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Proteoglycan Synthesis by Normal and Neoplastic Human Transitional Epithelial Cells

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Metabolically ^{35}S-labeled proteoglycans were isolated from cell-associated matrices and media of confluent cultures of human normal transitional epithelial cells and HCV-29T transitional carcinoma cells. On Sepharose CL-4B columns, the cell-associated proteoglycans synthesized from both cell types separated into three identical size classes, termed CI, CII, and CIII. Normal epithelial cell C-fractions eluted in a 22:34:45 proportion and contained 64%, 64%, and 72% heparan sulfate, whereas corresponding HCV-29T fractions eluted in a 29:11:60 proportion, and contained 91%, 77%, and 70% heparan sulfate, respectively. Medium proteoglycans from normal cells separated into two size classes in a proportion of 6:94 and were composed of 35% and 50% heparan sulfate. HCV-29T medium contained only one size class of proteoglycans consisting of 23% heparan sulfate. The remaining percentages were accounted for by chondroitin/dermatan sulfate. On isopycnic CsCl gradients, proteoglycan fractions from normal cells had buoyant densities that were higher than the corresponding fractions from HCV-29T cells. DEAE-Sephacel chromatography showed that cell and medium associated heparan sulfate from HCV-29T cells was consistently of lower charge density (undersulfated) than that from normal epithelial cells. In contrast, the chondroitin/dermatan sulfate of HCV-29T was of a charge density similar to that of normal cells. These as well as other structural and compositional differences in the proteoglycan may account, at least in part, for the altered behavioral traits of highly invasive carcinoma cells.

Key words: heparan sulfate, chondroitin sulfate, GAG chain size, undersulfation, HCV-29T, carcinoma cells

Glycoconjugates are important components of the cell-associated matrix of a wide variety of cells. The predominant glycoconjugates present in most noncartilagenous, pericellular matricies are sulfate-containing proteoglycans composed of heparan sulfate and chondroitin/dermatan sulfate side chains covalently attached to a protein core [1–3]. These proteoglycans have been implicated in the regulation of cell adhesion [4–7], control of cell growth [8–11], maintenance of basement membranes [12–14], organization of pericellular matricies [15], and storage of bioreactive substances [1]. Compositional and structural modifications of proteoglycans may therefore explain some of the

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altered cellular behavioral traits of neoplastic cells. For example, undersulfation of heparan sulfate proteoglycans may contribute to decreased intercellular and cell–matrix adhesion and thus may cause tumor cell shedding from primary tumors [4,6]; secretion of increased amounts of chondroitin sulfate proteoglycans and accumulation of these macromolecules in the tumor surrounding connective tissue have been shown to precede and promote tumor cell invasion [for review, see 1].

Several in vitro model systems have been used to characterize structural alterations of proteoglycans synthesized by various tumor cell types. Although the culture techniques and assay procedures for tumor cell lines are relatively standard, suitable "normal" cells for comparative studies are more difficult to establish. This is of added difficulty when working with normal human epithelial cell types. In this study, we have set up a system to compare structural and compositional characteristics of proteoglycan populations synthesized by primary cultures of normal human transitional epithelial cells and highly invasive human transitional carcinoma cells, both grown in collagen-coated culture dishes. Many of the structural features of proteoglycans derived from the two cell types were nearly identical. However, the heparan sulfate proteoglycans synthesized by the transitional carcinoma cells were consistently of lower charge density than the comparable proteoglycans synthesized by the normal epithelial cells. The reduction in charge density was observed exclusively in the heparan sulfate-containing proteoglycans, the other glycoconjugates being mostly unaffected.

MATERIALS AND METHODS

Reagents

Guanidine hydrochloride (GuHCl), cesium chloride (CsCl), papain, 6-amino-n-caproic acid, benzamidine HCl, phenylmethylsulfonylfluoride, EDTA, and CHAPS detergent were obtained from Sigma Chemical Co. (St. Louis, MO); N-ethylmaleimide from Calbiochem (La Jolla, CA); ultrapure urea from Schwartz/Mann Biotech (Cleveland, OH); carbodiimide from Aldrich Chemicals (Milwaukee, WI); Aquamix scintillation cocktail from West Chem. (San Diego, CA); and chondroitin ABC lyase (chondroitinase ABC) and heparitin sulfate lyase (heparitinase) from Miles (Elkhart, IN). PD-10 columns, Sepharose CL-4B and CL-6B, and DEAE Sephacel were all purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Aqueous solutions of carrier-free $[^{35}S]Na_2SO_4$ (1,236 Ci/mmole) were obtained from Amersham-Searle (Arlington Heights, IL). Nutrient mixture F-12 (Ham's) was from Gibco (Grand Island, NY). Monomer D1 proteoglycan, prepared from Swarm rat chondrosarcoma, was a gift from Dr. James Kimura, Rush-Presbyterian-St. Luke's Medical Center, Chicago. Vitrogen-100 was purchased from Collagen Corp. (Palo Alto, CA).

Cell Culture

Normal transitional epithelial cells were prepared from human ureters (3 × 5 mm) of kidney transplant patients as described by Resnikoff et al. [16]. The transitional epithelium was dissected from underlying connective tissue, minced into 1 mm^2 sheets, and placed into 100 mm dishes coated with a film of carbodiimide cross-linked collagen [17]. Explants were grown in supplemented Ham's F-12 medium supplemented with 2 mM L-glutamine, 1 μg/ml hydrocortisone, 5 μg/ml transferrin, 10 μg/ml insulin, 2.7 mg/ml dextrose, 0.1 mM ethanolamine, 0.1 mM phosphoethanolamine, 1% nonessential

amino acid mixture, 100 U/ml penicillin and steptomycin, and 0.5% fetal bovine serum [16]. Outgrowth of polygonal epithelial cells from the explants was allowed for 7 days, after which time explants were removed from the culture dishes to prevent fibroblast contamination. Cell proliferation continued at a steady rate until a cobblestone-like, contact-inhibited monolayer was formed, usually within 3–4 weeks after starting the explant cultures. HCV-29T carcinoma cells derived from an invasive, high-grade transitional cell carcinoma of the human urinary bladder were obtained from Dr. Jorgen Fogh, Sloan-Kettering Institute for Cancer Research (Rye, NY). Tumor cells were seeded at a density of 1×10^6 cells/100 mm collagen-coated dish. They formed a continuous monolayer within 4–5 days of incubation. Both normal and neoplastic epithelial cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2/95% air. Media were changed every 48 h.

