 M Tris-HCl buffer, pH 7.3, containing: (*a*) 0.1 M NaCl, (*b*) 3.0 M NaCl, (*c*) 3.0 M NaCl and 1% Triton X-100, and (*d*) 0.4% deoxycholate. This elution procedure resulted in the recovery of at least 95% of the radioactivity applied to the column.

Affinity Chromatography on Fibronectin-Sepharose: Affinity chromatography of radiolabeled proteoglycans and isolated polysaccha-

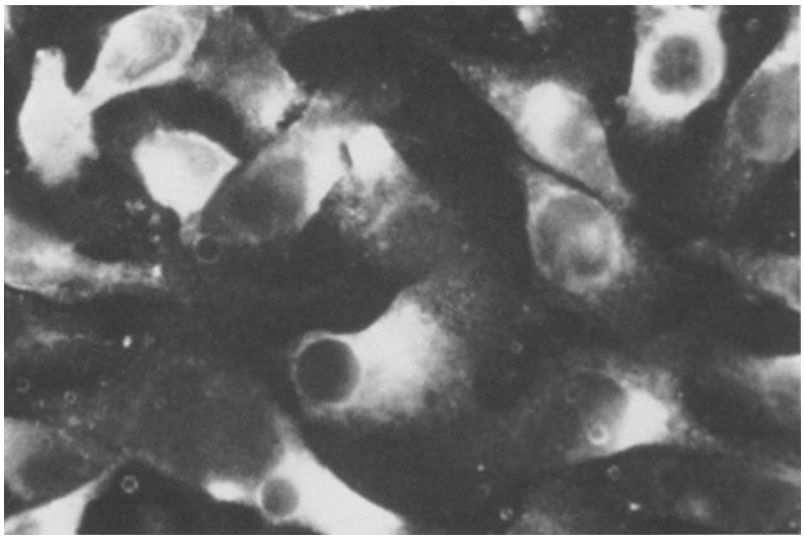

FIGURE 1 Immunofluorescent staining of hepatoma cells for heparan sulfate proteoglycans. Hepatoma cells were stained for heparan sulfate proteoglycan using an antibody raised against basement membrane heparan sulfate proteoglycan. × 1,100.

ride chains on columns of fibronectin-Sepharose was performed as previously described (7).

Binding of Exogenous Radiolabeled Glycosaminoglycans to Hepatoma Cells: The binding of added radiolabeled glycosaminoglycans to cultured hepatoma cells was investigated using a technique previously described (5).

RESULTS

Distribution of Heparan Sulfate Proteoglycans and Fibronectin in Rat Hepatoma Cells

Immunofluorescent staining of cultures of rat hepatoma cells using anti-rat fibronectin indicated that the extracellular fibronectin-containing matrix produced by cultures of normal hepatocytes (32; S. Johansson and M. Höök, unpublished observation) is lacking in the hepatoma cell cultures (not shown). Staining for heparan sulfate proteoglycan (Fig. 1) indicated that this molecule is present on the hepatoma cells but not in an extracellular matrix. These results point to a concomitant loss of fibronectin and heparan sulfate from the extracellular matrix, which is in keeping with previous reports (8, 33).

Isolation of Proteoglycans Secreted by Cultured Hepatoma Cells

Media obtained from cultured hepatoma cells incubated for 48 h in the presence of [^{35}S]sulfate or [^{3}H]glucosamine were subjected to anion-exchange chromatography on columns of DEAE-Sephacel. Most of the ^{35}S radioactivity was washed through the column, but some of the ^{35}S-labeled material bound to the DEAE-Sephacel and eluted as an asymmetrical peak in the NaCl gradient (Fig. 2). When [^{3}H]glucosamine was used as the radioisotope, two peaks of radioactivity were eluted in the NaCl gradient. The first peak, which appeared shortly after the start of the gradient, was not analyzed further. The elution position of the second peak essentially coincided with that of the ^{35}S-labeled material (data not shown). Heparan sulfate constituted the major portion (~80%) of the labeled material in this peak, as shown by the susceptibility of the material to nitrous acid deamination (Table I).[1] Most of the remaining material was susceptible to digestion with chondroitinase ABC and is tentatively identified as chondroitin sulfate (Table I). The heparan sulfate proteoglycans were isolated after digestion of the proteoglycan mixture with chondroitinase ABC.

