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Altered Expression and Distribution of Heparan Sulfate Proteoglycans in Human Gliomas[1]

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ABSTRACT

The expression of heparan sulfate proteoglycans (HSPGs) by human glioma cells was examined by biochemical and immunological methods *in vitro* and *in vivo*. Chondroitin sulfate was shown to represent the major [^{3}H]glucosamine-labeled glycosaminoglycan synthesized by cultured normal brain cells. However, high-grade glioma-derived cells were shown to express significantly increased quantities of hyaluronic acid and heparan sulfate and approximately equal amounts of chondroitin sulfate compared with normal glial cells. To investigate further the differential expression of HSPGs, proteoglycans were isolated from glioma cells and were used as an immunogen to generate monoclonal antibodies (MAbs). One of these MAbs, 39H (an IgM), was shown to bind more to high-grade glioma-derived cells then to low-grade glioma or normal brain cells *in vitro*. MAb 39H was also observed to bind to isolated HSPGs but not to heparan sulfate glycosaminoglycan chains or trypsin-treated cells. Immunofluorescence staining of the cultured high-grade glioma cells revealed an intense diffuse cell surface staining pattern over the entire cell and also isolated footpads. In contrast, the low-grade tumor or normal glial cells showed a distinctive punctated staining. A similar differential staining of MAb 39H was most prominent between tissue sections of glioblastoma multiforme and anaplastic astrocytomas *versus* low-grade astrocytomas and normal brain. The low grade gliomas exhibited a weak punctated staining, whereas the high-grade gliomas showed significantly more intense staining, particularly along the apical regions of the cells. These results suggest that altered expression of HSPGs may be related to the malignant transformation or growth potential of glial-derived cells.

INTRODUCTION

The ECM[3] has been observed to modulate the biological responses of a number of cell types through a diverse series of effects that has been shown to include cellular proliferation, migration, and differentiation (1–3). An ECM constituent that has been implicated as playing important roles in some of these biological processes is HSPGs, primarily due to their proposed adhesive properties (4, 5). HSPGs were diverse structures consisting of protein cores and covalently linked heparan sulfate GAG chains that allow multiple binding interactions with cells (6, 7) and other ECM components including fibronectin, collagen, and laminin (8–11). The multifunctional adhesive properties of HSPGs suggest that they may have important roles in tumor cell adhesion to its ECM and in tumor cell invasion (12, 13).

A number of investigations have focused on quantitative and qualitative modulation of GAGs and PGs in tumor cells compared with their normal cell counterparts (14). Some observed differences could be related to cell growth (15, 16); however, cellular transformation has been shown to affect GAG synthesis (17–19). For instance, it has been shown that the degree of sulfation of HS in tumorigenic cell lines or cell clones is decreased from that in their parental cells (20, 21). Furthermore, the degree of sulfation of the HS chains may have biological significance as the undersulfated HSPGs had a lower binding affinity for fibronectin or to hepatocytes as compared to the parental cells' HSPGs (20). However, the diversity of alterations in PG expression appears dependent, at least in part, on the particular cell type under investigation.

Altered expression of GAGs has been observed between primary brain tumor cells and normal brain; however, consistent differences have not been demonstrated, particularly for *in vitro* grown cells (22, 23). The majority of studies have focused on the expression of CSPGs and HSPGs expressed by normal brain (24, 25). Recent *in vivo* studies by Bertolotto and coworkers (26–28) suggest that the expression of CSPGs decreases as the histological grade of gliomas increases, thus showing increased malignant characteristics. Our recent investigations have focused on HSPGs expressed by glial-derived cells. In this report, we describe the use of a combination of biochemical and immunological methods to examine the expression and distribution of HSPGs by glial-derived cells *in vitro* and *in vivo*.

MATERIALS AND METHODS

Cells and Cell Culture. The human glial (MM), high-grade glioma-derived cells (EFC-2, LG, KE), low-grade glioma-derived cells (PL-1, NG-1), and medulloblastoma cells (RB2, DF) were obtained from surgical specimens of primary brain tumors of different histological grades or from normal brain tissue of patients with unrelated trauma as described (29, 30). The cells were characterized for the presence of glial acidic fibrillary protein, karyotype, and tumorigenicity in nude mice (*nu/nu*, female, 25 to 40 days; Harlan, TX). Normal rat brain cells enriched for type 1 astrocytes (RA) were obtained as previously described (31), and the rat glioma cells (C6) were obtained from Dr. D. Bigner (Duke University, Durham, NC). Cells were routinely grown in a mixture (1:1) of DME/Ham's F12 medium containing 10% FBS (Hyclone Lab, Logan, UT). The majority of cells were used at low *in vitro* passages (<20). The mouse myeloma SP2/0 (kindly provided by Dr. C. Reading, M. D. Anderson Cancer Center) was maintained in high glucose DME, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, plus 10% FBS. All cells are routinely assayed and found to be free of *Mycoplasma* contamination.

The cells were metabolically radiolabeled by addition of [^{3}H]glucosamine (10 μCi/ml), [^{35}S]sulfate (50 μCi/ml), or [^{3}H]serine (20 μCi/ml; ICN Pharmaceuticals, Irvine, CA) to the cell cultures for 24 h containing 5% dialyzed FBS. For the latter two radioactive precursors, the culture medium was depleted to one-tenth the usual concentration of serine or $MgSO_4$.

Preparation and Characterization of Proteoglycan Fractions. Cell-associated PGs from cultured cells or from tumor pieces from human glioma cells (32) were extracted in 4 M GdnHCl:4% Zwittergent 3–12:0.1 M sodium acetate, pH 6.0, in the presence of protease inhibitors

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[3] The abbreviations used are: ECM, extracellular matrix; HSPG, heparan sulfate proteoglycan; GAG, glycosaminoglycan; PG, proteoglycan; CSPG, chondroitin sulfate proteoglycans; DME, Dulbecco's modified minimal essential medium; FBS, fetal bovine serum; GdnHCl, guanidine hydrochloride; MAb, monoclonal antibody; SDS, sodium dodecyl sulfate; TPBS, phosphate-buffered saline containing 0.05% Tween 20; BSA, bovine serum albumin; PBS, phosphate-buffered saline; HS, heparan sulfate.