Metabolic Labeling of Cells

In each experiment, five confluent cultures of normal epithelial cells or HCV-29T carcinoma cells were changed to medium containing 100 μCi/ml of [^{35}S]Na_2SO_4 and incubated for 24 h at 37°C. Following this incubation period, the labeling medium was removed, pooled with two phosphate-buffered saline (PBS) rinses of the monolayers, and centrifuged at 500*g* for 15 min at 4°C, and the supernatants were brought to dissociative conditions by addition of 0.53 gm/ml solid GuHCl and 10 mM N-ethylmaleimide [18]. Washed cell layers were extracted for 60 min at 4°C with 4 M GuHCl in 0.05 M sodium acetate buffer, pH 5.8, containing 0.3% CHAPS (buffer A) as well as a standard mixture of protease inhibitors (0.1 M 6-aminohexanoic acid, 5 mM benzamidine HCl, 50 mM EDTA, 10 mM N-ethylmaleimide, 0.5 mM phenylmethylsulfonylfluoride) [19]. After scraping of the remaining cell layer from the growth surface with a rubber policeman, extracts were clarified by centrifugation at 10,000*g* for 5 min at 4°C and stored at −80°C for subsequent analysis. This extraction procedure was found to solubilize approximately 99% of the cell-associated radioactivity and only minimal amounts of exogenous collagen. Subsequent extraction of the culture dishes with 0.5 N NaOH for an additional 24 h at room temperature recovered a residual [^{35}S]Na_2SO_4-labeled fraction equivalent to 1% of the total cell-associated radioactivity.

Sizing and Characterization of Proteoglycans

Aliquots of the medium or cell-associated GuHCl extracts, representing approximately one-fortieth of the total extract (20 ml), were mixed with 100 μg each of rat chondrosarcoma D1 monomer proteoglycan and heparin, as carriers, and applied to an analytical 0.8 × 100 cm Sepharose CL-4B column. The column was eluted in buffer A containing 5 mM EDTA and 5 mM benzamidine HCl [20]. Fractions of 1.0 ml were collected at a flow rate of 4 ml/h. Void and total volumes were determined by elution of [^{3}H]hyaluronate (prepared in our laboratory as described previously [21]) and [^{35}S]Na_2SO_4, respectively. Fractions were mixed with Aquamix cocktail and counted. Recovery from these columns was greater than 80%. Preparatory CL-4B columns (1.5 × 100 cm) were then used to load larger volumes of sample. Three milliliter fractions were collected, with an aliquot being removed for counting and the remaining appropriate fractions pooled, dialyzed, and lyophilized for further analyses. Elution patterns were identical to the analytical column profiles.

Individual glycosaminoglycan chains were released from the proteoglycans by treatment with papain (30 μg/ml) in 0.1 M sodium acetate (pH 7.0) containing 5 mM EDTA and 5 mM cysteine for 4 h at 65°C [22]. The reaction was terminated by boiling for 10 min. The papain-released GAG chains were then mixed with 100 μg/ml each of chondroitin sulfate and heparin as carriers and applied to a 0.8 × 65 cm Sepharose CL-6B column. Samples were eluted with 50 mM Tris buffer (pH 8.0) containing 100 mM NaCl and 1% SDS [20]. Fractions of 0.5 ml were collected and aliquots were counted. Glycosaminoglycan (GAG) fractions were pooled, dialyzed, and lyophilized for subsequent analysis.

Ion Exchange Chromatography

Proteoglycans were separated on the basis of their charge density on DEAE Sephacel columns [19,23]. Proteoglycan fractions from CL-4B columns were applied to a 2 ml DEAE Sephacel column equilibrated with 50 mM Tris buffer, pH 7.5, containing 6 M urea, 2 mM EDTA, 0.1 M NaCl, and 0.3% CHAPS (buffer B). Unbound material was eluted with 7 column volumes of buffer B. Bound material was then eluted with 100 ml of a linear NaCl gradient (0.1 to 0.8 M NaCl) in buffer B. One milliliter fractions were collected at a flow rate of 4.5 ml/h. Recovery of radioactive material was 75–80%. Aliquots were removed for scintillation counting, and the proteoglycan fractions were pooled, dialyzed, and lyophilized for subsequent analyses.

Isopycnic Density Gradient Centrifugation

Lyophilized fractions from CL-4B columns were redissolved in buffer A containing the standard protease inhibitor mixture. Samples were adjusted to a density of 1.45 gm/ml by addition of solid CsCl, then centrifuged at 100,000*g* at 10°C for 65 h. Four equal fractions, designated D1, D2, D3, and D4 (bottom to top), were prepared using a tube slicer or gradient separator. Aliquots of these fractions were then mixed with scintillation cocktail and counted.

