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Altered Expression of Glycosaminoglycans in Metastatic 13762NF Rat Mammary Adenocarcinoma Cells†

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ABSTRACT: A difference in the expression and metabolism of sulfated glycosaminoglycans between rat mammary tumor cells derived from a primary tumor and those from its metastatic lesions has been observed. Cells from the primary tumor possessed about equal quantities of chondroitin sulfate and heparan sulfate on their cell surfaces but released fourfold more chondroitin sulfate than heparan sulfate into their medium. In contrast, cells from distal metastatic lesions expressed approximately 5 times more heparan sulfate than chondroitin sulfate in both medium and cell surface fractions. This was observed to be the result of differential synthesis of the glycosaminoglycans and not of major structural alterations of the individual glycosaminoglycans. The degree of sulfation and size of heparan sulfate were similar for all cells examined. However, chondroitin sulfate, observed to be only chondroitin 4-sulfate, from the metastases-derived cells had a smaller average molecular weight on gel filtration chromatography and showed a decreased quantity of sulfated disaccharides upon degradation with chondroitin ABC lyase compared to the primary tumor derived cells. Major qualitative or quantitative alterations were not observed for hyaluronic acid among the various 13762NF cells. The metabolism of newly synthesized sulfated glycosaminoglycans was also different between cells from primary tumor and metastases. Cells from the primary tumor continued to accumulate glycosaminoglycans in their medium over a 72-h period, while the accumulation of sulfated glycosaminoglycans in the medium of metastases-derived cells showed a plateau after 18–24 h. A pulse–chase kinetics study demonstrated that both heparan sulfate and chondroitin sulfate were degraded by the metastases-derived cells, whereas the primary tumor derived cells degraded only heparan sulfate and degraded it at a slower rate. These results suggested that altered glycosaminoglycan expression and metabolism may be associated with the metastatic process in 13762NF rat mammary tumor cells.

The dissemination of tumor cells to distant sites involves a multitude of complex interactions between the tumor cells and the host environment (Nicolson & Poste, 1983; Nicolson, 1984). An essential aspect of the metastatic process is the tumor cell's ability to migrate and invade the surrounding host tissue. Although the exact mechanisms of cellular locomotion are unknown, the process is thought to consist of a coordinate sequence of cell adhesions and dissociations (Heaysman, 1978; Weiss & Ward, 1983). Proteoglycans and glycosaminoglycans (GAGs)[1] have been proposed to play key functional roles in the adhesive and migratory properties of cells (Lark & Culp, 1982, 1983, 1984; Hook et al., 1984). In addition to modulating its own matrix, malignant cells have been shown to degrade the host's surrounding basal laminae (Jones, 1979; Sloane et al., 1981; Kramer et al., 1982; Nakajima et al., 1984).

A number of investigations have focused on qualitative and quantitative modulation of GAGs between transformed or tumor cells and their normal counterparts [reviewed by Kraemer (1979) and Hook et al. (1984)]. Differential expression of GAGs has been observed in virally or chemically transformed cells (Underhill & Toole, 1982; Angello et al., 1982a; Shanley et al., 1983). In addition, structural heterogeneity of GAGs has also been demonstrated in several transformed cells and hepatomas, in which heparan sulfate (HS) was shown to have a lower degree of sulfation than normal cells (Underhill & Keller, 1975; Keller et al., 1980; Winterbourne & Mora, 1981; Nakamura & Kajima, 1981; Robinson et al., 1984). The decreased sulfated HS was shown to be synthesized by mouse embryo clones with greater tu-

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[1] Abbreviations: GAG, glycosaminoglycans; HS, heparan sulfate; CS, chondroitin sulfate; HA, hyaluronic acid; AMEM, α-modified minimum essential medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; ΔDi-0S, 2-acetamido-2-deoxy-3-*O*-(α-D-gluco-4-enepyranosyluronic acid)-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(α-D-gluco-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(α-D-gluco-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; TRU, turbidity-reducing units.

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morigenicity than low tumorigenic clones (Winterbourne & Mora, 1981) and to have a lower binding affinity for fibronectin than HS (Robinson et al., 1984). GAGs also appear to play a role in growth regulation, as altered GAG expression and structure have been observed for growing vs. nongrowing cells (Underhill & Keller, 1976, 1977; Preston et al., 1985). Furthermore, the modulation of cellular proliferation by GAGs, especially HS, has been reported (Castellot et al., 1981; Kawakami & Terayama, 1981; Ratner et al., 1985).

Several studies have focused on GAGs synthesized by normal and neoplastic mammary cells. Using cultured human cells, Chandrasekaran and Davidson (1979) reported that the carcinoma cells synthesized mainly chondroitin sulfate (CS) and HS, whereas a normal cell line expressed predominantly hyaluronic acid (HA). In contrast, Angello et al. (1982a,b) observed that highly tumorigenic mouse mammary adenocarcinoma sublines synthesized significantly more HA than HS, while sublines of lower tumorigenicity expressed higher quantities of HS. Also, mouse mammary carcinoma cells with high lung-colonizing potential were shown to incorporate labeled precursor into HA at higher rates than cells with low experimental lung-colonizing potential (Kimata et al., 1983).

We have been investigating the expression of various cell surface components by cell lines and clones derived from the primary tumor or from spontaneous metastatic lymph node and lung lesions of the rat 13762NF mammary adenocarcinoma (Neri et al., 1981, 1982; Steck & Nicolson, 1983). To ascertain the possible roles, if any, of GAGs in breast cancer metastases, we have analyzed the distribution and composition of GAGs expressed by the rat 13762NF tumor cells. We describe here the differential expression and metabolism of GAGs between tumor cells derived from the primary tumor and those from spontaneous metastatic lesions.

Experimental Procedures

Sodium [^{35}S]sulfate (carrier free) and D-[6-^{3}H]glucosamine (20–30 Ci/mmol) were purchased from ICN Pharmaceuticals (Irvine, CA). *Streptomyces* hyaluronidase, chondroitin ABC lyase, chondroitin AC lyase, chondroitin 4-sulfatase, chondroitin sulfate (type C), hyaluronic acid, and standard disaccharide kit (ΔDi-0S, ΔDi-4S, ΔDi-6S) were obtained from ICN Biochemicals (formerly Miles Laboratories, Lisle, IL). Trypsin (crystallized 3 times) was from Worthington Biochemicals (Freehold, NJ), Pronase from Calbiochem-Behring (La Jolla, CA); α-modified Eagle's medium (AMEM) from Gibco (Grand Island, NY), fetal bovine serum (FBS) from Sterile Systems (Logan, UT), and DE-52 DEAE-cellulose from Whatman Chemicals (Clifton, NJ). Prepacked disposal Sephadex G-25 (PD-10) columns, Sephadex G-50 (20–80 μm), DEAE-Sephadex A-25, and Sephacryl S-300 and S-1000 were obtained from Pharmacia (Piscataway, NJ). Bio-Gel P-2 and Bio-Gel P-10 (both 200–400 mesh) were purchased from Bio-Rad (Richmond, CA).