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(0.1 M 6-aminohexanoic acid, 10 mM EDTA, 5 mM *N*-ethylmaleimide, 5 mM phenylmethylsulfonyl fluoride, 10 milliunits/ml of aprotinin, and 1 μg/ml of leupeptin) (32, 33). The extracts were passed through Sephadex G-50 equilibrated in 4 M GdnHCl and 0.1 M sodium acetate, pH 6.0. The void volume fractions were pooled and made to a final density of 1.47 g/ml by addition of solid CsCl (0.55 g/g of solution). Dissociative density gradients (4 M GdnHCl in 4.3 M CsCl) were formed by centrifugation at 150,000 × *g* for 60 h in a Beckman Ti 50.2 rotor at 9°C. The gradients were then fractionated into 10 equal volume fractions, and the density and radioactivity of each fraction were determined. The density fractions were subjected to gel filtration chromatography on Sepharose CL-4B columns (0.8 x 100 cm) in 4 M GdnHCl, 0.1% 3-3-cholamidopropyldimethylammonio-1-propane sulfonate (CHAPS), 50 mM Na_2SO_4, and 0.1 M sodium acetate, pH 5.8. The different PG classes were pooled, dialyzed extensively, and lyophilized. For certain preparations, the PG samples were further analyzed by ion exchange chromatography on DEAE-Sephacel in 8 M urea, 0.5% CHAPS, 20 mM NaCl, and 50 mM sodium acetate, pH 6.0 (34).

The PGs released into culture medium were fractionated by DEAE-Sephacel ion exchange chromatography (20) after addition of protease inhibitors (5 mM phenylmethylsulfonyl fluoride, 5 mM *N*-ethylmaleimide, 10 milliunits/ml of aprotinin). The various PG fractions were pooled, diluted, concentrated on a small DEAE-Sephacel column (34), and then fractionated by size on a Sepharose CL-4B column as described above.

The biochemical properties of PGs were investigated by various treatments. The GAGs from PGs or various cellular fractions were liberated by treatment with Pronase (1 mg/ml) at 37°C for 24 h, followed by identification by ion exchange chromatography or cellulose acetate electrophoresis and by sensitivity to chondroitinase ABC, chondroitinase AC, or nitrous acid as previously described (21, 35, 36). The treatment of the intact proteoglycan fractions with heparitinase or chondroitinase ABC (ICN Biochemicals, Lisle, IL) was performed in the presence of protease inhibitors as described (37) with no observed protein degradation. Also, the various GAGs were characterized further as described (35). Gel filtration chromatography of the isolated GAGs was performed on calibrated Sephacyl S-300 or S-1000 (1 x 100 cm) columns in 0.15 M sodium chloride, 50 mM Tris-HCl, pH 7.4, and 5 mM EDTA.

Preparation of Monoclonal Antibodies. Isolated individual PG fractions (100 μg) prepared as described above were injected i.p. into BALB/c mice with an equal volume of Freund's complete adjuvant. Each animal received 2 additional identical injections, at 2-wk intervals, except that Freund's incomplete adjuvant was used. For hybridoma production, a single cell suspension of mouse spleen cells was mixed with the mouse myeloma cells SP-2/0 at a ratio of 2:1, pelleted, and resuspended in the presence of 50% Polyethylene Glycol 4000 (38). The hybrids were then grown in DME/Ham's F12 medium containing 20% FBS and hypoxanthine, aminopterin, and thymidine (10^{-9} M, 4×10^{-7} M, 1.6×10^{-5} M, respectively) as described (39). Hybridoma supernatants were tested for the production of specific MAbs by use of an enzyme-linked immunosorbent assay against a panel of target cells grown 24 h in 96-well microtest plates. Fixed cells (0.5% glutaraldehyde) were incubated for 1 h at room temperature with 100 μl of hybridoma supernatant, followed by 3 washes with biotinylated anti-mouse immunoglobulin (heavy + light) (Vector Laboratories). The bound antibody was quantitated after 3 washes by incubation with avidin-peroxidase complex for 0.5 h, 3 additional washes, and development with *O*-phenylenediamine (1 mg/ml) in 0.1 M citrate buffer, pH 4.5, containing 0.012% hydrogen peroxide. After 15 min, the absorbance was determined at 450 nm by a Titertek Multiscanner. Hybridoma production was considered positive if their supernatants had an absorbance over twice the standard deviation above the background. Selected hybridomas were cloned by limiting dilution. The antibody isotype was determined by an enzyme-linked immunosorbent assay using anti-mouse IgG, IgG2a, IgG2b, IgG2c, IgA, and IgM according to the manufacturer's instructions (Bio-Rad, Richmond, CA).

Immunotechniques. Selected MAbs were assayed for their reactivity against PG fractions by 2 assays. The first method was a solid-phase radioimmunoassay (9). Briefly, IgG goat/anti-mouse IgM (Cappel; 0.1 ml) was incubated overnight at 4°C in each well of a 96-well Immunolon II plate. The wells were washed 3 times with TPBS containing 1% BSA (TPBS-BSA) followed by addition of culture supernatant or control antibody preparations in TPBS-BSA (0.1 ml). After an 8-h incubation at room temperature, metabolically ^{35}S-labeled PG fractions or chemically iodinated ^{125}I-labeled fractions (40) in TPBS-BSA (0.1 ml) were added to the individual wells. If the individual PG fractions were subjected to enzymatic treatments, they were incubated for 1 h at 37°C followed by boiling for 5 min before addition to the wells. The GAGs were administered at the same time as the PG fractions to the various wells. The plates were incubated overnight at 4°C followed by four washings with TPBS-BSA, and the radioactivity remaining in each well was determined after solubilization in 1% SDS, 50 mM NaOH, and 0.2% mercaptoethanol. All assays conditions were performed using quadruplicate wells.