Compositional Analysis of GAGs

Fractions isolated from the CL-4B, CL-6B, or ion exchange columns were treated with chondroitinase ABC, nitrous acid, or heparitinase to determine their compositional content of chondroitin sulfate/dermatan sulfate and heparan sulfate. Pooled, lyophilized fractions to be analyzed were redissolved in distilled water, and aliquots were mixed with the appropriate 10× digestion buffer. For the specific degradation of chondroitin sulfate/dermatan sulfate, samples were digested with 0.1 U/ml chondroitinase ABC [24] in enriched Tris buffer (0.25 M Tris, pH 7.4, 0.29 M sodium acetate, 0.25 M NaCl, and 0.05% bovine serum albumen) for 3 h at 37°C. Heparan sulfate was degraded chemically by the low-pH nitrous acid treatment of Lindahl et al. [25] or enzymatically with heparitinase (0.1 U/ml in 50 mM Tris, pH 7.0, 5 mM calcium acetate, and 0.5 mg/ml bovine serum albumin; 3 h at 37°C) [20]. The percent of the total GAG fraction susceptible to a particular digestion procedure was determined by the loss of voided material on Sepharose CL-6B columns. Similar percentages were also found with quantification of the increase in degraded material eluting in the total volume of these columns.

RESULTS

Distribution and Fractionation of [^{35}S]Sulfate-Labeled Macromolecules

Confluent cultures of human HCV-29T transitional carcinoma cells metabolically labeled with sodium [^{35}S]sulfate for 24 h contained approximately twice the cell layer-associated and six times the media-associated counts of comparable primary normal human transitional epithelial cell cultures (confluent monolayer of 78 cm^2). The cell layer-associated glycoconjugates accounted for 46% (±7%, n = 5) of the total counts in normal epithelial cells and 24% (±8%, n = 4) in carcinoma cells. The remainder of the labeled macromolecules, 54% in normal epithelial cell cultures and 76% in HCV-29T carcinoma cultures, were secreted into the medium (Table I). Both the cell layer-associated and secreted ^{35}S-labeled glycoconjugates were subsequently subjected to Sepharose CL-4B chromatography for molecular sizing. The cell-associated ^{35}S-labeled macromolecules from both cell types eluted in three molecular size classes, termed CI, CII, and CIII (Fig. 1A,B). CI, CII, and CIII from normal epithelial cells contained 22%, 34%, and 45% of the total cell-associated, ^{35}S-labeled glycoconjugates, whereas the corresponding size classes from HCV-29T carcinoma cells contained 29%, 16%, and 60%, respectively (Table I). Similar elution patterns for both cell types were obtained after 48 and 72 h of labeling (data not shown). These Sepharose CL-4B elution profiles remained unchanged when extracts were treated with chloroform/methanol prior to chromatography or when the cell-associated fractions were rechromatographed in the presence of 1% Triton X-100, both designed to prevent possible associations of proteoglycans with endogenous lipids. To identify the GAG species within CI, CII, and

TABLE I. Composition of Proteoglycans Isolated From Sepharose CL-4B Columns

Cell type	CL-4B[a] fraction	cpm[b] present	(%)	Percent total GAG[c]	K_{av}[d]	Glycosaminoglycan composition %HS[e]	%CS[f]
Normal	CI	19,700	(22)	11	0.00	—(65)	59
epithelial	CII	30,700	(34)	17	0.37	64(—)	29
cells	CIII	41,000	(45)	22	0.80	72(—)	33
	MI	5,600	(6)	3	0.00	35(—)	65
	MII	85,900	(94)	49	0.36	50(—)	56
HCV-29T	CI	60,900	(29)	8	0.00	91(92)	17
carcinoma	CII	23,400	(11)	3	0.34	73(77)	33
cells	CIII	126,300	(60)	17	0.71	66(70)	44
	MII	541,300	(100)	72	0.37	23(27)	83

[a]Cell-associated (CI, CII, CIII) and medium (MI, MII) pooled column fractions (see Fig. 1).
[b]Total cpm present in each pooled fraction isolated from the Sepharose CL-4B column; values in parentheses represent percentage of cell or medium associated counts from an individual chromatographic profile.
[c]Percent of the total counts (cell + medium) from normal or neoplastic transitional epithelial cells.
[d]K_{av} is defined as (Ve − Vo)/(Vt − Vo) of proteoglycans eluted on Sepharose CL-4B columns (Fig. 1).
[e]Percent of total cpm susceptible to nitrous acid treatment (or heparitinse; values in parenthesis) as determined by inclusion on Sepharose CL-6B columns. (—), Values not analyzed.
[f]Percent of total cpm susceptible to treatment by chonroitinase ABC as determined by inclusion on Sepharose CL-6B columns.