Cell Culture. Rat 13762NF tumor cells were obtained from the primary tumor of the mammary fat pad (MTPa, tumor explant; MTC and MTF7, cell clones) or from spontaneous metastases to the regional lymph node (MTLY, tumor explant) and lung (MTLn2 and MTLn3, cell clones) (Neri et al., 1982). In this study the cells were used between cell culture passages 6–10 for MTPa; 12–19 for MTC, MTF7, MTLY, and MTLn3; and 39–43 for MTLn2. Routine screening of the cells showed that they were free of mycoplasmal and viral contamination.

The cells were grown on 100-mm tissue culture plates (Corning, Corning, NY) containing 10 mL of 10% FBS in AMEM and no antibiotics in a 5% CO_2–humidified air mixture at 37 °C. The cells used in these experiments were in exponential growth; these tumor cells detach after the cultures become confluent so that stationary-phase cells cannot be obtained. For the various experiments, the cells were seeded into 100-mm culture plates [(2.5–5) × 10^5 cells for experiments of more than 49 h or (1–2) × 10^6 cells for experiments of less than 48 h] and allowed to attach overnight. Metabolic radiolabeling of the cells was accomplished by removing the medium, washing the cells twice with phosphate-buffered saline (PBS; free of Ca^{2+} and Mg^{2+}), and then adding 10 mL of medium containing 10% FBS and 10 μCi/mL [^{3}H]glucosamine or 20–50 μCi/mL [^{35}S]sulfate. The AMEM used in the labeling with [^{35}S]sulfate contained one-tenth the usual concentration of $MgSO_4$ (80.9 μmol) and was replaced with additional $MgCl_2$. Pulse labeling (4 h) was accomplished with prewarmed AMEM (4 mL) containing 100 μCi/mL [^{35}S]-sulfate and devoid of FBS and $MgSO_4$. The pulse-labeling medium was then aspirated, the cells were washed 3 times with warm PBS, and then 10 mL of growth medium (AMEM containing 10% FBS) was added.

Isolation of Glycosaminoglycans. GAGs from various cellular fractions (medium, cell surface, and cellular) were harvested following the procedure of Vogel and Kendall (1980). Briefly, the medium was collected, the cells in each of four 100-mm tissue culture plates were washed individually with 5 mL of PBS, and this wash was added to the medium (extracellular fraction), which was then centrifuged (1000*g*, 5 min) to remove cellular debris. The cells were incubated with PBS (5 mL) containing 10 μg/mL trypsin and 5 mM ethylenediaminetetraacetic acid (EDTA) for 15 min at 37 °C on a rotary shaker (80 rpm). The cells for all plates were collected and separated from the cell surface fraction by centrifugation (1000*g*, 5 min). After removal of the supernatant (cell surface fraction), the pelleted cells [(5–10) × 10^6] were suspended in 2.5 mL of PBS containing 0.1% CHAPS. Aliquots were removed from the cellular fraction for protein determination according to manufacturer's instructions (BCA protein assay, Pearce, Rockford, IL). In several experiments the extracellular matrix fraction was obtained in a similar manner by removing the medium and then collecting the cells for each of the culture plates with 5 mL of 5 mM EDTA in PBS with shaking for 15 min at 37 °C. The plates were checked by microscopy after two washes with PBS to assure that all cells were removed from the culture dish; the residual material on the culture plate was termed the matrix fraction. Cell surface and cellular fractions were then harvested by trypsin treatment as described previously. Carrier hyaluronic acid and chondroitin sulfate (50 μg each) were added to the various fractions, except if the specific activity of incorporation of the various precursors into GAGs was to be determined. The various cellular fractions were then digested with Pronase and trypsin (1 mg/mL each) for 24 h at 37 °C, followed by two subsequent 24-h incubations after addition of Pronase (0.5 mg/mL). The fractions were then lyophilized, redissolved in 1.5 mL of water, applied to a PD-10 column equilibrated with water, and eluted with 1 mL of water; collection of macromolecular material was in the next 3.5 mL of eluant. The majority (>90%) of dextran sulfate, M_r ~5000, was recovered in the macromolecular fraction by this procedure. For experiments on quantitative determinations of macromolecular material, the next 3.1 mL of eluant was collected in order to exclude unincorporated radioactive label. Recovery from the PD-10 columns was greater than 95%.

The macromolecular material was then fractionated by either DEAE-cellulose ion-exchange chromatography (Lau &

Ruck, 1983) or cellulose acetate electrophoresis (Cappellitti et al., 1979). For ion-exchange chromatography, the macromolecular material was adjusted to 10 mM Tris-HCl, pH 7.2, and then applied to a DE-52 column (1 × 5 cm) equilibrated in the same buffer. After a 15-mL wash, the column was eluted with a linear gradient of NaCl (0–0.8 M, 160 mL), and the individual peaks were then pooled. Alternatively, the quantitation of GAGs was performed by electrophoresis (Cappellitti et al., 1979) on cellulose acetate sheets (Titan III, Helena Laboratories, Beaumont, TX) with the following modifications. Separation of the GAGs by net charge utilized 50 mM propylenediamineacetate, pH 9.0, with electrophoresis at 120 V for 1 h. The cellulose acetate sheets were then stained with toluidine blue and destained, and the radioactivity associated with the individual GAGs was determined by harvesting sequential 1-mm segments of the sheet, followed by scintillation counting. The migration was compared to that of standard GAGs.

Analytical Methods. The GAGs were identified by their sensitivities to the following various treatments. HA was characterized by its sensitivity to *Streptomyces* hyaluronidase (25 TRU/mL) in 0.15 M sodium chloride, 0.1 M sodium acetate, and 100 μg/mL BSA, pH 5.0. CS was identified by treatment with chondroitin ABC and chondroitin AC II lyase digestions (Saito et al., 1968): 0.1 unit/mL enzyme in 50 mM Tris-HCl, pH 8.0, 50 mM sodium acetate, 50 μg/mL BSA, and 500 μg chondroitin sulfate. Identification and separation of disaccharides from enzymatic digestions of CS or HA were performed by thin-layer chromatography on Avicel plates (Analtech, Newark, NJ). The plates were desalted (butanol/ethanol/H_2O, 52:32:16) and then developed (butanol/acetic acid/1 M NH_4OH, 2:3:1), followed by detection of the UV light absorbing areas (Wasserman et al., 1977). Quantitation of the radioactivity was accomplished by scraping 0.4-cm segments into 0.5 mL of 1 M HCl and incubation with shaking at 37 °C for 18 h, followed by centrifugation to remove the silica and scintillation determination of equal aliquots of the supernatant (Vogel & Kendall, 1980). Uronic acid assays were done according to the method of Bitter and Muir (1962).