Alternatively, immunoblotting with the various PG fractions was performed. The isolated PG fractions were subjected to electrophoresis in 2% agarose in 0.1% SDS, 0.1 M Tris base, 0.1 M borate, and 5 mM EDTA, pH 8.2, and then they were electrophoretically transferred to nitrocellulose paper (0.1 μm) at 40 V for 5 h. The immunoblot was incubated overnight in TPBS, 3% BSA, and 0.1% goat serum, followed by a 3-h incubation with the hybridoma supernatant. The membrane was washed 3 times with TPBS, and then biotinylated anti-mouse IgM was added in TPBS, for 1 h, followed by 3 additional washes and addition of Vectastain ABC complex. The antigen-antibody complex was then visualized by incubation of substrate according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA).

Immunostaining of human cells cultured on coverslips or cryostat sections of human surgical specimens (obtained from the Department of Pathology, M. D. Anderson Cancer Center) were performed by similar methods. The coverslips were washed with PBS, fixed with 4% formaldehyde for 30 min, and then washed again with PBS. To permeabilize the cells for internal staining, the after fixation sections were dipped sequentially in acetone H_2O (1:1) at 4°C for 2 min, acetone at −20°C for 5 min, and acetone:H_2O (1:1) for 2 min, drained, and then washed with PBS. Appropriately diluted antibody culture supernatant or normal mouse serum was applied to the various coverslips for 3 h at 37°C. Cryostat sections were incubated overnight. The slides were washed 6 times (5 min), and the fluorescein isothiocyanate-conjugated goat anti-mouse IgM (1:30) in PBS was applied for 1 h at 37°C. The slides were washed an additional 6 times, mounted, and viewed with epifluorescence on a Nikon Optiphot microscope. To semiquantitate the intensity of fluorescence of the various sections, the required exposure time as determined by an automatic Microflex UFX photomicrograph attachment was recorded. The relative intensity was determined dividing nonspecific control section exposure time by the 39H section exposure time (quotients less than 2 = +; 2–3 = ++; 3–4 = +++; and greater than 4 = ++++).

RESULTS

Expression of Glycosaminoglycans. The composition of [^3H]-glucosamine-labeled, protease-resistant, macromolecular material from various cellular fractions of glial and glioma cells was analyzed by an ion exchange chromatography and cellulose acetate electrophoresis. The synthesized GAGs were identified by their migration compared with standard GAGs and by their sensitivities to various GAG-degradative enzymes and were identified as glycopeptides HA, HS, and CS. The glioma cells (EFC-2) were observed to express significantly increased quantities of the total amount of GAGs in both the medium and cell surface fractions (167.9 and 6.9 ng of uronic acid/μg of protein) compared with their normal counterparts (MM) (78.5 and 4.5 ng of uronic acid/μg of protein, respectively) in culture (Table 1). The increase was particularly significant for HA and HS, where EFC-2 cells displayed approximately 3 and 4 times more of each GAG, respectively, than did MM cells. In contrast, MM cells were observed to express significantly more high-

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Table 1 *Composition of [³H]glucosamine-labeled glycosaminoglycans in various cellular fractions*

Cell line (cellular fraction)	cpm/μg protein		ng uronic acid/μg protein	
	Glycopeptides	Hyaluronic acid	Heparan sulfate	Chondroitin sulfate
Medium				
MM	1046[a]	99 (13.4)[b]	83 (19.0)	450 (46.1)
EFC-2	348	243 (40.3)	664 (77.4)	451 (50.2)
Cell surface				
MM	515	3 (0.4)	5 (1.0)	28 (3.1)
EFC-2	146	8 (1.0)	25 (2.9)	25 (3.0)

[a] [³H]Glucosamine incorporated in macromolecular material. All cells were labeled simultaneously and processed at the same time under identical conditions. The individual labeled components were determined by ion exchange columns. Recoveries of columns averaged 87% (79 to 96%).

[b] The specific activities of incorporation of [³H]glucosamine into GAGs were assayed by the method of Bitter and Muir (57). The conversion factor between hyaluronic acid and glucuronolactone was determined to be 2.3. The total protein in the cellular extracts of the different cells was determined by the method of Lowry *et al.* (58).

molecular-weight glycopeptides, but both cell lines displayed about equal quantities of CS.

Structural analyses were performed on the various GAGs. The distribution of *N*-sulfation of glucosamine residues on HS, as determined by treatment with nitrous acid and gel filtration chromatography on Bio Gel P10, was similar for HS derived from EFC-2 or MM cells (data not shown). Also, the CS disaccharides resulting from treatment of CS with chondroitinase ABC were shown to contain about an equal mixture of chondroitin 4-sulfate and chondroitin 6-sulfate for both cell lines as determined by quantitation of ³H-labeled disaccharides after separation by their layer chromatography (41). Furthermore, the sizes of the various GAGs were examined by gel filtration chromatography on a Sephacryl S-1000 column for HA and a Sephacryl S-300 column for HS and CS. The relative molecular weights of the sulfated GAGs were similar for the various cells (22,000 ± 5,000 for HS and 48,000 ± 8,000 for CS). In contrast, the HA obtained from EFC-2 cells was significantly smaller than that from MM cells (K^{av}, 0.50 *versus* 0.32, respectively).