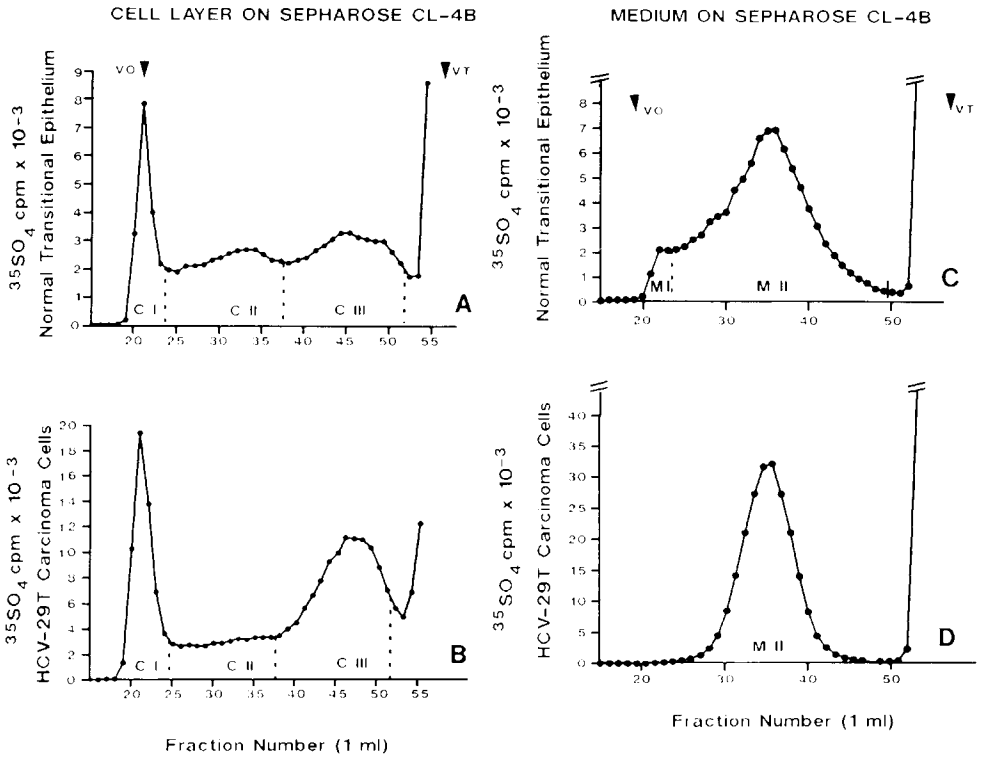

Fig. 1. Sizing of proteoglycans by Sepharose CL-4B chromatography. Aliquots of ^{35}S-labeled glycoconjugates isolated from the cell-associated (**A,B**) or medium (**C,D**) compartments were applied to 0.80 × 100 cm Sepharose CL-4B columns and eluted in buffer A containing 5 mM EDTA and 5 mM benzamidine HCl. One milliliter fractions were collected and analyzed by liquid scintillation counting. Elution pattern of normal transitional epithelial cells are depicted in A and C and those of HCV-29T transitional carcinoma cells in B and D. Fractions pooled and designated as cell-associated (CI, CII, CIII), or medium (MI, MII) are delineated by vertical dotted lines. Void volume (VO) and total volume (VT) positions are depicted by arrowheads. Profiles shown represent one example of an elution pattern that was identical in four separate experiments.

CIII, samples were individually digested with nitrous acid (or heparitinase) or chondroitinase ABC, then rechromatographed on Sepharose CL-4B. The results showed that both normal and neoplastic transitional epithelial cells deposited primarily heparan sulfate into their cell-associated matricies (Table I). The ratios of cell surface heparan sulfate to chondroitin/dermatan sulfate were higher in the HCV-29T CI-glycoconjugate fraction than in the corresponding CI fraction from normal epithelial cells (Table I, $P < 0.01$, data not shown). The ratios in the CII and CIII fractions between the two cell types were not statistically different from each other.

The molecular sizes of the secreted ^{35}S-labeled macromolecules were distinctly different between the normal and neoplastic transitional epithelial cells. Normal epithelial cells secreted two different sizes of glycoconjugates into their medium (termed MI and MII), whereas HCV-29T carcinoma cells released only one size class, which consisted of molecules of the same size as those in normal epithelial MII (Fig. 1C,D). The secreted normal glycoconjugates contained approximately equal parts of heparan

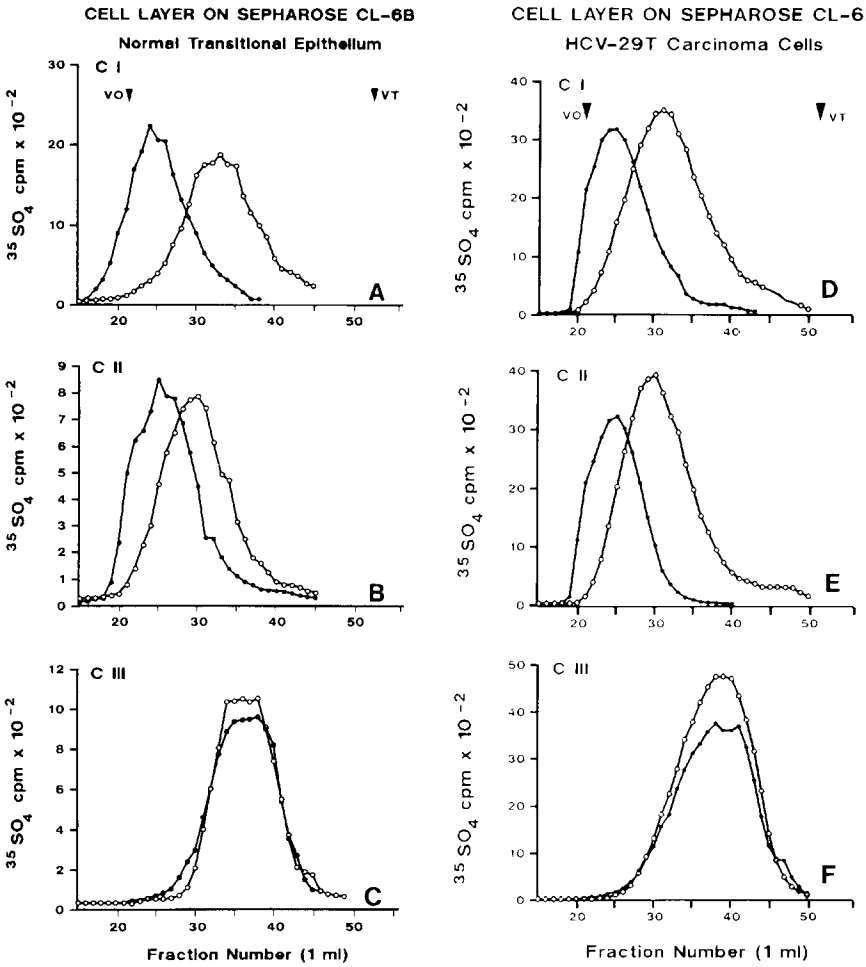

Fig. 2. Sizing of cell-associated glycosaminoglycans with Sepharose CL-6B chromatography. Aliquots of pooled, cell-associated Sepharose CL-4B fractions depicted in Figure 1, before (●) and after (○) exhaustive papain digestion as described in Materials and Methods, were applied to 0.80 × 65 cm Sepharose CL-6B columns and eluted in 50 mM Tris buffer, pH 8.0, containing 100 mM NaCl and 0.1% sodium dodecyl sulfate (SDS). One milliliter fractions were collected and analyzed by liquid scintillation counting. Elution patterns of normal and tumor cell-associated bands CI, CII, and CIII are depicted in **A, B, C** and in **D, E, F,** respectively. Void volume (VO) and total volume (VT) positions are depicted by arrowheads. Profiles shown represent one example of an elution pattern that was identical in four separate experiments.

sulfate and chondroitin/dermatan sulfate. In contrast, the tumor-derived secreted glycoconjugates consisted predominantly of chondroitin/dermatan sulfate proteoglycans (approximately 60% of the total ^{35}S-labeled glycoconjugates).