HS degradations were performed by the low-pH method as described by Shively and Conrad (1976) to achieve quantitative release of N-sulfated glucosamine residues. Alternatively, samples were treated as described for reaction A (Lindahl et al., 1973). The frequency and distribution of N- and O-sulfated residues in HS chains were determined by two methods. The lyophilized HS was then degraded by treatment with nitrous acid (Shively & Conrad, 1976), and the resulting residues were subjected to gel filtration chromatography on a calibrated Bio-Gel P-10 column. The quantity of glucosamine residues terminating as anhydromannose residues was used to calculate the frequency and distribution of N-sulfated residues by summation of the amount of activity (a_n) in an oligosaccharide made of n disaccharide units divided by n, as a percentage of the summation of total activity, $(\sum a_n/n)/(\sum a_n)$ (Winterbourne & Mora, 1981). Alternatively, purified ^{35}S-labeled HS was used to determine the percentage of O- and N-sulfated residues as described by Keller et al. (1980). Nitrous acid treated HS was applied to a Bio-Gel P-2 column, and subsequent void volume and included fractions were pooled separately. The included fractions were then applied to a Sephadex A-25 column (1 × 10 cm) equilibrated in 10 mM Tris-HCl, pH 7.2, and eluted with a linear sodium chloride gradient (120 mL) to 1.5 M. The various column profiles were then used to determine the percentage of N-sulfation (the radioactivity included on the Bio-Gel P-2 column times the percentage of radioactivity eluting at an identical position as inorganic sulfate standard on the Sephadex A-25 ion-exchange column) and O-sulfation [the radioactivity migrating in void volume fractions plus the percentage of radioactivity not migrating as inorganic sulfate by ion-exchange chromatography (Keller et al., 1980)].

Gel filtration chromatography was performed on Bio-Gel P-2 or P-10 columns (0.8 × 120 cm, 200–400 mesh) equilibrated with 100 mM pyridinium acetate, pH 5.8, or on Sephacryl S-300 or S-1000 (1 × 100 cm) in 0.15 M sodium chloride and 50 mM Tris-HCl, pH 7.4. The Bio-Gel columns were calibrated with oligosaccharides generated by the degradation of HA (Winterbourne & Mora, 1981) and inorganic sulfate. Calibration of the Sephacryl columns was performed with dextrans (M_r 2×10^6, 5×10^5, 1.5×10^5, 7×10^4, 4.2×10^4, 1.8×10^4, and 9×10^3) and with CS and heparin (M_r 6.8×10^4 and 1.2×10^4, respectively).

Results

The accumulation of ^{35}S-labeled macromolecular material into the various cell fractions (medium, cell surface, extracellular matrix, and intracellular) with continuous exposure to the radioactive precursor is depicted in Figure 1. The cells derived from the primary tumor (MTPa, MTC, and MTF7; top panels in Figure 1) showed a continuous accumulation of labeled material into their medium over the 72 h of the experiment. In contrast, the metastases-derived cells (MTLY, MTLn2, and MTLn3; bottom panels in Figure 1) showed a plateau in their accumulation of ^{35}S-labeled material released into the medium after 12–24 h. In all other cellular fractions, radioactivity per milligram of protein reached a plateau after 6–12 h. Because the metastases-derived cells showed a plateau in released material after 24 h, most analyses were performed after 18–24 h of metabolic labeling. After 24 h of labeling, approximately 70% (68–75%) of the ^{35}S-labeled macromolecular material was released into the medium, 20% (14–21%) was found on the cell surface, and 5% (3–7%) each was associated with the matrix and intracellular fractions (Table I).

The composition of ^{35}S-labeled GAGs from the medium and cell surface fractions of the various rat mammary tumor cells indicated that only two sulfated GAGs were observed to be synthesized by these cells (Table I and Figure 2). From their migration on cellulose acetate electrophoresis compared to that of standard GAGs and their sensitivities to chondroitinase ABC and AC II or nitrous acid the GAGs were identified as heparan sulfate and chondroitin sulfate. The ratio of [^{35}S]HS to [^{35}S]CS was greater in the metastases-derived cells than in the primary tumor derived cells (Table I). Since all the cells incorporated approximately similar amounts of labeled precursor (on the basis of cpm/μg of protein) into the cell surface fraction and recoveries of all columns were about equal (83–97%) for the fractionation of GAGs, the HS/CS ratios reflect a differential expression of HS and CS between the primary tumor derived and metastases-derived tumor cells. In addition, the vast majority of ^{35}S-labeled macromolecular material (>95%) was observed to be associated with GAGs (Figure 2) so that the incorporation of [^{35}S]sulfate into macromolecules could be used to estimate the relative quantities of GAGs. However, since the sulfate label can be incorporated differentially into the various GAGs, quantitative alterations and specific activity of incorporated precursor were also determined with [^3H]glucosamine.

Analysis of [^3H]glucosamine-labeled GAGs indicated that the primary tumor cells displayed in their cell surface fractions about equal quantities of ^3H-labeled HS and CS, whereas the metastases-derived cells expressed at least fivefold more HS