Isolation and Characterization of PG. Cell-associated PGs from EFC-2 cells were fractionated by dissociative isopyknic centrifugation and gel filtration chromatography on Sepharose CL-4B columns (Fig. 1). Two major radioactive and protein peaks were observed in the column profiles for the D1 through D4 density fractions, one near the void volume of the column and another included further into the column (K^{av}, 0.65). Both peaks could be further degraded by protease treatment. The low density D5 fraction contained the majority of proteinaceous material (>90%) and one major sulfated peak consisting of mainly HS and CS GAG chains as assessed by their similar elution on gel filtration columns with or without Pronase treatment. Further purification of the PGs, in some cases, was accomplished by ion exchange chromatography on DEAE-Sephacel columns, although relatively homogeneous preparations of the PG were obtained from the CL-4B columns. Analysis of the PG peaks (D1 to D4) indicated the larger molecular weight fractions contained mainly CS GAG chains, whereas the included fractions consisted predominantly of HS chains (Table 2). Henceforth, the V_0 eluting peak will be referred to as CSPG and the latter peak will be HSPG fractions. Similar fractionation of PGs was obtained from normal glial-derived ells (MM); however, the amount of high density (D1 to D3) HSPG was decreased.

Generation and Screening of Monoclonal Antibodies. Isolated HSPG or CSPG fractions (D1 to D4) from EFC-2 cells were used as an immunogen to generate a panel of MAbs that reacted against cultured human normal and tumor cells. The several hybrids were generated from each PG fraction and were subsequently further subcloned, expanded, and then evaluated. The reactivity of a MAb, 39H, produced against HSPG fractions is shown in Fig. 2. MAb 39H was observed to react to a significantly (2 SDs) greater extent to glioma cells derived from high-grade tumors than to glial-derived cells from low-grade tumors or normal brain of human or rat origin (Fig. 2*a*). Furthermore,

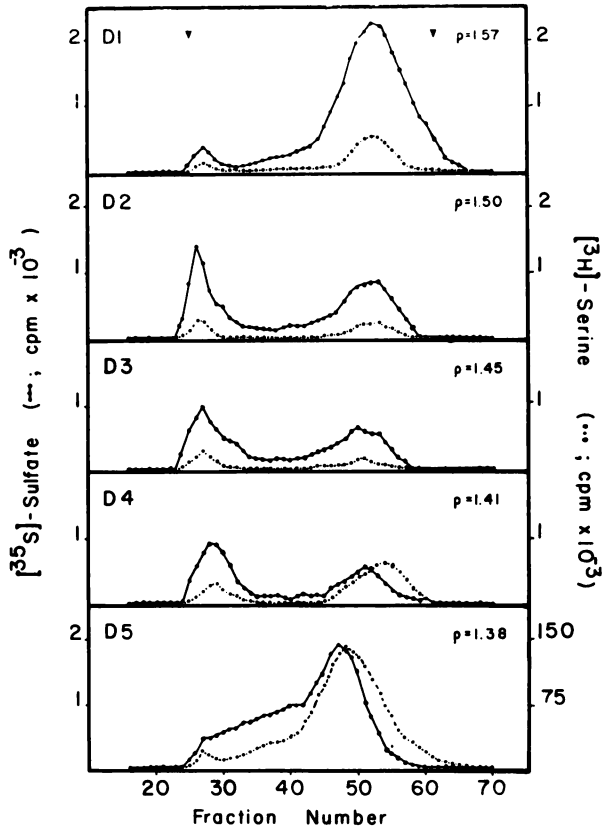

Fig. 1. Gel filtration profiles of macromolecules metabolically radioactively labeled with [³H]serine or [³⁵S]-SO_4^{-2} from EFC-2 cells after dissociative CsCl density gradient ultracentrifugation. The cellular extracts were fractionated by centrifugation in 4 M GdnHCl and 4.3 M CsCl, and the resulting density fractions (denoted by *P* values) were subjected to Sepharose CL-4B column chromatography. The *arrows* represent the void volume (*left*) and total volume (*right*) elution fraction of the column.

Table 2 *Composition of [³⁵S]sulfate-labeled cell-associated PG fractions from EFC-2 cells*

Density fraction (CL-2B elution)	Density	Composition[a]	
		CS[b] (%)	HS (%)
D1 (24–31)	1.57	95[c]	5
D1 (44–62)	1.57	5	95
D2 (23–32)	1.50	95	5
D2 (43–60)	1.50	5	95
D3 (23–34)	1.45	95	5
D3 (41–56)	1.45	5	95
D4 (24–34)	1.41	80	20
D4 (43–57)	1.41	10	90
D5 (23–34)	1.38	70	30
D5 (41–57)	1.38	20	80

[a] The composition of ³⁵S-labeled GAGs from the various PG fractions was determined by cellulose acetate electrophoresis as described in "Materials and Methods." The SE of each determination was ± 5%.

[b] CS, chondroitin sulfate.

[c] The recoveries ranged from 79 to 96% for individual assays.

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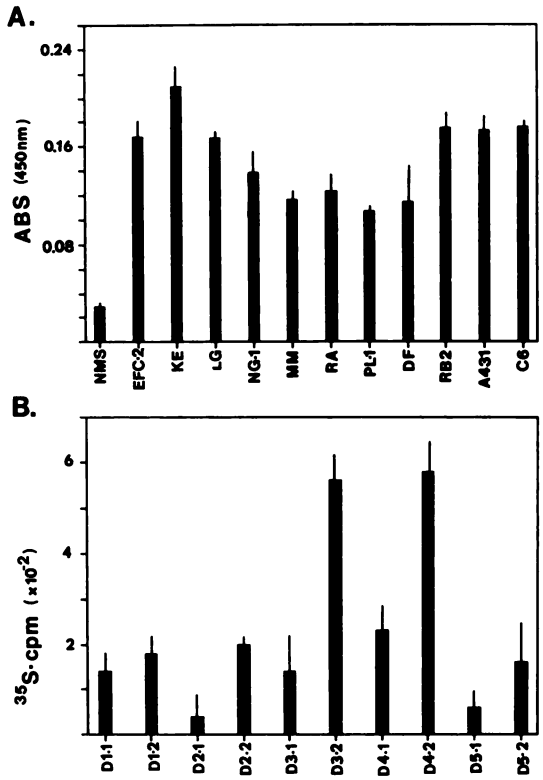

