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# Influence of collagen substrata on glycosaminoglycan production by B16 melanoma cells

(extracellular matrix)

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**ABSTRACT** **A cloned metastatic murine melanoma cell line exhibited similar growth characteristics when propagated on either type I collagen, type IV collagen, or plastic. However, cells grown on both types of collagen exhibited an altered cellular morphology and on type IV collagen only, an increased substrate adhesiveness, relative to those maintained on a plastic substratum. Incorporation of [$^3$H]glucosamine and [$^{35}$S]sulfate into glycosaminoglycans (GAGs) of cells grown on collagen substrates was 20% and 40% less, respectively, than cells grown on plastic, whereas degradation of cell-associated [$^{35}$S]sulfate-labeled GAGs was similar in cells grown on collagen or plastic. Although the composition of GAGs was similar in all cultures, consisting of approximately 60% chondroitin and 40% heparin or heparan sulfate, the degree of sulfation of the heparin or heparan sulfate molecules was markedly decreased in cultures grown on collagen. The results indicate that the composition of the extracellular matrix influences the biological behavior of B16 melanoma cells, in part by altering the amount and nature of the GAG molecules produced.**

Glycosaminoglycans (GAGs) are long-chain polyanionic carbohydrates that include hyaluronic acid, chondroitin 4- and 6-sulfates, keratan sulfate, dermatan sulfate, heparin, and heparan sulfate (1). These macromolecules are found in most mammalian cells (2) and are believed to play an important role in migration (3–6), maintenance of morphogenetic structure (7), and cellular differentiation (8, 9). Despite intensive study, little is known about the factors that regulate the biosynthesis of these polysaccharides. However, previous studies have suggested that the extracellular environment can influence the rate of synthesis and secretion of these macromolecules (10, 11).

Collagen is a major constituent of the extracellular matrix. Type I collagen is found predominently in bone, dermis, and tendon, whereas type IV collagen is found in basement membranes (12). Various types of collagen bind to GAGs both in their native proteoglycan state and in the form of polysaccharide chains (13–18). For this reason, the coordinated biosynthesis of collagen and proteoglycan has been investigated (19–21); both of these kinds of macromolecules appear to be produced in the same cytoplasmic region (22), and exogenous proteoglycan has been found to depress both proteoglycan and collagen synthesis by chondrocytes (23).

Changes in GAG synthesis of cells exposed to collagen substrata have been reported for various nonmalignant lines, including corneal epithelial cells from chicken embryos (24), chicken embryo somites (25), mammary epithelia (26, 27), and human skin fibroblasts (28). All of these studies have shown that cells grown on collagen substrata incorporate greater amounts of [$^{35}$S]sulfate into GAGs than do cells propagated on plastic. A collagen-mediated reduction in the rate of cell-associated GAG degradation rather than an actual change in the rate of synthesis of these macromolecules has been postulated (26, 27). However, mammary epithelial cells that have spontaneously transformed to a malignant phenotype are less effective than normal cells in decreasing proteoglycan degradation in response to collagen (29). Other malignant lines have been reported to show alterations in both the synthesis of cell surface proteins and their interaction with matrix proteins (30, 31). Because malignant melanoma cells are exposed to type I collagen during local invasion into the dermis and are in contact with type IV collagen during metastatic spread into the vasculature, we have investigated GAG production by a cloned malignant B16 melanoma line grown on type I collagen, type IV collagen, or plastic to gain information on the influence of these collagen substrata on the formation of these polysaccharides.

## MATERIALS AND METHODS

**Cell Culture.** A B16 melanoma clone (B16/YM1) was derived from a lung nodule resulting from an intravenous injection into a C57BL mouse of the B16/F1 murine melanoma obtained from I. J. Fidler. This line had metastatic potential, with 74% of mice injected with $2 \times 10^4$ cells forming lung colonies. Cells were maintained in Eagle's minimal essential medium with Hanks' salts (MEMH, GIBCO) supplemented with 10% fetal calf serum, L-glutamine, nonessential amino acids, sodium pyruvate, and minimal essential medium vitamin solution. The original cloned cell line was passed several times in tissue culture and then frozen. Cell cultures were reinitiated from frozen stocks every 3 wk.