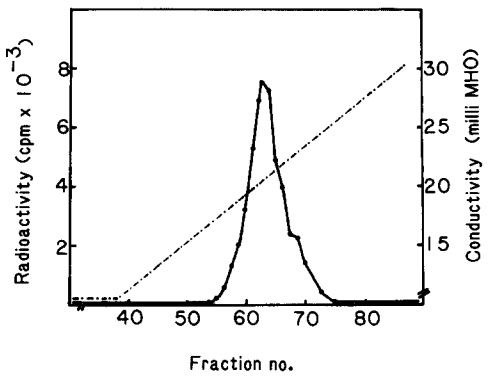

FIGURE 2 Ion-exchange chromatography of ^{35}S-labeled medium from hepatoma cell cultures. 5 ml of conditioned medium from [^{35}S]sulfate-labeled hepatoma cultures were made up to 30 ml with 0.35 M NaCl in 0.05 M Tris-HCl, pH 8.0, and then applied to a column (3 × 1 cm) of DEAE-Sephacel, which had been equilibrated with the pH 8.0 buffer. The column was washed with 40 ml of 0.35 M NaCl in 0.05 M acetate buffer, pH 4.0, and eluted with a linear gradient (150 ml) from 0.35 to 1.5 M NaCl in acetate buffer, pH 4.0. Fractions of 2.0 ml were collected and 0.05-ml samples were analyzed for ^{35}S radioactivity (●——●); conductivity (-·-·-).

Structural Comparison of Heparan Sulfate Proteoglycans Isolated from Cultured Hepatoma Cells and from Rat Liver Microsomes

SIZE: The molecular sizes of ^{35}S-labeled heparan sulfate proteoglycans isolated from the hepatoma cell culture medium and from rat liver microsomes were compared by chromatography on a column of Sepharose CL-4B eluted with 4 M GuHCl. Both proteoglycans eluted as fairly broad peaks, which indicates considerable heterogeneity in molecular size (Fig. 3). The elution profile of the hepatoma proteo-

[1] When [^{3}H]glucosamine is used for labeling of proteoglycan, the isotope is presumably incorporated into oligosaccharides as well as into polysaccharides. These oligosaccharides are presumably not degraded by nitrous acid treatment of digestion with chondroitinase ABC and would therefore be classified as "other" in Table I.

TABLE I

^{35}S- or ^{3}H-labeled Proteoglycans Produced by Hepatoma Cells

	Proportions of radioactivity (% total cpm) in		
	Heparan sulfate	Chondroitin sulfate	Other
Medium			
[^{35}S]Sulfate	80	19	1
[^{3}H]Glucosamine	81	6	13
Cell layer			
[^{35}S]Sulfate	71	25	4
[^{3}H]Glucosamine	65	14	21

Newly synthesized proteoglycans were labeled with ^{3}H or ^{35}S by incubation of hepatoma cells in medium containing [^{3}H]glucosamine or [^{3}H]sulfate. After 48 h incubation, the medium was removed and the cell layer was solubilized as described in Materials and Methods. Radiolabeled proteoglycans from the media and the cell extracts were isolated by chromatography on DEAE-Sephacel. The proteoglycans were identified as heparan sulfate or chondroitin sulfate proteoglycan on the basis of their susceptibility to chondroitinase ABC digestion and nitrous acid deamination, respectively. Radioactive material that resisted both treatments was designated "other."[1]