Fig. 2. The binding of MAb 39H to glutaraldhyde-fixed cell lines (*A*) and to various macromolecular pools from the gel filtration of the density gradient fractions as depicted in Fig. 1 (*B*). Antibody culture supernatant (10 μg/ml) was placed for 1 h on the fixed monolayer culture of the various cells. The binding of 39H was determined by enzyme-linked immunosorbent assay. The origin of the various cells is described in "Materials and Methods." Alternatively (*B*), the binding of the various ^{35}S-labeled macromolecular fractions was assessed by a solid-phase radioimmunoassay as described in the text. The designations of the various macromolecular pools are *D1* through *D5* for the different density fractions followed by *1* or *2* for gel filtration Fractions 24–35 and 43–55, respectively, from the Sepharose CL-4B columns (see Fig. 1). *Bars*, SE.

most cultured cells showed significant binding of MAb 39H, particularly against established human cell lines obtained from cervical and colon carcinomas and melanoma, but not against normal fibroblasts or mammary carcinoma cells. Since MAb 39H bound differentially to cells derived from glioblastomas and to those from low-grade tumors or normal brain, most of our subsequent efforts were focused on this MAb. MAb 39H was shown to be an IgM.

The MAb was assayed for its specificity against various PG fractions from EFC-2 cells by several independent methods. Using a solid-phase immunoassay, in which the various PG fractions were incubated with 39H previously immobilized on microtiter plates (Fig. 2*b*), MAb 39H was observed to predominantly bind to isolated HSPG preparations in the D3 and D4 fractions. Alternatively, the PG fractions were immobilized on poly-L-lysine-precoated microtiter plates, followed by incubation with hybridoma culture supernatants. After washing, bound MAb was detected by addition of ^{125}I-anti-mouse IgM and was compared to wells incubated in the presence of normal mouse serum or another mouse IgM MAb (4100 ± 436 *versus* 1860 ± 306, 1700 ± 260, respectively, for binding to HSPG fractions, and 1990 ± 482, 1650 ± 281, and 1870 ± 327 for CSPG fractions). The results are similar to those from the solid-phase immunoassay. To further characterize the antigenic determinants, several variations of solid phase immunoassay were performed. First, the binding of ^{35}S-labeled PGs was not inhibited by the addition of cold CS or HS; however, no binding was observed if the PG fractions were preincubated with protease (Table 3). These results suggest that the MAbs were direct against the PG protein cores and not GAG chains. Also, treatment of HSPG fractions with chondroitinase ABC (0.1 unit) in the presence of protease inhibitors did not affect the binding of ^{35}S-labeled material to MAb 39H. In contrast, binding was not observed for heparatinase treatments of ^{35}S-labeled HSPG to MAb 39H, indicating that the MAbs were not binding to contaminating PGs in the various PG fractions. Furthermore, the binding of ^{125}I-labeled HSPG fractions was not affected by either glycolytic treatment but was inhibited by protease treatment (Table 3).

The reactivity of MAb 39H was also demonstrated to ion exchange-purified PG fractions that had been subjected to electrophoretic analysis in agarose gels and then transferred to nitrocellulose paper. The immunostaining with MAb 39H recognized M_r ~220,000 to 150,000 antigens from the HSPG-

Table 3 *Binding of various PG fractions to MAb 39H in a solid-phase radioimmunoassay*

Treatments[a]	cpm bound[b]			
	^{35}S-CSPG	^{35}S-HSPG	^{125}I-CSPG	^{125}I-HSPG
None	90 (11)[c]	764 (100)	250 (5)	5130 (100)
+HS (1 mg/ml)	110 (15)	805 (105)	340 (7)	5650 (71)
+CS[d] (1 mg/ml)	110 (15)	796 (104)	370 (7)	3700 (72)
+Chondroitinase ABC (0.1 unit)	60 (8)	840 (109)	400 (8)	4270 (83)
+Heparanitinase (50 microunits)	80 (10)	160 (20)	300 (6)	4830 (94)
+Trypsin (100 μg/ml)	90 (12)	70 (9)	300 (6)	1260 (24)

[a] The solid-phase radioimmunoassay and the various incubations with the individual GAGs or degradative enzymes were performed as described in "Materials and Methods."
[b] The bound radioactivity was determined after solubilization from the wells with 1% SDS, 50 mM NaOH, and 0.2% β-mercaptoethanol and subjected to analysis with a scintillation or gamma counter. All assays were performed in quadruplicate, and the SDs averaged 12% (range, 3 to 27%).
[c] Numbers in parentheses, percentage of control; the binding of radioactively labeled HSPG to 39H with no treatment was assigned a value of 100% binding.
[d] CS, chondroitin sulfate.