Collagen type I was isolated from mouse tails according to the procedure of Michalopoulos and Pitot (32). Type IV collagen, obtained from a transplantable murine sarcoma, was provided as a gift from Joseph Madri and Heinz Furthmayr of this institution. Tissue culture dishes were coated with various concentrations of collagen in 0.5 M acetic acid, and the dishes were then air-dried at room temperature and rinsed with serum-free medium to remove the acetic acid; thus, these collagen preparations represent molecules that are partially degraded (33).

For measurements of the rates of cellular proliferation, B16/YM1 cells were plated at a density of $3.5 \times 10^3$ cells per cm$^2$ on untreated or collagen-treated plastic dishes. At various times thereafter, the medium was removed and cells were detached by exposure to 0.25% trypsin for 3 min at 37°C, followed by gentle pipetting. The cell numbers were determined by using a model ZBI Coulter Particle Counter. Cultures were photographed with an inverted microscope with a phase-contrast objective.

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Abbreviation: GAG, glycosaminoglycan.

Susceptibility to detachment with EDTA was measured 12 hr after B16/YM1 cells were seeded. This was accomplished by removal of medium and incubation of plates with 2 mM EDTA for 10 min at 37°C. Medium was then added, the supernatant removed, and the number of cells therein determined. Residual cells attached to the plates were removed by treatment with 0.25% trypsin and their numbers ascertained as described above.

**Radiolabeling, Compartmentalization, and Quantitation of GAGs.** Incorporation of radioactive precursors into GAGs was measured by plating cells in culture cluster wells at an initial density of $3.5 \times 10^3$ cells per $cm^2$. Wells were either uncoated or coated with 0.7 or 7 $\mu$g of collagen I or collagen IV per $cm^2$. After 24 hr, cultures were incubated with 9 $\mu$Ci of [$^3$H]glucosamine (22.6 Ci/mmol; 1 Ci = $3.7 \times 10^{10}$ Bq) per ml and 18 $\mu$Ci of [$^{35}$S]sulfate (1 Ci/mmol) per ml in Eagle's minimal essential medium as modified by Joklik (MEMJ, GIBCO) for an additional 24 hr. At the end of each incubation period, radiolabeled GAGs were monitored in the medium and cell-associated fraction of each well. Initial experiments yielded essentially identical results with both 0.7 and 7 $\mu$g of collagen per $cm^2$. Therefore, these results were pooled for analysis and subsequent experiments were performed only with the higher concentration. Relative acid-soluble precursor pool sizes were determined by precipitation with 1% phosphotungstic acid in 0.5 M HCl, followed by two extractions with 10% trichloroacetic acid, and were found to be equal by 24 hr for both precursors. For chase experiments, cells were seeded for 24 hr at an initial density of $7 \times 10^3$ cells per $cm^2$ in uncoated plastic dishes and in dishes coated with collagen. After an additional 12-hr period during which time cells were exposed to 18 $\mu$Ci of [$^{35}$S]sulfate per ml, medium was removed and replaced with MEMJ/10% fetal calf serum containing no radioisotopes, and incubations were continued for various periods of time.

For measurement of the compartmentalization of GAGs, media and a phosphate-buffered saline washing of the cellular monolayer were combined and designated as the medium compartment. The cells were detached by treatment with 0.1% trypsin for 3 min at 37°C and their numbers determined. The medium compartment, the cell suspension, and the culture well were each treated with 0.5 mg of collagenase (235 units/mg, type IV, Sigma) suspended in serum-free medium at 37°C for 12 hr. All material was then heated to 50°C for 1 hr to denature any remaining collagen. The cell suspension was treated with an equal volume of 3 mM $MgCl_2$ containing DNase at 0.08 mg/ml (34). The culture wells, cell suspensions, and medium were then incubated at 37°C for 12 hr with 0.1 mg of proteinase K. The wells were washed with distilled water and this material was added to the cell suspension and referred to as the cell-associated fraction. Each compartment was adjusted to pH 12 with 1 M NaOH to remove possible peptide residues from the linkage region and 1 hr later samples were neutralized and 200 $\mu$g of chondroitin sulfate was added. Because heavily sulfated GAGs have been reported to bind to collagen with high affinity (14), collagen-coated and uncoated plastic plates were incubated with 0.2% $NaDodSO_4$ for 1 hr at 37°C to ensure that all of the radiolabeled GAGs were being analyzed. Under these conditions, <10% of the incorporated cellular radioactivity remained on the substratum after removal of cells.