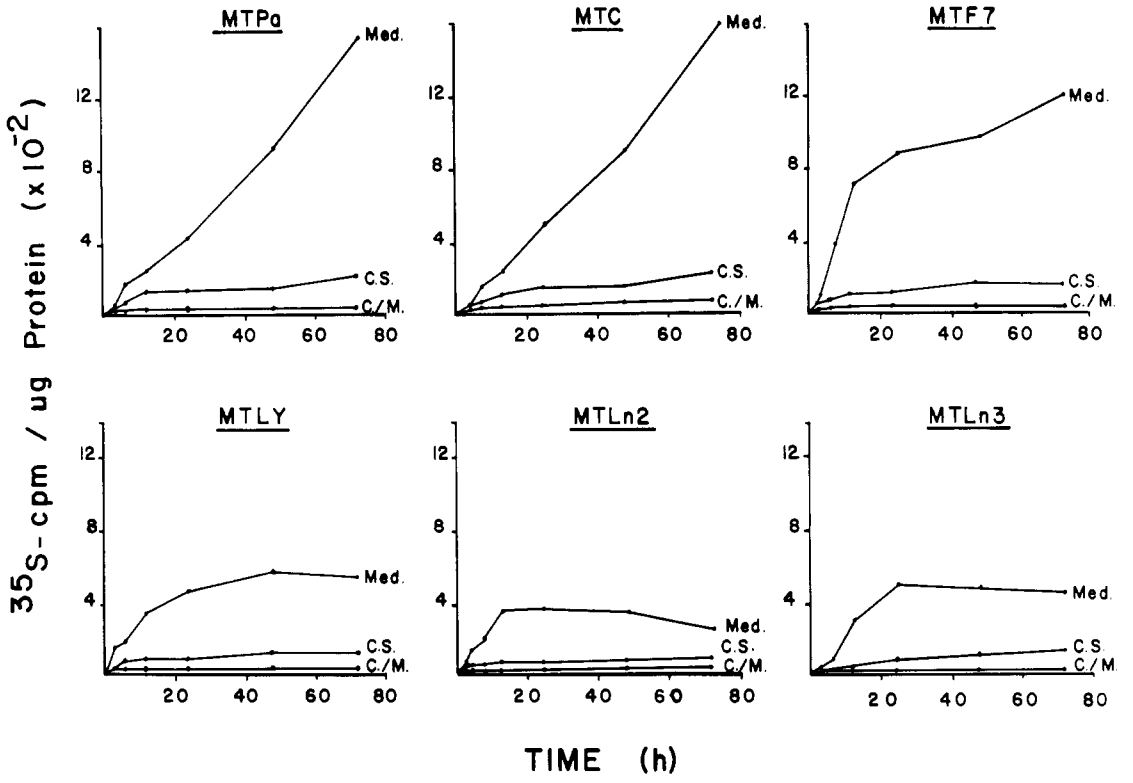

FIGURE 1: Time course of incorporation of [^{35}S]sulfate into GAGs per microgram of protein by cultures of 13762NF mammary adenocarcinoma sublines. The primary tumor derived cells are depicted in the top panels (MTPa, MTC, and MTF7), and the metastases-derived cells (MTLY, MTLn2, and MTLn3) are shown in the bottom panels. The quantity of ^{35}S-labeled macromolecular material in the various cellular fractions (medium, Med.; cell surface, C.S.; matrix, M.; and cell associated, C.) was determined as described under Experimental Procedures.

Table I: Composition of ^{35}S-Labeled Glycosaminoglycans in 13762NF Metastatic Mammary Tumor Cells

		medium[b]				cell surface			
cell line	anatomic location[a]	cpm/μg of protein	% of total[c]	HS (%)[d]	CS (%)[d]	cpm/μg of protein	% of total	HS (%)	CS (%)
MTPa	P	435	70.3	21	79	109	17.6	32	68
MTC	P	507	69.5	27	72	131	18.0	30	70
MTF7	P	725	78.6	18	82	109	11.8	45	55
MTLY	M	378	68.7	77	23	110	20.0	81	19
MTLn2	M	362	68.0	74	26	113	21.0	73	27
MTLn3	M	518	75.1	78	22	101	14.6	72	28

[a] Anatomic location from which tumor cells were derived: P, primary tumor, or M, metastasis. [b] Cells were incubated with [^{35}S]sulfate, and glycosaminoglycans were isolated from different cellular locations as described in text. [c] The percentage of total radioactivity incorporated into macromolecular material (Sephadex G-50 void volume fractions) based on cpm/μg of protein determined for the cell fraction. The remaining approximately 10% of incorporated radioactivity was found in matrix and cellular fractions. [d] Percentage represents the amount of total radioactivity associated with the separated GAGs after cellulose acetate electrophoresis. Abbreviations used are HS for heparan sulfate and CS for chondroitin 4-sulfate.

Table II: Composition of [^{3}H]Glucosamine-Labeled Glycosaminoglycans on the Cell Surface (cpm/μg of protein)

cell line	glycopeptides	hyaluronic acid	heparan sulfate	chondroitin sulfate
MTPa	223[a] (46%)[b]	71 (16%)	62 (18%)	48 (20%)
MTC	244 (54%)	68 (21%)	60 (11%)	54 (14%)
MTF7	684 (70%)	131 (10%)	103 (10%)	50 (10%)
MTLY	668 (71%)	110 (5%)	152 (22%)	29 (2%)
MTLn2	325 (64%)	116 (21%)	51 (11%)	7 (3%)
MTLn3	566 (65%)	93 (11%)	141 (20%)	19 (3%)

[a] [^{3}H]Glucosamine incorporated into macromolecular material per microgram of protein in cellular fractions. All cells were labeled simultaneously and processed at the same time under identical conditions. [b] Relative percentage of individual components based on ion-exchange column profiles (average of three independently processed samples). Recoveries of the columns averaged 93% (83–97% range).

Table III: Composition of [^{3}H]Glucosamine-Labeled Glycosaminoglycans in the Medium (cpm/μg of protein)

cell line	glycopeptides	hyaluronic acid	heparan sulfate	chondroitin sulfate
MTPa	1592[a] (60%)[b]	612 (25%)	50 (2%)	195 (13%)
MTC	543 (68%)	213 (18%)	23 (2%)	118 (12%)
MTF7	976 (61%)	346 (19%)	40 (3%)	295 (17%)
MTLY	830 (82%)	93 (6%)	123 (11%)	10 (1%)
MTLn2	628 (56%)	286 (33%)	93 (9%)	18 (2%)
MTLn3	738 (59%)	428 (24%)	249 (15%)	33 (2%)

[a] [^{3}H]Glucosamine incorporated into macromolecular material. All cells were labeled simultaneously and processed at the same time under identical conditions. [b] Relative percentage of individual components based on ion-exchange column profiles (average of three independently processed samples). Recoveries of the columns averaged 83% (68–97% range).

than CS (Table II). The macromolecular components found in the medium fractions also showed an altered expression after 24 h of metabolic labeling (Table III). For the primary tumor derived cells, the quantity of glycopeptides and hyaluronic acid showed a two- to three-fold increase, CS a fourfold increase, and HS no change in GAGs released into the medium compared with GAGs expressed on the cell surface. In contrast, the metastases-derived cells only showed a slight increase in