Determination of the Proteoglycan Nature and GAG Chain Size

The proteoglycan nature of the radiolabeled glycoconjugates in each of the pooled CL-4B fractions was determined by their elution profiles on CL-6B columns before and after exhaustive papain digestion. The CL-6B chromatographic profile of fractions CI,

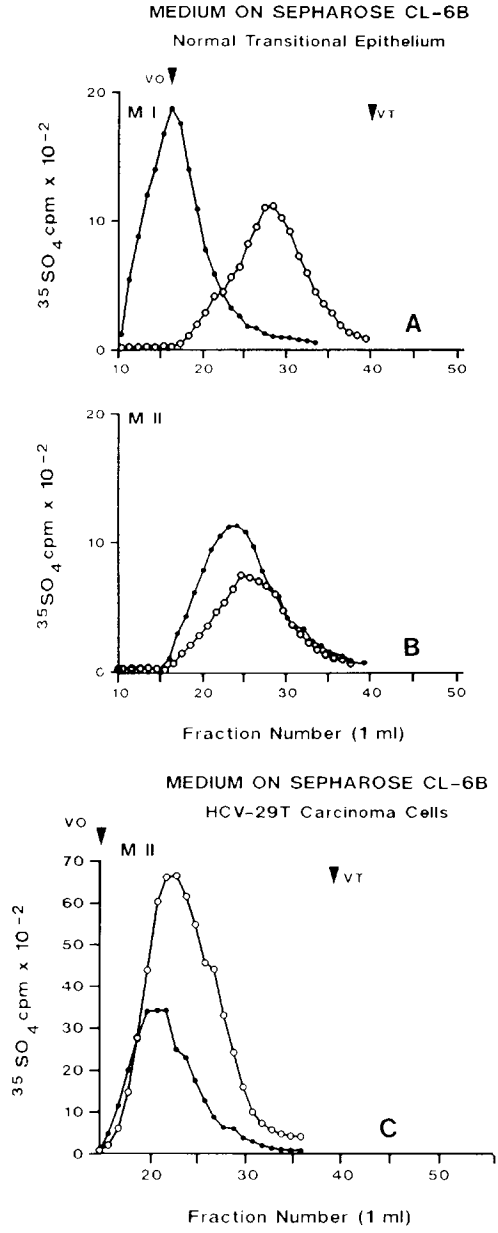

Fig. 3. Sizing of medium glycosaminoglycans by Sepharose CL-6B chromatography. Aliquots of pooled, medium Sepharose CL-4B fractions depicted in Figure 1, before (●) and after (○) exhaustive papain digestion as described in Materials and Methods, were applied to 0.80 × 65 cm Sepharose CL-6B columns and eluted in 50 mM Tris buffer, pH 8.0, containing 100 mM NaCl and 0.1% SDS. One milliliter fractions were collected and analyzed by liquid scintillation counting. Elution patterns of medium MI and MII, derived from normal transitional epithelial cells, are depicted in **A** and **B**, respectively. The HCV-29T transitional carcinoma cell elution pattern of medium MII is depicted in **C**. Void volume (VO) and total volume (VT) positions are depicted by arrowheads. Profiles shown represent one example of an elution pattern that was identical in four separate experiments.

TABLE II. Molecular Weights of Glycosaminoglycans Chromatographed on Sepharose CL-6B Columns

Cell type	Fraction[a]	K_{av}[b]	Approximate molecular weight[c]
Normal	CI	0.38	38,000
epithelial	CII	0.32	55,000
cells	CIII	0.52	19,000
	MI	0.48	22,000
	MII	0.34	46,000
HCV-29T	CI	0.32–0.33	48,000–55,000
carcinoma	CII	0.32–0.33	48,000–55,000
cells	CIII	0.58	16,500
	MII	0.32	48,000

[a]Cell-associated (CI, CII, CIII) and medium (MI, MII) fractions from Sepharose CL-4B column (see Fig. 1).
[b]K_{av} is defined as (Ve – Vo)/(Vt – Vo) of glycosaminoglycans chromatographed on Sepharose CL-6B columns (Fig. 2).
[c]Approximate molecular weights were assigned following Sepharose CL-6B column standard calibration described by Wasteson [26].

CII, MI, and MII, derived from both normal and neoplastic transitional epithelial cells, shifted to lower molecular weight positions following papain digestion, indicating prior association of GAGs with a core protein (Figs. 2, 3). In contrast, the cell-associated macromolecules in CIII displayed little or no shift in molecular weight following papain digestion, suggesting that these molecules were free GAG chains (Fig. 2C,F). The molecular weights of the papain-released GAG chains were estimated by comparison of their K_{av} values with those determined by Wasteson [26]. As is shown in Table II, the

TABLE III. Distribution of Proteoglycans on Isopycnic CsCl Density Gradients

		Gradient fraction[b]			
Cell type	Fraction[a]	D4 (1.39)	D3 (1.44)	D2 (1.51)	D1 (1.62)
Normal	CI	42	13	17	28
epithelial	CII	3	7	23	67
cells	CIII	22	6	6	66
	MI	37	34	11	18
	MII	7	10	16	67
HCV-29T	CI + CII[c]	53	23	18	6
carcinoma	CIII	41	25	11	23
cells	MII	29	14	21	36

[a]Cell-associated (CI, CII, CIII) and medium (MI, MII) fractions from Sepharose CL-4B columns (see Fig. 1).
[b]D4, D3, D2, and D1 designate quarter fractions along the CsCl density gradient, with D4 representing the top fraction (most bouyant) and D1 representing the bottom fraction. Values are given as percentage of total cpm distributing in the four fractions. The average density (gm/ml) of each isolated fraction is given in parenthesis.
[c]Fractions CI and CII were combined in these experiments because the amount of radioactivity in band CII was too small to run separately.