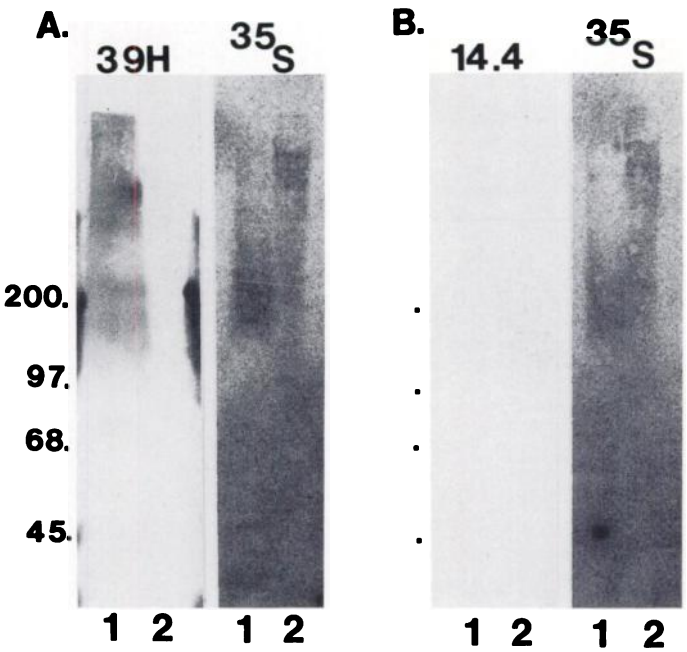

Fig. 3. Immunostaining of ^{35}S-labeled macromolecular fractions after separation in 3% agarose with MAb 39H. *A*, immunostaining of MAb 39H and autoradiography of ^{35}S-labeled HSPG and CSPG fractions (*Lanes 1* and *2*, respectively). *B*, same conditions as previously described except a mouse IgM MAb (1.14.4) generated *2* against a cell surface antigen of human mammary carcinoma. The prestained molecular weight markers (×10^{-3}, *left*) and immunostaining were differentiated by their blue and red coloring, respectively.

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isolated fractions. A similar distribution for ^{35}S-labeled material was observed as for the immunostaining (Fig. 3). No significant binding of MAb 39H was observed to CSPG fractions or if whole cell lysates were electrophoresed and immunoblotted. Similarly, no binding to either HSPG or CSPG fractions was seen utilizing an unrelated mouse IgM.

Cellular Localization of Antigens. The distribution of the antigens recognized by MAb 39H was examined by indirect immunofluorescence on cultured cells and frozen sections of human surgical specimens. The immunostaining of the *in vitro* grown cells was performed with and without acetone permeabilization representing internal and cell surface staining, respectively. The normal glial and low-grade glioma cells revealed a punctated staining, particularly around the nucleus and apical regions, for cell surface reactivity of MAb 39H (Fig. 4). The internal staining of these cells also showed reactivity along fibril structures. In contrast, cells derived from high-grade gliomas exhibited a more intense and homogeneous cell surface immunostaining that at higher magnification was shown to be a result of numerous punctated areas. For internal staining, punctated and fibril immunoreactive structures were still observed (Fig. 4). Furthermore, a similar differential staining was observed between cells derived from normal fetal rat brain enriched for type 1 astrocytes (RA) (31) and rat C6 glioma cells (see Fig. 2*a*).

Examination of the reactivity of MAb 39H on cryostat sections also revealed a differential staining between normal tissue and low-grade gliomas and those from high-grade gliomas (Fig. 5). Sections from normal brain and low-grade tumors exhibited a low level of punctate staining. In contrast, sections of glioblastomas showed significantly more intense and apical staining of the cells. The nuclear regions were generally devoid of reactivity; however, disorganized cell surface fibril areas were apparent (Fig. 5). Cross-reactivity with basal lamina of endothelial cells was observed with MAb 39H. In addition, several other tumor types were also examined (Table 4). In general, the hypercellular regions of the tumor sections exhibited the most intense staining, but no staining was observed with breast carcinoma brain metastases.

DISCUSSION

Heparan sulfate proteoglycans have been observed to reside predominantly on the cell surface and in the basal lamina of numerous cell types. A number of recent studies have demonstrated that HSPGs play important biological roles in several cellular activities including permeability of basement membranes, modulation of blood coagulation, and cellular adhesion, migration, and growth regulation (4, 42). Furthermore, several independent species of HSPGs have been identified according to differences in protein cores (43) and GAG chains (5), thus, suggesting that this class of macromolecules may have a wide range of possible functional roles. A number of studies have demonstrated alterations in GAGs and PGs during transformation; however, consistent results have been shown in only a few systems (14, 22, 23). We have used a combination of