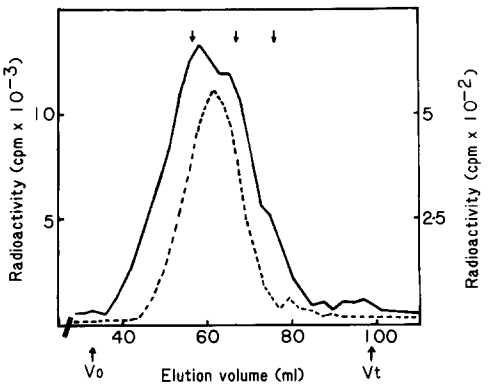

FIGURE 3 Gel chromatography of ^{35}S-labeled heparan sulfate proteoglycans purified from hepatoma cell culture medium and rat liver microsomes. Samples of hepatoma heparan [^{35}S]sulfate proteoglycan from the culture medium (——; cpm × 10^{-2}) and rat liver heparan [^{35}S]sulfate proteoglycan (– – –; cpm × 10^{-3}) were applied to a column (98 × 1.2 cm) of Sepharose CL-4B equilibrated with 4 M GuHCl in 0.05 M Tris-HCl, pH 8.0, containing protease inhibitors. The column was eluted with the same buffer at a flow rate of 4 ml/h. Fractions of 1.5 ml were collected and 0.5-ml samples were analyzed for ^{35}S radioactivity.[2] The arrows indicate the elution positions of the protein standards (phosphorylase *b*, 94,000 mol wt; α-chain of fibrinogen, 70,000 mol wt; and the heavy chains of IgG, 53,000 mol wt, respectively).

glycans indicates the presence of two poorly separated subpopulations: the peak of the major population eluted as a broad peak at K_{av} of 0.39 compared with a K_{av} of 0.44 for the peak fraction of the rat liver proteoglycan.[2] By comparing the elution positions of the ^{35}S-labeled proteoglycans with those of the standard proteins, the relative molecular weights of the rat liver proteoglycans and the hepatoma heparan sulfate proteoglycans can be estimated to be ~80,000 and ~95,000, respectively. When analyzed on a column (1 × 93 cm) of Sepharose CL-4B, the major population of the hepatoma proteoglycan eluted at a volume of 62 ml, which is comparable to the elution position of heparan sulfate proteoglycans synthesized by cultured hepatocytes (see Fig. 3; reference 5).

The hepatoma heparan sulfate proteoglycans were degraded by incubation with papain or alkali/borohydride, which is consistent with a proteoglycan structure. Alkali/borohydride treatment, which releases the labeled polysaccharide chains from the core protein, yielded a product that was somewhat smaller than that obtained after papain digestion, which cleaves in the core protein. The elution positions after gel chromatography on Sepharose CL-4B corresponded to K_{av} = 0.69 and K_{av} = 0.62 for [^{35}S]polysaccharides obtained after incubation of the hepatoma heparan sulfate proteoglycan with alkali/borohydride and papain, respectively (data not shown).

On Sephadex G-200, the alkali-released polysaccharides from the HTC medium heparan sulfate proteoglycan eluted as a broad peak at a K_{av} of 0.29 compared with a K_{av} of 0.32 obtained for heparan sulfate chains from normal rat liver (data not shown). These elution positions correspond to those of chondroitin sulfates with molecular weights of 19,000 and 16,000, respectively (29).

POLYANIONIC PROPERTIES: The polyanionic properties of heparan sulfate proteoglycans isolated from rat liver microsomes, cultured hepatocytes and cultured hepatoma cells, respectively, were compared by anion-exchange chromatography. Tritium-labeled heparan sulfate proteoglycans isolated from conditioned media of the hepatoma cultures