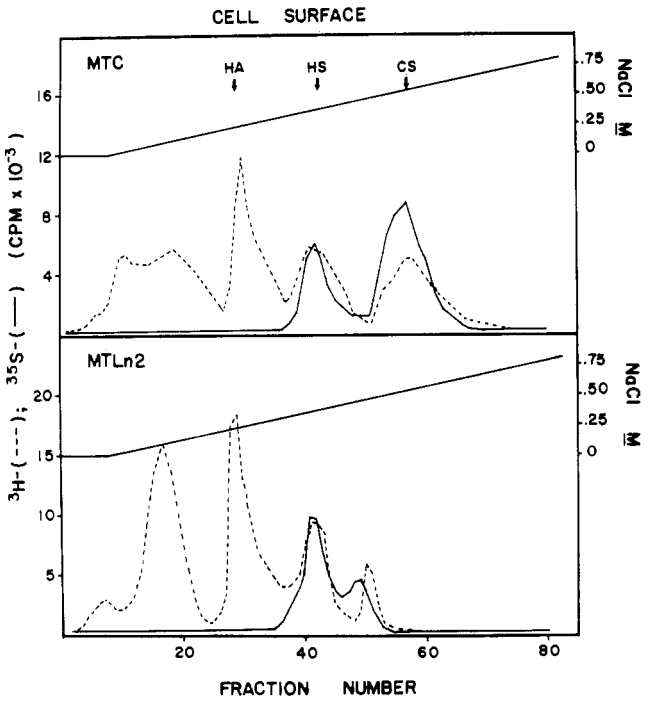

FIGURE 2: Chromatography elution patterns on DEAE-cellulose of GAG-associated macromolecular material, labeled for 24 h with [^{3}H]glucosamine (---) or [^{35}S]sulfate (—). The top panel shows the elution of GAG from MTC cells, and the bottom panel shows the GAG elution from MTLn2 cells. The GAGs were applied to a 5 × 1 cm column of DE-52 in 10 mM Tris-HCl, pH 7.2, and eluted with a linear gradient of NaCl (0.8 M). The arrows marked HA, HS, and CS represent the elution positions of standard hyaluronic acid, heparan sulfate, and chondroitin sulfate.

the quantity of all labeled released components to the cell surface fraction (Table III). All cells from the various cell lines were metabolically labeled at the same time, and all material was processed under identical conditions, so that the most direct comparisons could be made among the different cells (Vogel & Campbell, 1985). The percentages in Tables II and III represent an average of at least three independent determinations. The specific activities of incorporation of [^{3}H]glucosamine per microgram of uronic acid among the various cells were similar except for MTPa cells (specific activities of HA = 15 500 ± 2500 cpm/μg of uronic acid, HS = 28 700 ± 3600 cpm/μg of uronic acid, and CS = 36 000 ± 4000 cpm/μg of uronic acid, with the correlation coefficient >0.96). For MTPa cells, which represent a heterogeneous population of cells from the primary tumor, the specific activity was about threefold lower (HA = 5500 ± 600, HS = 9400 ± 600, and CS = 10 300 ± 700; Table IV). These data indicate differences in the chemical quantities of HS and CS synthesized by the various cells, not altered incorporation of the radiolabeled precursor.

CS from the metastases-derived cells consistently eluted from the ion-exchange columns at lower salt concentrations compared with the CS from the primary tumor cells and standard CS (Figure 2). Therefore, structural characterizations of the CS were undertaken. Individual GAGs were pooled from the DEAE-cellulose columns and subjected to enzymatic or chemical treatments (HS was incubated with chondroitinase ABC, CS and HA were treated with nitrous acid, and CS was subsequently treated with *Streptomyces* hyaluronidase). The isolated GAGs were purified by passage through PD-10 columns. CS from the various cells was then treated with chondroitinase ABC, and the fractions were subjected to thin-layer chromatography to separate the resulting disaccharides. Analysis of the CS disaccharides revealed that CS was sulfated almost exclusively at the 4-position (chondroitin 4-sulfate) by their migration with authentic ΔDi-4S and sensitivity to chondroitin 4-sulfatase. Of the [^{3}H]glucosamine-labeled disaccharides from the primary tumor derived cells, 96 ± 1% comigrated with standard ΔDi-4S and the remainder with ΔDi-0S in four independent experiments. In contrast, decreased sulfation of CS was observed in the metastases-derived cells MTLY, MTLn2, and MTLn3 as the quantity of ΔDi-0S increased to 16%, 14%, and 18% (±2%), respectively. No significant differences were demonstrated between CS obtained from either the cell surface or the medium fractions.

Table IV: Hyaluronic Acid—Specific Activity and Chain Size

cell line	sp act. (cpm/μg of uronic acid)	cell surface K_{av}	medium K_{av}
MTPa	4100[a]	0.38[b]	0.42 + 1.0[b]
MTC	13 800	0.32	0.61 + 1.0
MTF7	14 800	0.42	0.52 + 1.0
MTLY	17 700	0.45	0.83 + 1.0
MTLn2	15 200	0.35	0.56 + 1.0
MTLn3	15 900	0.35	0.52 + 1.0

[a] The specific activity of incorporation of [^{3}H]glucosamine into isolated hyaluronic acid was assayed by the method of Bitter and Muir (1962). All cells were metabolically labeled and processed independently, but at the same time and under similar conditions. The conversion factor between hyaluronic acid and glucuronolactone was determined to be 2.2. [b] The relative elution profiles of HA from the various cells were determined on a calibrated Sephacryl S-1000 column (1000 × 0.8 cm). The column was equilibrated with 0.2 M NaCl, 50 mM sodium acetate, 5 mM EDTA, and 0.05% sodium azide, pH 6.0, and eluted at a flow rate of 8 mL/h, and recoveries ranged from 83% to 94%. The column was calibrated with high molecular weight DNA and sodium sulfate.

The N-sulfation of glucosamine residues on HS was determined by treatment with nitrous acid followed by gel filtration on Bio-Gel P-10 columns (Figure 3). HS from 13762NF cells resulted in similar Bio-Gel P-10 elution profiles, and calculations estimated that 46.3 ± 1.3% of the glucosamine residues were N-sulfated (Winterbourne & Mora, 1981). The ratio of N- to O-sulfated residues was examined by using [^{35}S]heparan sulfate according to the method described by Keller et al. (1980). The mean percentage of O-sulfation was 51.9 ± 3.8, whereas that of the N-sulfation was 49.4 ± 3.3 for all 13762NF cells. Furthermore, no qualitative differences in the elutional profiles of the O-sulfated diamination fragments of HS were observed on Bio-Gel P-10 Columns. The majority of O-sulfated degradation products were eluted in the position of di- and tetrasaccharides. In addition, the remainder of glucosamine residues that were not N-sulfated were N-acetylated, since significant degradation was not observed under conditions that degrade unsubstituted residues (reaction B; Lindahl et al., 1973). From the percentage of N-sulfation and ratio of N- to O-sulfation, we estimated that from 90% to 95% of the sugar residues of HS are sulfated.