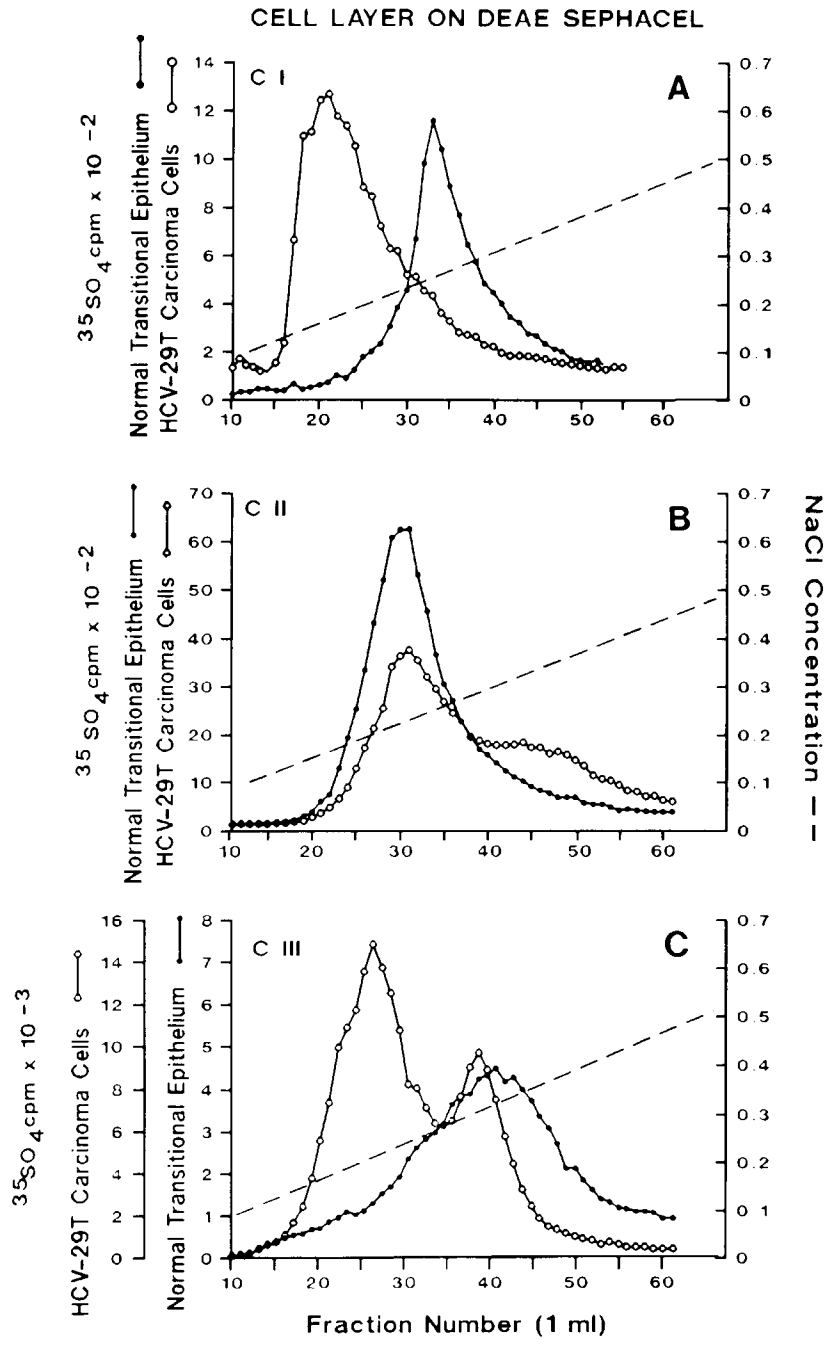

Fig. 4. Ion-exchange chromatography of proteoglycans on DEAE Sephacel. Aliquots of pooled, cell-associated (**A, B, C**) or medium (**D, E**) fractions isolated from Sepharose CL-4B columns depicted in Figure 1 were applied to a 2 ml DEAE Sephacel columns equilibrated in buffer B and eluted by a linear NaCl gradient (0.1 to 0.8 M NaCl). One milliliter fractions were collected and analyzed by liquid scintillation counting. Elution patterns of cell-associated CI, CII, and CIII, derived from normal transitional epithelial cells (●) and HCV-29T carcinoma cells (○) are depicted in A–C. Elution patterns of normal epithelium medium MI and MII (●) and HCV-29T medium MII (○) are depicted in D and E. Profiles shown represent one example of an elution pattern that was identical in four separate experiments.