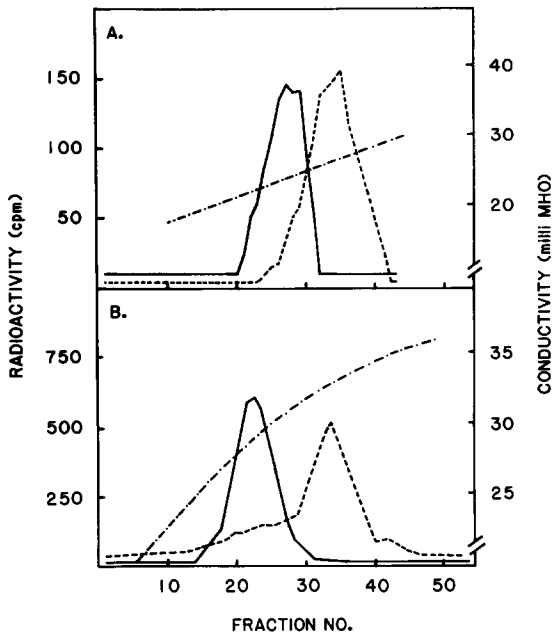

FIGURE 4 Ion-exchange chromatography of heparan sulfate proteoglycans purified from hepatoma cell culture medium, rat liver microsomes, and cultured rat hepatocytes. A sample of [^{3}H]heparan sulfate proteoglycan purified from hepatoma cell culture medium was mixed with a sample of heparan [^{35}S]sulfate proteoglycan purified from rat liver microsomes (*A*) or from cultured rat hepatocytes (*B*) and applied to a column (1 × 3 cm) of DEAE-Sephacel equilibrated with 0.35 M NaCl in 0.05 M Tris-HCl, pH 8.0. The column was washed with 20 ml of 0.35 M NaCl in 0.05 M acetate buffer, pH 4.0, and eluted with a linear gradient (150 ml) from 0.35 to 1.2 M NaCl in acetate buffer, pH 4.0. Fractions of 2.0 ml were collected and 0.5-ml samples were analyzed for ^{3}H radioactivity (——) and ^{35}S radioactivity (– – –); conductivity (–·–·–).

[2] A degradation of hepatoma heparan sulfate proteoglycans was observed when culture media containing [^{35}S]proteoglycans were incubated at 37°C for long periods of time. It is therefore possible that the second peak represents a slightly degraded form of the native proteoglycans.

were mixed with heparan [^{35}S]sulfate proteoglycans purified from rat liver microsomes (Fig. 4*A*) or from cultured hepatocytes (Fig. 4*B*) and applied to a column of DEAE-Sephacel. The column was eluted with a linear gradient of NaCl. The hepatoma derived [^{3}H]heparan sulfate proteoglycans were eluted earlier and well separated from the rat liver [^{35}S]-proteoglycans regardless of whether these were labeled in vivo or in vitro, which demonstrates a difference in polyanionic properties between the two proteoglycan species.

Sulfate and uronic acid residues constitute the negatively charged groups in heparan sulfate, whereas glucosamine units carrying unsubstituted amino units represent potential positive charges. Since the density of uronic acid units in heparan sulfate is fixed, variations in polyanionic properties must reside in differences in the number of sulfate groups and/or exposed amino groups on glucosamine units.

The distribution of *N*-deacetylated glucosamine residues in the heparan sulfate chains was investigated by treating the ^{3}H-labeled samples with nitrous acid under conditions that cleave both *N*-sulfated and *N*-unsubstituted glucosamine units, whereas *N*-acetylated glucosamine units are left intact (see Materials and Methods). The deamination mixtures were fractionated on a column of Sephadex G-50. As shown in Fig. 5, nitrous acid deamination of ^{3}H-labeled heparan sulfate isolated from rat liver microsomes resulted in the conversion of the polysaccharide to fragments of low molecular weight. On the other hand, degradation of the hepatoma heparan sulfate with nitrous acid yielded, in addition to low molecular weight fragments, oligosaccharides of intermediate size. This suggests that there is a higher content of *N*-acetylated glucosamine units in the hepatoma heparan sulfate compared with normal liver heparan sulfate. Consequently, the hepatoma heparan sulfate must have a lower density of sulfamino groups compared with that of the polysaccharide isolated from normal liver.