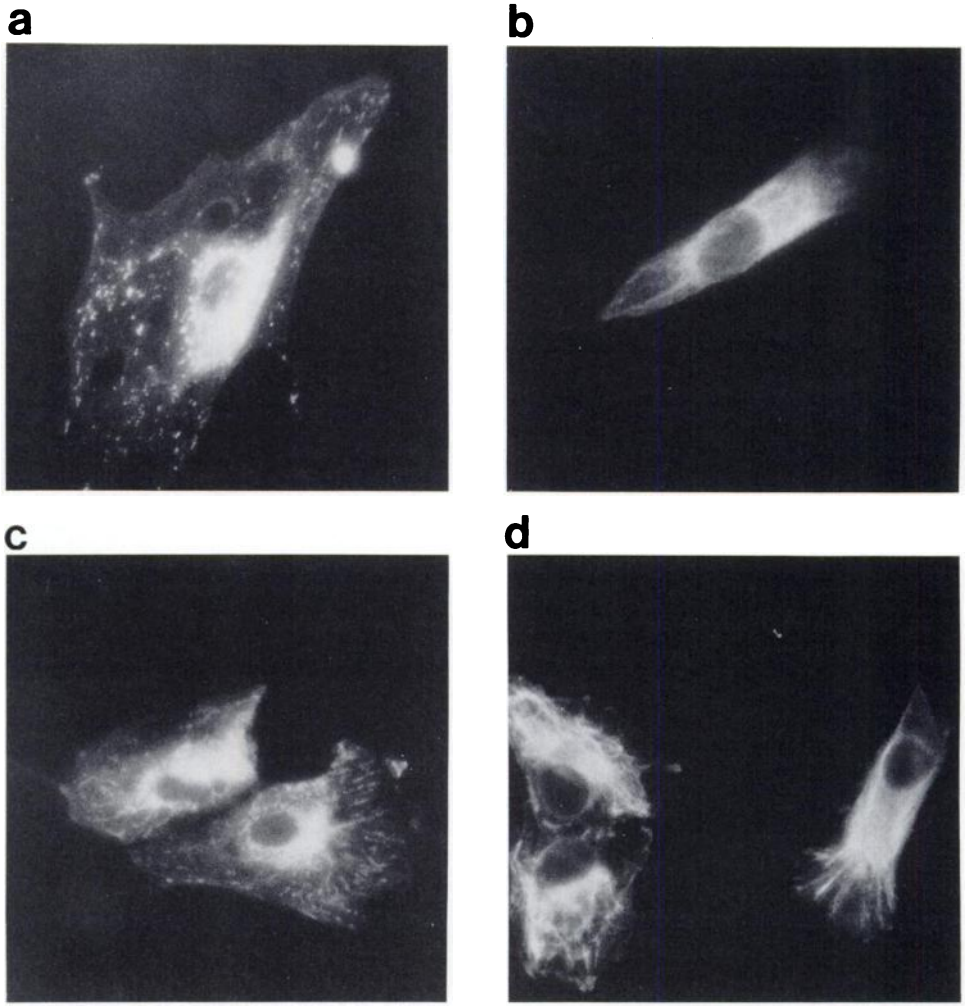

Fig. 4. Immunofluorescence staining with MAb 39H of cultured low-grade tumor-derived glioma cells, PL-1 (*a* and *c*), and high-grade glioma cells, EFC-2 (*b* and d). The cell surface reactivity is depicted in *a* and *b*, and the acetone-permeabilized cells are shown in *c* and *d*. The MAb was incubated with the cells for 3 h, and reactivity was detected with fluorescein isothiocyanate-anti-mouse IgM. Control Mab was a nonspecific IgM or normal mouse serum, and neither revealed any reactivity. × 150.

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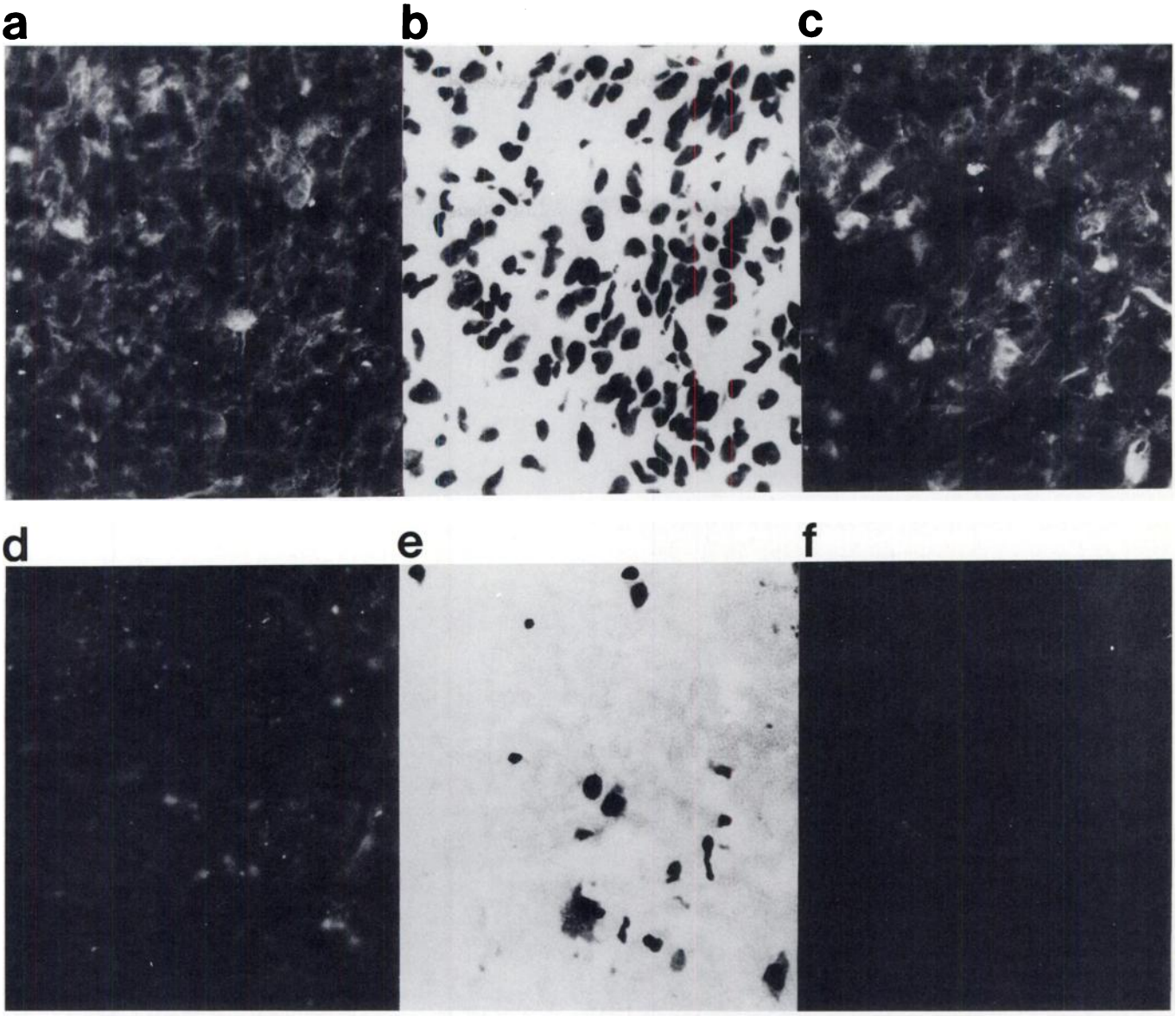

Fig. 5. Reactivity of MAb 39H as detected by immunofluorescence (*a*, *c*, *d*, and *f*) on frozen tissue sections of glioblastoma multiforme (*a*, *b*, *c* and *f*) and low-grade glioma (*d* and *e*). *b* and *c* were H&E-stained adjacent tissue sections of immunofluorescent-stained sections *a* and *d*, respectively. *f* shows control nonspecific MAb immunostaining. × 100.