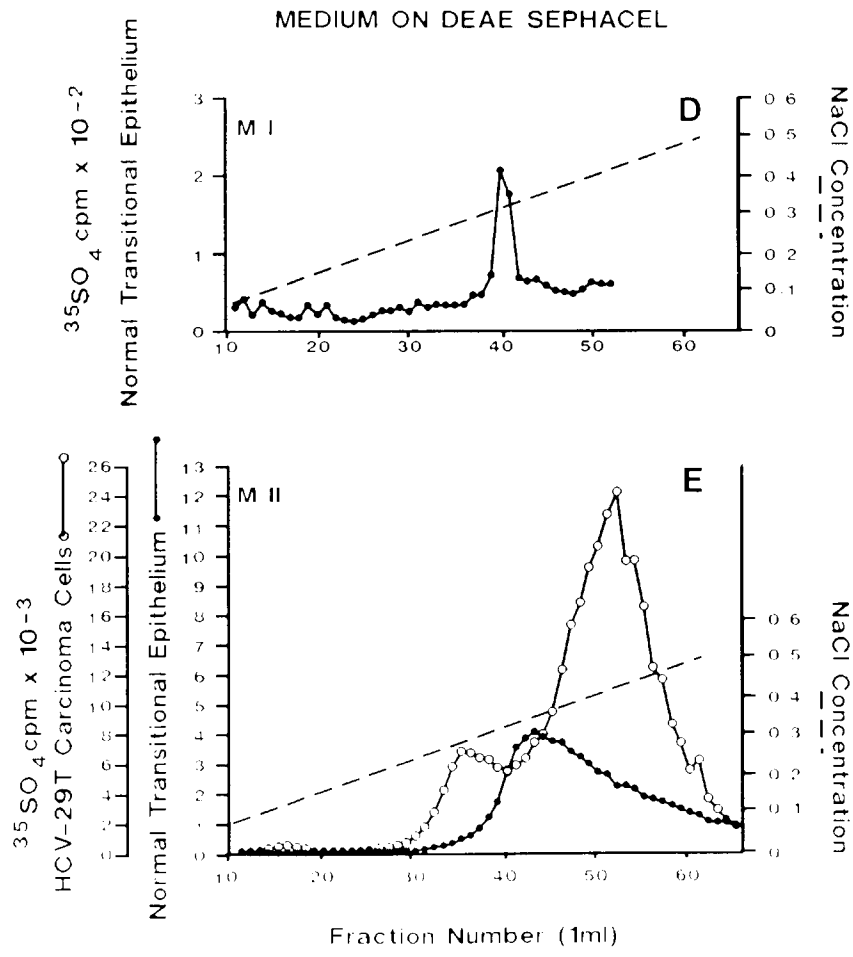

Figure 4

GAG chains isolated from CI proteoglycan of normal epithelial cells had a molecular weight of 38 kD, whereas that from normal CII proteoglycans and tumor CI and CII proteoglycans fell all within a molecular weight range of 48–55 kD. The GAGs isolated from CIII had a molecular weight of 19 kD for normal epithelial cells and 16.5 kD for HCV-29T carcinoma cells. The GAG of the medium proteoglycans exhibited the following molecular weights: 22 kD for MI and 48 kD for MII proteoglycans of normal epithelial cells and 46 kD for MII proteoglycans of HCV-29T carcinoma cells (Table II). The compositional heterogeneity of the proteoglycans (i.e., heparan sulfate vs. chondroitin/dermatan sulfate) had little effect on their elution patterns. Papain-released GAG chains subsequently digested with either chondroitinase ABC or heparitinase and rechromatographed on the CL-6B columns exhibited elution profiles similar to those shown in Figures 2 and 3 (data not shown).

Isopycnic Density Gradient Centrifugation

To estimate the ratio of protein to GAG, pooled proteoglycan fractions from CL-4B columns were brought to dissociative conditions with 4 M GuHCl, mixed with

TABLE IV. Composition of Proteoglycans Eluted From DEAE-Sephacel Columns*

Cell type	Fraction	Glycosaminoglycan composition	
		%HS	%CS
HCV-29T	CIIIa	105	0
carcinoma	CIIIb	15	85
cells	MIIa	76	24
	MIIb	43	57

*Footnotes are the same as for Table I.

CsCl, and centrifuged to equilibrium. In normal transitional epithelium, the majority of the CI and MI proteoglycans floated on the gradient (D4 fraction), whereas CII, CIII, and MII proteoglycans demonstrated a much higher buoyant density, with approximately 67% of the material sedimenting into the D1 fraction (Table III). The tumor-derived proteoglycans demonstrated a tendency to distribute into the upper portions of the CsCl gradients. In light of the fact that we could not detect any noncovalent associations of these proteoglycans with lipid, these results suggest a higher core protein/GAG ratio. The high degree of undersulfation observed in HCV-29T GAGs (discussed below) might also have contributed to their lower buoyant densities. This is evident in the cell-associated CIII material, in which little shift in molecular weight was observed after papain digestion (Fig. 2F), yet 41% of these macromolecules distributed into the D4 fraction.

Determination of Charge Density by Ion Exchange Chromatography

Samples from each of the pooled CL-4B fractions were chromatographed on DEAE Sephacel columns (Fig. 4). Proteoglycans from normal transitional epithelial cells eluted as a unimodal species. CI and CII proteoglycans eluted at 0.25 M NaCl (Fig. 4A,B), whereas CIII, MI, and MII proteoglycans eluted at approximately 0.35 M NaCl (Fig. 4C–E). The elution profiles of the unimodal peaks, such as normal CI and CII, which contained a mixture of heparan sulfate and chondroitin/dermatan sulfate proteoglycans (see Table I), were unaltered when the proteoglycans were predigested with either chondroitinase ABC or heparitinase prior to their chromatography on the DEAE columns (data not shown). We thus could not confirm or deny the existence of hybrid heparan/chondroitin sulfate proteoglycan on these columns. Tumor-derived CI and CII proteoglycans eluted from DEAE columns as single peaks (Fig. 4A,B), whereas CIII and MII proteoglycans eluted as two distinct peaks (Fig. 4C,E). The tumor CI peak expressed a significantly lower charge density than that of the corresponding normal peak (Fig. 4A). Tumor-derived CIII resolved into a DEAE peak with lower charge density and a DEAE peak with identical charge density to that of normal CIII (Fig. 4C). The two DEAE peaks of tumor MII expressed either a higher or lower charge density than the single DEAE peak from normal MII (Fig. 4E), indicating that the proteoglycans in the second DEAE peak of MII were slightly oversulfated.