Mechanism of Cell Association of the Hepatoma Heparan Sulfate Proteoglycan

A considerable amount (~20%) of the radiolabeled glycosaminoglycans remained associated with the cell layer after removal of the medium from ^{35}S-labeled hepatoma cultures.

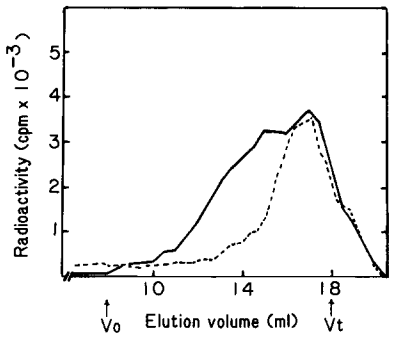

FIGURE 5 Gel chromatography of nitrous acid deaminated ^{3}H-labeled heparan sulfate proteoglycans. Samples of [^{3}H]heparan sulfate proteoglycans purified from rat liver (- - -) and from hepatoma cell culture medium (——) were subjected to the combined nitrous acid treatment described in Materials and Methods. The mixtures were then neutralized and applied to a column (62 × 0.6 cm) of Sephadex G-50 equilibrated with 1.0 M NaCl. The column was eluted with the same buffer at a flow rate of 3 ml/h. Fractions of 0.4 ml were collected and analyzed for ^{3}H radioactivity.

To isolate these cell-associated proteoglycans, the cells were solubilized in a buffer containing detergent and chromatographed on a column of DEAE-Sephacel. An asymmetrical peak of radioactivity was eluted at an ionic strength comparable to that required for the elution of the medium proteoglycans. The compositional analysis of the cell-associated glycosaminoglycans indicated that heparan sulfate was the predominant (71%) glycosaminoglycan (Table I).

SUSCEPTIBILITY TO TREATMENT WITH HEPARIN OR TRYPSIN: To analyze the mode of association of heparan sulfate proteoglycans with the hepatoma cell surface, cells were incubated with heparin to displace receptor-bound proteoglycan or with trypsin to release proteoglycans anchored in the membrane via their core proteins. Less than 5% of the cell-associated proteoglycans was released by heparin. It appears, therefore, that there are few or no heparan sulfate proteoglycans bound to polysaccharide receptors on the hepatoma cells. On the other hand, up to 45% of the ^{35}S-labeled glycosaminoglycans associated with the cell layer could be released by incubation of the cells at 37°C with 100 μg/ml of trypsin for 5 min. After this incubation period, the cells had rounded up and a large proportion had detached from the substratum. Samples of the trypsin-released material were subjected to chondroitinase ABC digestion and nitrous acid deamination, and the results indicated that ~80% of the ^{35}S radioactivity was associated with heparan sulfate.

HYDROPHOBIC CHROMATOGRAPHY ON OCTYL-SEPHAROSE: Radiolabeled heparan sulfate proteoglycans purified from the medium of cultured hepatoma cells, from detergent-solubilized cells, and from the material released from the cells by trypsin digestion were transferred to a buffer composed of 2% cholate in 0.02 M Tris-HCl, pH 7.3, and applied to columns of octyl-Sepharose. The columns were eluted in a stepwise manner with Tris-HCl buffers containing salt and/or detergents. The results are illustrated in Fig. 6. Most of the medium- and trypsin-released heparan sulfate proteoglycans (90 and 86%, respectively) were eluted from the octyl-Sepha-