The sizes of the various GAGs were examined by gel filtration on a Sephacryl S-1000 column for HA (Table IV) and a Sephacryl S-300 column for CS and HS (Table V). In general, the primary tumor derived GAGs processed a larger relative molecular weight than those of the metastatic lesions. This difference was most pronounced for the cell surface CS from metastases-derived cells, which were almost half the size of the CS from primary tumor cells [average M_r 36 300 vs. M_r ~54 400 for primary cells (approximately a 1.5-fold difference; Table V)]. However, this difference in GAG chain length could not solely account for the quantitative differential expression of CS between the primary tumor derived and

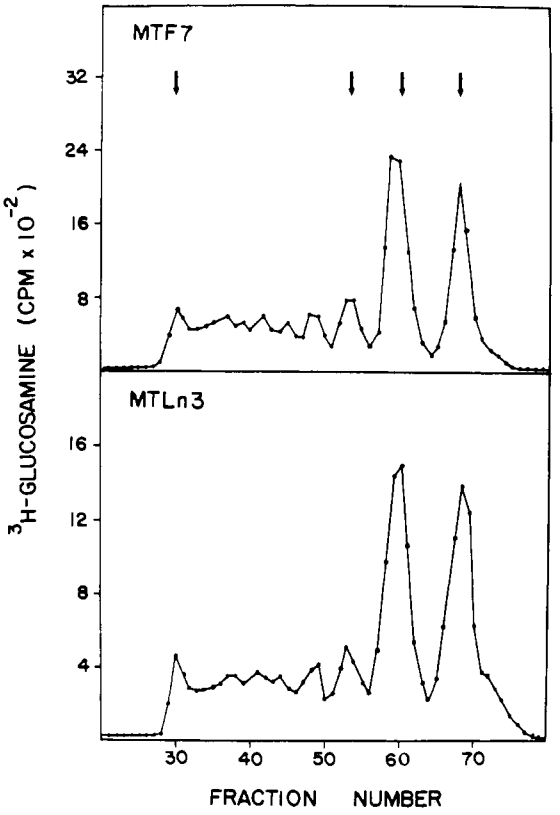

FIGURE 3: Chromatography on Bio-Gel P-10 of low-pH nitrous acid degradation products of HS labeled with [^{3}H]glucosamine from MTF7 and MTLn3 cells. The samples were applied to a column (1000 × 0.5 cm) and eluted with 0.1 M pyridinium acetate, pH 5.8. The arrows, from left to right, indicate the elution positions of the void volume fraction (blue dextran), hexasaccharides, tetrasaccharides, and disaccharides, generated by the degradation of HA with hyaluronidase, as described under Experimental Procedures.

Table V: Glycosaminoglycan Chain Size

	heparan sulfate		chondroitin sulfate	
cell line	medium	cell surface	medium	cell surface
MTPa	28 000[a]	28 000	63 000	63 000
MTC	38 000	38 000	50 000	50 000
MTF7	50 000	50 000	50 000	50 000
MTLY	21 000	21 000	33 000	38 000
MTLn2	33 000	38 000	33 000	38 000
MTLn3	33 000	33 000	33 000	33 000

[a] The relative molecular weights of GAG chains were determined on a calibrated Sephacryl S-300 column (1000 × 0.5 cm). The average range of error of estimated M_r was 3000. The column was equilibrated with 0.2 M NaCl, 50 mM sodium acetate, and 5 mM EDTA, pH 6.0, and eluted at a flow rate of 20 mL/h, and recoveries ranged from 80% to 90%. The column was calibrated with chondroitin sulfate (M_r ~ 60 000) and heparin (M_r ~11 000), which was parallel to a calibration curve generated by using dextrans.

metastases-derived cells. The average cpm of CS/μg of protein on the cell surface fraction for MTC and MTF7 cells was 52 (Table II), and the adjusted average based on size was 34.5 (52/1.5), whereas for metastases-derived cells, the average was 18.3 for CS chains. This calculation suggests there are about twice the number of CS chains on the primary tumor derived cells as compared with metastases-derived cells. Alternatively, on the basis of percentage of [^{3}H]glucosamine incorporated, CS represents 14.6% or 8.6% (14.6/1.5), including GAG chain length difference, for the primary tumor derived cells compared to 2.3% for metastases-derived cells (Table II).

The GAG medium fraction also shows an increased differential expression of CS compared to the cell surface fraction (Tables II and III). Furthermore, the CS of the metastases-derived cells was smaller in the medium fraction than on the cell surface fraction, suggesting that some degradation may have occurred. The HA released into the medium separated into two peaks eluting at K_{av} = 0.42–0.82 and 1.0, which also suggested that some degradation of HA may have occurred (Table IV). Both HA peaks were mostly degraded by treatment with *Streptomyces* hyaluronidase.

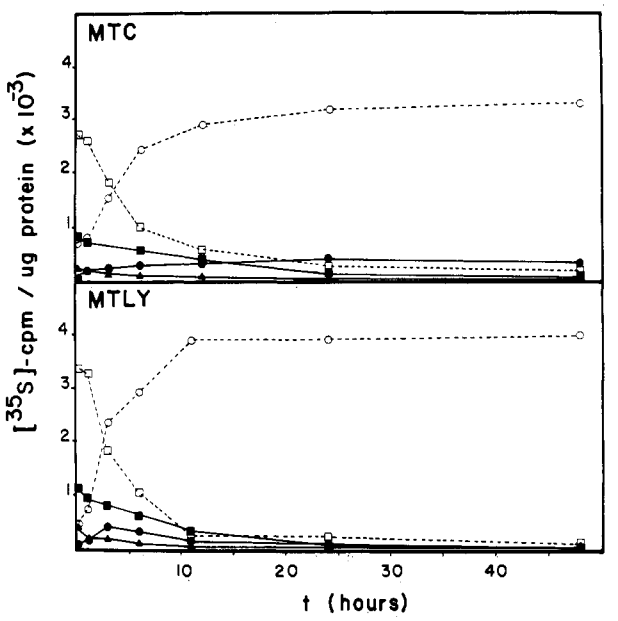

FIGURE 4: Change of ^{35}S-labeled material in the various cellular fractions of MTC cells (top panel) and MTLY cells (bottom panel) during periodic chase times following a 4-h pulse labeling with [^{35}S]sulfate. The total radioactivity (○, □) and macromolecular radioactivity (●, ■, ▲) associated with the various cellular fractions are shown: extracellular medium (○, ●), cell surface (□, ■), and cellular (▲). The quantity of total radioactivity found in the cellular fractions was similar to that of the macromolecular determinations. The amount of ^{35}S-labeled materials was assayed as described under Experimental Procedures.