To study the composition of the biphasic DEAE peaks from the tumor-derived CIII and MII proteoglycans, the DEAE peaks were concentrated and subjected to nitrous acid digestion prior to rechromatography on DEAE Sephacel. As is shown in Table IV, the lower charge density peak, termed CIIIa, was composed entirely of heparan sulfate and thus was completely degraded by nitrous acid, whereas the majority of the higher charge density peak, termed CIIIb, was composed of 85% chondroitin/

dermatan sulfate. Similar results were obtained with tumor-derived MII. The majority of the lower charge density material was heparan sulfate, and the higher charge density material was chondroitin/dermatan sulfate. Thus the tumor cells appeared to fail differentially to complete sulfation of their heparan sulfate chains, while allowing for normal or slightly increased sulfation of their chondroitin/dermatan sulfate.

DISCUSSION

Normal and neoplastic human transitional epithelial cells synthesized glycosaminoglycan-containing proteoglycans that were either part of the cell-associated matrix or were secreted into the medium. In our model system, the amounts of $^{35}SO_4$-labeled proteoglycans present in these two compartments was consistently higher in neoplastic cell cultures. The cell-associated proteoglycans consisted of three similar size classes in both cell types. These size classes corresponded to those observed in other epithelial cell systems [27–29]. For example, mouse mammary epithelial cells synthesized nearly identical size classes of proteoglycans. However, the mammary proteoglycans with the highest molecular weight, which was equivalent to that in our CI glycoconjugate fraction, was found to be an aggregate of two lower molecular weight proteoglycan species with endogenous lipid [27]. Although the possibility that our CI proteoglycans represent some form of aggregate cannot be conclusively ruled out, we did not find the CI proteoglycan aggregated with lipid molecules. Preextraction of CI with chloroform/methanol or rechromatography of the CI fraction on Sepharose CL-4B in the presence of 1.0% Triton X-100 had no effect on the gel filtration profiles.

The proteoglycans partition into the cell-associated and media compartments and the GAG composition of the various proteoglycan fractions varied markedly between normal transitional epithelial cells and HCV-29T carcinoma cells. Normal cells deposited approximately equal parts of proteoglycans into the pericellular matrix and the medium, whereas HCV-29T carcinoma cells secreted most of their proteoglycans into the medium. The GAGs of the cell-associated proteoglycans from both normal and neoplastic cells were predominantely heparan sulfate. The medium proteoglycans consisted of equal parts of heparan sulfate and chondroitin/dermatan sulfate in normal cells and approximately 80% chondroitin/dermatan sulfate in neoplastic cells. These compositional ratios varied significantly in the different cell systems. For example, malignant human mammary MDA/MD/231 epithelial cells secreted, in a fashion analogous to our carcinoma cells, 80% chondroitin/dermatan sulfate and 20% heparan sulfate [30]. However, mouse mammary epithelial cells produce 20% chondroitin/dermatan sulfate and 80% heparan sulfate [31], whereas human WiDr colon carcinoma cells synthesized only heparan sulfate [32]. A more consistent finding was the reduced accumulation of proteoglycans in the cell-associated matrix. Similar to HCV-29T carcinoma cells, transformed mouse mammary epithelial cells had a much lower capacity to accumulate their proteoglycans in stable collagen-associated matricies than their normal counterparts [28]. Increased synthesis and secretion of chondroitin sulfate proteoglycans into the extracellular compartment was shown to contribute significantly to the invasive growth of many carcinoma cells [for review, see 1].

The molecular sizes of the GAG chains were similar in normal and neoplastic human transitional epithelial cells. However, they proved to be larger than those reported for mouse mammary epithelial cells (31–39 kD [28]), rat hepatoma cells (16–19 kD [33]), and human mammary epithelial cells (30–38 kD [34]) but equal to or

smaller that those described for human neuroblastoma cells (K_{av} values 0.230–0.317, molecular weight 75–55 kD [29]) and mouse EHS sarcoma (60 kD [34]). It is interesting to note that the size of the chondroitin/dermatan sulfate side chains was identical to that of the heparan sulfate chains. Such similarities were also observed in human mammary epithelial cells [30].

The most dramatic differences between the proteoglycans from normal and neoplastic transitional epithelial cells was observed in their charge densities. A large proportion of proteoglycans derived from HCV-29T carcinoma cells eluted from DEAE ion exchange columns at a lower ionic strength than comparable proteoglycan fractions from primary cultures of normal transitional epithelial cells. This lower ionic strength was associated exclusively with heparan sulfate proteoglycans and was not observed in chondroitin/dermatan sulfate proteoglycans. Differential reduction in the charge density of heparan sulfate-type proteoglycans was observed by several investigators in various neoplastic cell systems and was commonly attributed to undersulfation of the GAG chains [27,33,35,36]. These finding were at variance with those of a recent publication that detected no differences in the charge densities of heparan sulfates from normal and neoplastic human mammary epithelial cells [30]. However, this study might have suffered from the fact that the "normal" mammary epithelial cells were at passage 23 and thus might have undergone in vitro transformation.

Reduced charge densities of heparan sulfate (undersulfation) in tumors might have several important consequences that might explain altered cellular behavior. For example, the production of heparan sulfate species with reduced charge density might significantly decrease intercellular and cell–matrix adhesion. Heparan sulfate undersulfation was found to reduce the binding of heparan sulfate to other heparan sulfate chains by weakening the capacity for lateral self-association [37] as well as the binding to other, heterologous matrix macromolecules such as fibronectin [33]. Reduced adhesions between tumor cells would in turn facilitate tumor cell shedding at the tumor invasion zone and allow tumor cells with increased motility to invade extracellular matricies. Heparan sulfate has also been implicated in the control of cell growth. The heparan sulfates associated with cultured cells in the proliferative or log phase are often less highly charged than those associated with quiescent, contact-arrested cells [38]. These findings demonstrate that alterations in the cell-associated matrix may critically influence the behavior of malignant cells.

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