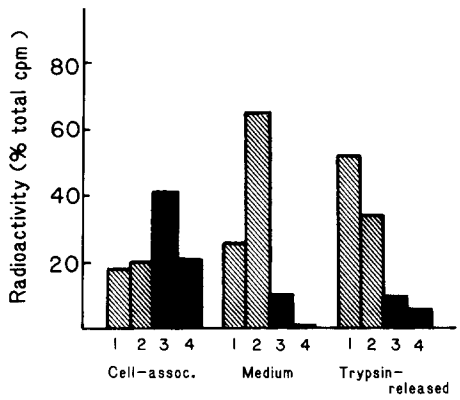

FIGURE 6 Hydrophobic chromatography of [^{3}H]heparan sulfate proteoglycans synthesized by hepatoma cells. Samples of [^{3}H]-heparan sulfate proteoglycans purified from the hepatoma cell culture medium, the cell layer, and released by mild trypsin treatment of the cells were applied to a column (3 × 1 cm) of octyl-Sepharose. The column was eluted in a stepwise manner with 25-ml aliquots of 0.02 M Tris-HCl buffers, pH 7.3, containing salt (striped bars: *1*, 0.1 M NaCl; *2*, 3.0 M NaCl) and then buffers supplemented with detergent (black bars: *3*, 3.0 M NaCl and 1% Triton X-100; *4*, 0.4% deoxycholate). The amount of ^{3}H radioactivity eluted is expressed as a percentage of the total radioactivity recovered from the column.

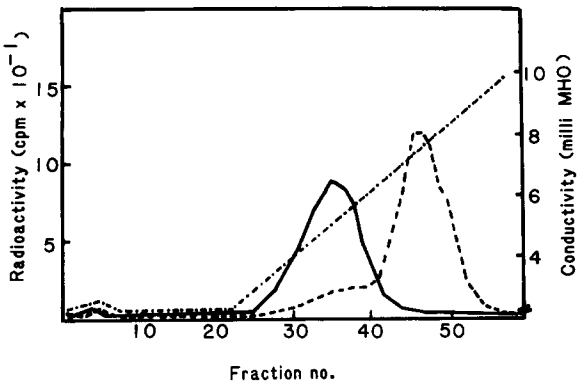

FIGURE 7 Affinity chromatography of labeled heparan sulfate proteoglycans on columns of fibronectin-Sepharose. A sample of [^{3}H]heparan sulfate proteoglycan purified from rat liver was mixed with a sample of heparan [^{35}S]sulfate proteoglycan purified from hepatoma cell culture medium and applied to a column (3 × 1 cm) of fibronectin-Sepharose equilibrated with 0.04 M CsCl in 0.01 M Tris-HCl, pH 7.5.[3] The column was washed with 20 ml of the same buffer and then eluted with a linear gradient of 0.04–0.5 M CsCl in 0.01 M Tris-HCl, pH 7.5. Fractions of 1.0 ml were collected and analyzed for ^{3}H radioactivity (- - -) and ^{35}S radioactivity (——); conductivity (-·-·-).

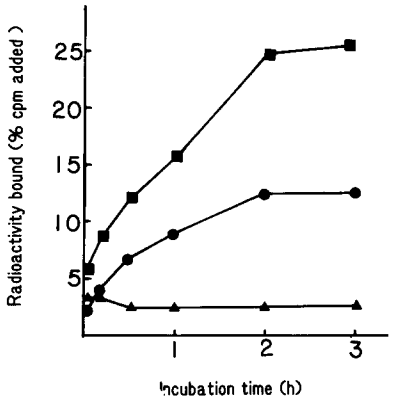

FIGURE 8 Binding of labeled glycosaminoglycans and hepatoma heparan sulfate proteoglycans to hepatoma cells. Hepatoma cells were suspended in 0.5 ml of medium and incubated at 37°C with [^{3}H]heparin (■, ~12,000 cpm), [^{3}H]heparan sulfate from bovine liver (○, ~17,000 cpm), or heparan [^{35}S]sulfate proteoglycan from hepatoma cell culture medium (▲, ~10,000 cpm) in a rotational shaking incubator. The mixtures were incubated for the indicated time periods and cells present in 0.2 ml of the suspensions were isolated by centrifugation through a Percoll gradient medium and assayed for associated radioactivity. The amount of radioactivity that bound to the cells is expressed as a percentage of the total radioactivity added to the cell suspension.

rose column in buffers that disrupt ionic but not hydrophobic interactions. In contrast, detergent was required to displace the major proportion (62%) of cell-associated heparan sulfate proteoglycan from the column. The finding that the detergent-solubilized but not the trypsin-released proteoglycans bound to the octyl-Sepharose by hydrophobic forces is consistent with the hypothesis (see reference 6) that the cell-associated heparan sulfate proteoglycans are anchored in the plasma membrane via a hydrophobic segment of the core protein that is cleaved off by trypsin.