We then examined the metabolism of endogenous GAGs. The primary tumor derived cells showed a continuous accumulation of GAGs into their medium, whereas the metastases-derived cells showed a plateau after 24 h (Figure 1). MTF7 cells, the primary tumor derived cells that display the greatest metastatic potential (Neri et al., 1982), also approached a stable level of accumulation of GAGs in the medium, although this plateau was not as pronounced as that for the metastases-derived cells. Pulse–chase kinetic experiments were done to determine the GAGs' stability and degradation of the GAGs (Figure 4). The primary tumor derived MTC cells accumulated ^{35}S-labeled macromolecular and total labeled material into their medium over a 48-h chase period, with an accompanying loss of radioactivity from cell surface and cellular fractions after 4 h of metabolic labeling (pulse period). In contrast, the metastases-derived MTLY cells accumulated macromolecular material into their medium only during the first 6 h after the pulse, and this was followed by a loss of the sulfated macromolecular material. The total radioactivity distribution for MTPa and MTF7 cells was similar to that observed for the MTC cells (Figure 4) except that the MTF7 cells' ^{35}S macromolecular GAGs began to decrease after about 24 h. The MTLn2 and MTLn3 cells' kinetic distribution of radioactivity paralleled that seen for MTLY. These data agree with those seen for the continuous-label accumulation studies. Furthermore, during the 4-h pulse period, more radioactive sulfate was incorporated into GAGs from the metastases-derived cells than the primary tumor derived cells (930 ± 140 cpm/μg of protein compared with 1200 ± 200 cpm/μg of

Table VI: Turnover and Composition of Glycosaminoglycans after 4-h Labeling and Chase Intervals

		composition[b]							
		0 h		6 h		12 h		24 h	
cell line	half-life[a]	HS (%)	CS (%)	HS (%)	CS (%)	HS (%)	CS (%)	HS (%)	CS (%)
MTPa									
cell surface	7.5	63	37	65	35	69	31	nd[c]	nd
medium	12			57	43	53	47	28	72
MTC									
cell surface	10	46	54	35	65	30	70	nd	nd
medium	9.5			65	56	30	70	26	74
MTF7									
cell surface	7	67	33	69	31	69	31	nd	nd
medium	3.5			65	35	59	41	53	47
MTLY									
cell surface	6.5	90	10	93	7	90	10	nd	nd
medium	1.5			96	4	90	10	87	13
MTLn2									
cell surface	6	91	9	92	8	92	8	nd	nd
medium	1			91	9	91	9	86	14
MTLn3									
cell surface	3.25	94	6	84	16	85	15	nd	nd
medium	0.75			94	6	94	6	83	17

[a] The half-life in hours was determined by the loss of 50% of the ^{35}S-labeled macromolecular material from the cell surface fraction. The half-life of medium macromolecular material was calculated by the time required to accumulate 50% of the GAGs into the medium. Both determinations were performed by computer programs. [b] The composition of ^{35}S-labeled GAGs in the various cellular fractions was determined by cellulose acetate electrophoresis as described under Experimental Procedures after various chase periods. [c] Not determined.

protein, respectively). When the half-lives of macromolecular sulfated GAGs were determined for the loss of GAGs from the cell surface fraction and their accumulation in the medium (Table VI), the half-lives of GAGs in both cellular fractions were shorter for metastases-derived cells than primary tumor derived cells. The differential metabolism of GAGs is particularly interesting for the GAGs in the medium because their half-lives are lower in cells with the greater metastatic potential (Neri et al., 1982).

The composition of the sulfated GAGs in the medium and cell surface fractions was determined at various times after the pulse period of 4 h (Table VI). For all cells the composition of the ^{35}S-labeled GAGs on the cell surface remained constant over the chase period, except for MTC cells in which the percentage of CS increased (54–70% in 12 h). The composition of the medium was similar to that of the cell surface fraction through 12 h, when the percentage of CS increased for all cells, especially the primary tumor derived cells. Since the quantity of ^{35}S-labeled GAGs in the medium for the primary tumor derived cells remained relatively constant once the plateau was reached (Figure 4) and the composition also increased toward CS (Table VI), the data suggest that some HS degradation was occurring. This agrees with comparisons of the GAG composition between medium and cell surface fractions as presented in Tables II and III, which show that only a slight increase in the HS medium fraction was seen compared with HA and CS. In contrast, the metastases-derived cells showed a decrease in ^{35}S-labeled GAGs in the medium after an initial accumulation, but the ratio remained approximately the same (a slight increase toward CS was observed), suggesting that both HS and CS were being degraded. These data also agree with only a slight increase in released material over that observed in the cell surface fraction after 24 h of metabolic labeling (Tables II and III).

Discussion

Glycosaminoglycans, particularly HS, have been implicated in a number of cellular processes including growth regulation, adhesive properties, and modulation of cell surface receptors (Ohnishi et al., 1975; Castellot et al., 1981; Ratner et al., 1985; Lark & Culp, 1982, 1983, 1984; Kraemer & Smith, 1974). The majority of these cellular properties are altered by transformation, and these are generally accompanied by modulation of GAG expression and synthesis (Kraemer, 1979). Although a number of studies have examined the modulation of GAGs in cellular transformation or between tumor and normal cells, little attention has been focused on the characterization of GAGs synthesized by primary tumor cells and their metastases. A recent study (Turley & Tretiak, 1985) suggested that more HA and HS were released from B16 melanoma cells with enhanced metastatic capabilities but that they formed a less organized extracellular matrix than their low metastatic counterparts.