Table 4 *Immunohistochemical reactivity of MAb 39H with human brain tissue samples*

Tissue (diagnosis)[a]	No. of samples	Staining intensity[b]	Comment
Glioblastoma multiforme	9	++++ (6),[c] +++ (2), ++ (1)[d]	After XRT[d]
High-grade astrocytoma (II, III)	2	+++ (1), ++ (1)	
Oligodendroglioma	2	++ (2)	
Medulloblastoma	2	+++ (2)	
Low-grade/gliosis	1	+ (1) (P) +[e]	
Necrosis/gliosis	1	+ (1)	
Normal brain	5	+ (5) (P)	
Metastatic adenocarcinoma[f]	2	− (2), + (2) (P)	lesions (−) brains (+, P)

[a] The diagnosis was made from serial sections stained with hematoxylin-eosin.
[b] Relative immunofluorescent staining intensity; see "Materials and Methods" for quantitation.
[c] Numbers in parentheses, number of sections at each staining intensity.
[d] The patient received radiation therapy prior to surgical biopsy.
[e] (P) +, punctated.
[f] The metastatic lesions were from mammary adenocarcinoma tumors to the brain. 39H was not observed to bind to the tumor cells, but low punctated staining was seen for adjacent normal brain.

biochemical and immunological techniques to investigate the expression of HSPGs in human gliomas. Our results indicate an increased expression of HSPGs by gliomas compared with normal brain. First, a representative cultured glioma cell line was shown to have an increased synthesis of HS and HA GAG chains released into the medium and on the cell surface compared with normal cells. Second, this observation was supported by the increased binding of a MAb, 39H, generated against a glioma-derived HSPG, to glioma cells *in vitro*. However, the differential binding of 39H was most dramatically shown between tissue sections from glioblastoma multiforme and normal brain or gliosis, where reactive astrocytes could be identified. The 39H staining of astrocytomas was observed to be intermediate between the two previously mentioned conditions. These results suggest that the expression of HSPGs in astrocytic-derived primary brain tumors may correlate with the progression of these tumors *in vivo*.

The biochemical analysis of GAGs isolated from our representative glial and glioma cells *in vitro* revealed that the tumor cells expressed approximately 3-fold more HS and HA in their

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medium and on their cell surfaces than did the normal cells. The accumulation of GAGs by glioma cells appeared to be the result of increased synthesis and not the product of altered structures or modulations in the specific activity of radioactive precursors. We did not see any major structural changes of HS or CS GAG chains between the glioma or glial cells; however, the presence of minor modifications or altered subpopulations of GAG chains cannot be excluded (44). Glimelius *et al.* (23) examined a number of cultured human glioma and glial cells and reported a wide variability in the expression of GAGs; however, most of the glioma cells showed an increased accumulation of HA. Similarly, cultured tumor cells have been observed to express a hyaluronidase-sensitive "halo" that aids in their resistance to lymphocyte-mediated cytotoxicity (45). Using fresh surgical specimens, Bertotto and coworkers (26) demonstrated biochemically an increase in HS (~5-fold) and HA (~1.2-fold) in glioblastomas over those in normal brain. A smaller proportional increase of GAGs was also observed in astrocytomas but not in anaplastic astrocytomas. These data suggest that altered GAG expression may accompany transformation of astrocytic cells similar to that seen in other systems (17–21). However, an alternative possibility is that the increased expression of HSPG may be associated with the proliferation of the astrocytic cells, but since adult astrocytes rarely proliferate, the increased synthesis of HS would relate to an abnormal status of these cells whether due to transformation or growth.

The immunohistochemical results indicate a significant increase in the expression of the 39H epitope in high-grade, glioma-derived tumor cells over that in normal brain, especially *in vivo*. The MAb 39H was shown to bind to a HSPG species synthesized by glial-derived cells. Furthermore, the epitope appeared to be associated with the protein core rather than with the HS GAG chains because of the inability of isolated HS chains to inhibit the binding of 39H and the observation that trypsin treatment of HSPG abolished the binding to 39H in radioimmunoassay. Presently, we cannot ascertain if the epitope is proteinaceous or a glycoconjugate or both in molecular nature. In addition, the epitope is present on both normal and tumor cells, albeit at differential levels, as indicated by the reactivity of 39H to glial-origin cells *in vitro* and *in vivo*. However, the difficulty in obtaining normal adult human astrocytes should be pointed out, so direct comparisons of 39H antigen expression in isolated human glial and glioma cells was not possible, but differential levels of 39H binding were observed between rat astrocytes and glioma cells. Also, cross-reactivity of 39H with other cell types was observed particularly with cultured cells and with endothelial cell basement membranes *in vivo*. Furthermore, a correlation between the biochemical expression of HS and the binding of 39H was demonstrated for EFC-2 and MM cells.

The increased expression of HSPGs by glioma cells observed in this study contrasts with the reported alteration of GAG synthesis associated with a number of other tumor types. However, a large number of transformed cells display an increased synthesis of HA compared with their normal counterparts (21, 22, 46, 47), similar to that observed here. Furthermore, increased quantities of CS have been found in carcinomas of the breast, lung, liver, colon, and prostate as well as melanoma cells (48–53). However, decreased sulfation and/or lower production of HS has been associated with the transformation of cells (18, 54). In general, the majority of neoplasms appear to modulate their GAG metabolism during tumorigenesis; however, many of the alterations are cell type specific, and their functional consequences are unknown.

Some possible insights into the biological process and mechanisms of PGs and GAGs have been suggested by several recent growth-associated studies. Proliferating smooth muscle cells have been shown to synthesize 2 to 3 times more HSPG than quiescent cells; however, the growth-inhibitory activity associated with the HSPGs isolated from the latter cells was significantly more potent (55). Conrad and coworkers (41, 56) have shown that the appearance of a highly sulfated HS species in the nucleus of hepatocytes correlated with a decrease in the proliferation of those cells. Hepatomas have been shown to synthesize an undersulfated HSPG and to accumulate lower quantities of HS in their nuclei (41, 56).

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