Functional Differences between Heparan Sulfate Proteoglycans Isolated from Normal Rat Liver and from the Medium of Hepatoma Cultures

FIBRONECTIN INTERACTION: A sample of heparan [^{35}S]sulfate proteoglycans purified from hepatoma cell culture media was mixed with a sample of [^{3}H]heparan sulfate proteoglycans obtained from rat liver microsomes and applied to a column of fibronectin-Sepharose. All of the labeled material bound to the column and was eluted in a CsCl gradient (see Fig. 7). The ^{35}S-labeled hepatoma proteoglycan was eluted at a lower concentration of CsCl than the ^{3}H-labeled rat liver proteoglycan.[3] Heparan sulfate chains released from the core proteins by alkali/borohydride eluted from the fibronectin-Sepharose at the same salt concentration as their corresponding proteoglycans (data not shown). The observed difference in elution position between the proteoglycans from the two sources presumably reflects a difference in binding affinity.

BINDING TO HEPATOMA CELLS: Data described above indicate that the heparan sulfate associated with the surface of cultured hepatoma cells is not bound to the cells via a polysaccharide-receptor interaction. This observation may reflect an absence of functional receptors on the hepatoma cells and/or a structural defect in the heparan sulfate produced by the hepatoma cells (i.e., reduced sulfation).

To analyze these possibilities, hepatoma cells were incubated for various times in the presence of [^{3}H]heparin, [^{3}H]heparan sulfate isolated from bovine liver, and ^{35}S-labeled heparan sulfate proteoglycans purified from hepatoma culture media, respectively. The two ^{3}H-labeled glycosaminoglycans bound to the cells in a time-dependent manner, and maximal binding occurred after 2 h of incubation (see Fig. 8). In contrast, the hepatoma ^{35}S-labeled proteoglycan did not bind to the cells. To exclude the possibility that the lack of detectable cell binding of the hepatoma proteoglycan was a result of a low specific activity of the proteoglycans or was caused by the presence of an inhibitor in the hepatoma preparations which interfered with polysaccharide-receptor binding, cells were incubated with a mixture of ^{3}H-labeled heparan sulfate from bovine liver and heparan [^{35}S]sulfate proteoglycans derived from the medium of the hepatoma cell cultures. The presence of the hepatoma proteoglycan did not significantly affect the binding of the bovine liver [^{3}H]heparan sulfate to the cells. Furthermore, hepatoma heparan sulfate proteoglycans did not bind to isolated rat hepatocytes (L. Kjellén, unpublished observations). Hence, the hepatoma cells contain a receptor capable of binding polysaccharides but the heparan sulfate produced by these cells does not fulfill the structural requirements for receptor interaction, presumably because of its low sulfate content.

DISCUSSION

Previous studies (28) have shown that the rat liver heparan sulfate proteoglycan has a molecular weight of 80,000 and is composed of a core protein with a molecular weight of ~20,000 to which four polysaccharide chains are attached. In the present investigation we found that a heparan sulfate proteoglycan isolated from the medium of hepatoma cells cultures has essentially the same size (~95,000 compared with ~80,000) and that the polysaccharide chains isolated from rat

[3] The ionic strength of the buffer needed to elute [^{3}H]heparin from the fibronectin-Sepharose was the same whether the column was eluted with a gradient of CsCl or NaCl.

liver and hepatoma proteoglycans also are of similar size (~16,000 compared with ~19,000). It is therefore likely that the general structure of the hepatoma proteoglycan conforms to the model proposed for the rat liver proteoglycan. Previous studies (34) of a heparan sulfate proteoglycan isolated from an ascites hepatoma indicated that this proteoglycan contained three to four polysaccharide chains attached to the core protein.