We examined the expression and metabolism of GAGs of tumor cells derived from a rat mammary adenocarcinoma and its subsequent metastatic lesions. Of the sulfated GAGs, the metastases-derived cells (MTLY, MTLn2, and MTLn3 sublines) expressed a significantly higher percentage of HS on their cell surface and released more HS into the medium than did the primary tumor derived cells that expressed relatively more CS. This modulation of GAGs was observed to be the result of decreased synthesis of CS by the metastases-derived cells. Cell cycle variation of GAG synthesis has been described (Preston et al., 1985), yet all of the 13762NF carcinoma cells' doubling times were found to be similar (Neri et al., 1982), and the cells were in exponential growth during the course of our studies. Although decreased synthesis of CS and increased synthesis of HA have been reported upon transformation of chondroblasts and fibroblasts (Hopwood & Dorfman, 1977; Muto et al., 1977; Shanley et al., 1983), the metastatic properties of these cells were not examined. In our studies, altered GAG expression was demonstrated in the quantity of GAGs; it was not a result of altered incorporation of radioactive precursor, nor was it caused entirely by a decrease in the molecular weight of the CS chains. The specific activity of synthesized radioactive GAGs from the MTPa cells was about one-third that observed for the other cells, but the overall pattern of GAG expression was similar to that of the other primary tumor derived cells, MTC and MTF7. This different specific activity was probably a result of the heterogeneity of the MTPa cell line being derived from a primary tumor explant (Neri et al., 1982; Iozzo, 1985).

The decrease in the synthesis of CS in the metastases-derived 13762NF cells was accompanied by structural alterations, such as undersulfation and smaller size of CS chains. The CS consistently eluted from anionic ion-exchange columns at lower ionic strength compared with standard CS, and analysis of disaccharides generated by treatment with chondroitinase ABC or AC suggested that 12–18% were not sulfated. Undersulfation of CS has been reported for chondroblasts, although it was observed for both normal and transformed cells (Shanley et al., 1983). The CS chains in the metastases-derived cells were also demonstrated to have a significantly smaller molecular weight than the CS from primary tumor derived cells. The decreased length of CS may reflect their increased rates of synthesis (Robinson et al., 1975). This would agree with the increased incorporation of [^{35}S]-sulfate into macromolecular components during a 4-h pulse for the metastases-derived cells as compared with the MTC and MTF7 cells. Our preliminary studies also indicated that the altered expression of CS is reflected in the differential synthesis of chondroitin sulfate proteoglycans between the metastases-derived and primary tumor derived cells (Steck et al., 1985). We are currently investigating whether the altered CS chains are preferentially linked to different proteoglycans.

Altered sulfation of HS in transformed cells and cells with increased tumorigenicity has been reported (Keller et al., 1980; Winterbourne & Mora, 1981; Robinson et al., 1984). All the 13762NF cells' HS chains were sulfated at about half the glucosamine residues, and about three-fourths of these were found in di- and tetrasaccharides. The vast majority of HS residues also appeared to be either N-sulfated or N-acetylated. In addition, the proportion of O-sulfation to N-sulfation was about equal. The O-sulfation also was observed mainly on regions of high N-sulfation, consistent with findings of other investigators (Cifonelli & King, 1977; Winterbourne & Mora, 1981). These results suggest that no significant alterations were found in the structure of HS chains among the various 13762NF cells examined.

In contrast to other reports on mammary carcinoma cells, no significant variations in the expression of hyaluronic acid were observed among the 13762NF cells. The majority of HA was found to be released into the medium, especially for the primary tumor derived sublines. The metastases-derived cells released HA into their medium that had a smaller K_{av} on Sephacryl S-1000 columns than did primary tumor cells, although all cells had a peak near the total volume of the column, suggesting that some degradation has occurred. In addition, Underhill and Toole (1982) have shown that transformation of fibroblasts is accompanied by a decreased cellular coat of HA, and this variation is paralleled by an increase in hyaluronidase activity (Orkin et al., 1982). Although no major changes in HA expression were observed among the various 13762NF cells, differences in the metabolism of HA cannot be excluded.

We found that 13762NF cells possessed altered metabolism of the sulfated GAGs between the primary tumor derived and metastases-derived cells. The plateauing of accumulation of ^{35}S-labeled GAGs in the medium of metastases sublines suggested several possibilities. First, the cells released degradative enzymes, so the rate of synthesis and degradation of GAGs became similar; alternatively, GAG synthesis is repressed upon prolonged culturing of the metastases-derived sublines. The first possibility was supported by several studies. The pulse–chase kinetic experiment demonstrated that the primary tumor derived cells accumulated and maintained the GAGs in their medium after a 4-h pulse, whereas the metastases-derived cells displayed an initial accumulation followed by a loss of macromolecular GAGs in the medium. Furthermore, analysis of the GAG composition in the medium as compared to GAGs originally expressed on the cell surface showed little change for the metastases-derived cells, but the primary tumor sublines showed an increasing percentage of CS. Since the composition of GAGs in the metastases-derived cells was only slightly altered and yet the total quantity of labeled GAG was decreased, this suggested that both sulfated GAGs were degraded. Also, there was a constant decrease in radioactivity associated with the cellular fractions during the chase period that indicated the loss of released GAGs was not caused solely by reinternalization. In contrast, the primary tumor sublines displayed an increased percentage of CS in the medium while the total quantity of GAGs remained approximately constant, suggesting some degradation of HS. Furthermore, no significant differences in the synthesis of GAG were observed when [^{3}H]glucosamine was added to the cells after various periods in culture, suggesting that repression of GAG synthesis was not occurring. The combination of these results suggested that the 13762NF mammary tumor cells all express a heparanase activity, whereas the metastases-derived cells also display a chondroitin lyase activity.

However, the alteration in the expression of CS chains on 13762NF cells was probably not solely due to the chondroitinase activity, because after a 4-h pulse, there was an altered expression of CS on the cell surface of the metastases-derived cells compared to the primary tumor derived cells. If chondroitinase activity were solely responsible for such a variance, the differential expression of HS and CS would be expected on the cell surface fraction during the chase period, unless chondroitinase were acting on internally synthesized CS chains. However, no differences in the ratios of HS and CS expression were observed.

The roles of extracellular GAG degradatory activities in metastatic process are unknown. Heparanase activity has been suggested to play key roles in the invasion of extracellular matrix, particularly the basal laminae of endothelial cells (Kramer et al., 1982; Nakajima et al., 1984), and also in the migratory properties of cells (Lark & Culp, 1982, 1983). Although heparanase activity was observed for all the rat 13762NF tumor cells, the chondroitinase activity was most pronounced on the tumor cells derived from metastases. Since it is well recognized that metastasis is a complex and sequential processes (Nicolson & Poste, 1983; Nicolson, 1984), it is doubtful that these degradatory activities could be solely responsible for metastatic behavior. However, the alteration of mammary tumor GAGs, and enzymatic activities directed against the GAGs, may allow us to identify a few of the cellular properties important in metastasis.

Registry No. Heparan sulfate, 9050-30-0; chondroitin 4-sulfate, 24967-93-9; hyaluronic acid, 9004-61-9.

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