The heparan sulfate synthesized by transformed cells generally has a reduced sulfate content compared with the heparan sulfate produced by the untransformed counterparts. Studies on cultured mouse 3T3 cells and their SV40 transformants indicate a transformation-dependent change in the charge density of the heparan sulfate synthesized by these cells (14, 15), which correlates with expression of the SV40-specific T-antigen (16). Similarly, Nakamura et al. (35) have shown that the AH-130 hepatoma cells produce large quantities of a heparan sulfate with a low sulfate content. These observations are confirmed in the present study, in which cultured rat hepatoma cells are shown to synthesize a heparan sulfate that has a markedly reduced sulfate content compared with that isolated from normal rat livers or from cultured hepatocytes.

Attempts were made to identify the cause of the reduced sulfation in the hepatoma heparan sulfate. The hepatoma polysaccharide was found to contain an increased number of *N*-acetyl groups and a decreased number of *N*-sulfate units compared with rat liver heparan sulfate. The first stage in the biosynthesis of heparan sulfate-like polysaccharides is the formation of a polymer composed of alternating units of *N*-acetyl glucosamine and glucuronic acids (30 and references therein). Subsequent to, or along with, the formation of the carbohydrate backbone, the polysaccharide is subjected to a series of discrete polymer modification reactions initiated by an *N*-deacetylation of *N*-acetyl glucosamine units and followed by *N*-sulfation, glucuronic acid epimerization, and *O*-sulfation of hexosamine and iduronic acid units. In the hepatoma cells the polymer formation reactions appear to proceed normally but the *N*-deacetylase does not seem to operate as efficiently as in the hepatocyte. The *N*-deacetylase has tentatively been identified as a regulatory enzyme (30) and the extent of initial *N*-deacetylation appears to control the overall polymer modification. Impaired *N*-deacetylation in the biosynthesis of heparan sulfate in the hepatoma cells could therefore result in a low sulfate content of the final product.

The reduced sulfate content of the hepatoma heparan sulfate may affect the interactions of the proteoglycan with other molecules. The distribution of the heparan sulfate proteoglycans in the hepatoma cell layer appears to be restricted to the cells; no extracellular matrix is formed, as indicated by immunofluorescent staining. Previous studies have shown that highly sulfated heparan sulfate-like polysaccharides enhance the rate of binding of fibronectin to collagen (36) and stabilize the fibronectin-collagen complex (9). In the present study the hepatoma heparan sulfate was shown by affinity chromatography to have a markedly lower apparent affinity for fibronectin compared with heparan sulfate from normal rat liver. It therefore appears possible that heparan sulfate plays a role in the process of matrix formation and that successful matrix formation depends on a strong heparan sulfate-fibronectin interaction. However, the role of heparan sulfate in matrix formation remains speculative since it cannot be excluded that other matrix components produced by the hepatoma cells may also be defective.

A number of studies have implicated glycosaminoglycans in the regulation of cell proliferation (17–20). In particular, mucopolysaccharides and proteoglycans isolated from normal liver have been shown to reduce the rate of proliferation of cultured hepatoma cells (21, 37). In the present investigation heparan sulfate proteoglycans isolated from rat liver microsomes were found to bind directly to a component on the hepatoma cell. This interaction resembles that between heparan sulfate and specific polysaccharide receptors present on the surface of hepatocytes, which has previously been characterized in some detail (5, 38). Since the heparan sulfate proteoglycan produced by the hepatoma cells was unable to bind to the cell surface receptors, presumably because of its low sulfate content, it is tempting to suggest that the observed inhibitory effect of normal heparan sulfate on the proliferation of hepatoma cells involves the polysaccharide-receptor interaction.

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