GAGs were quantitated by precipitation with an equal volume of 80 mM $Na_2SO_4$ containing 2% cetylpyridinium chloride at 37°C for 1 hr. Precipitates were collected on 0.45-$\mu$m HA Millipore filters and washed with 15 ml of 1% cetylpyridinium chloride/40 mM $Na_2SO_4$. The washed filters were placed in scintillation vials, 10 ml of Biofluor (New England Nuclear) was added, and radioactivity was determined with an ambient temperature scintillation spectrometer.

Aliquots from the pooled medium and cell-associated compartments, representing the total GAGs of cells grown on untreated or collagen I- or collagen IV-treated plastic dishes, were applied to a column (2 × 100 cm) of DEAE-cellulose (DE52, Whatman) equilibrated with 0.1 M LiCl and eluted at 60°C with 1.5 M LiCl. The macromolecular material was collected and lyophilized. Portions were then treated with 50 units of neuraminidase from *Vibro cholerae* (Calbiochem–Behring) and applied to a Sephadex G-50 column (1 × 100 cm), eluted with 0.1 M ammonium acetate in 10% ethanol, and the macromolecular peak was collected and lyophilized. Equal amounts of the macromolecular peak were used without treatment or were treated with either hyaluronidase from *Streptomyces hyalurolyticus* (Miles) (35), chondroitin ABC lyase, (pH 8, Sigma) (36), or nitrous acid (33); these materials were reapplied to the Sephadex G-50 column. Duplicate experiments were performed by using a high-performance gel permeation chromatography system (TSK 3000, Toyasoda). The percentage of degradable material present in each sample was calculated for cells grown on untreated, collagen I-treated, or collagen IV-treated plastic dishes.

Ion-exchange elution profiles of GAG molecules were determined by applying aliquots of neuraminidase-treated macromolecular material to a DEAE-cellulose column, and elution was conducted with a 0.15–1.5 M LiCl linear gradient.

## RESULTS

The effects of collagen substrata on the proliferation of B16/YM1 melanoma cells in culture were measured (Fig. 1). The rate of replication of the murine melanoma cells was not influenced by the composition of the substratum upon which they were cultured. However, differences in cellular morphology were observed between cells grown on plastic and collagen-coated surfaces, as assessed by phase-contrast microscopy (Fig. 2). The altered cellular morphology, which occurred on both types of collagen substrata but was more pronounced on the collagen I substratum, was characterized by decreased spreading of the B16/YM1 cells. These differences persisted throughout the incubation period.

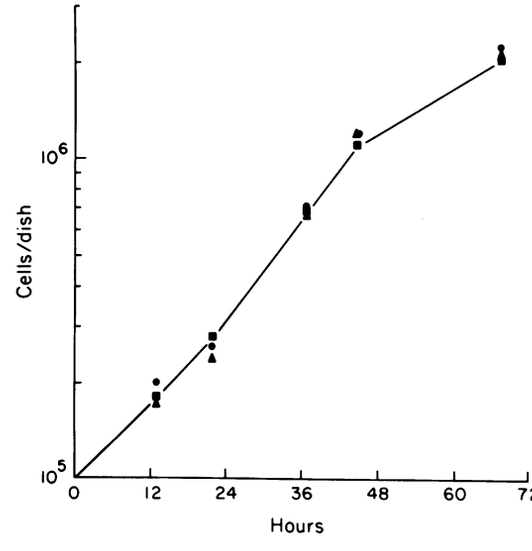


FIG. 1. The rate of replication of B16 melanoma cells on plastic, collagen I, and collagen IV substrata. Cells were plated at a density of $3.5 \times 10^3$ cells per $cm^2$ on untreated plastic tissue culture plates (■), plates coated with collagen I (●), or plates coated with collagen IV (▲). Cell numbers were determined with a model ZBI Coulter Particle Counter after their removal by treatment with trypsin. Deviation between flasks was <10%.

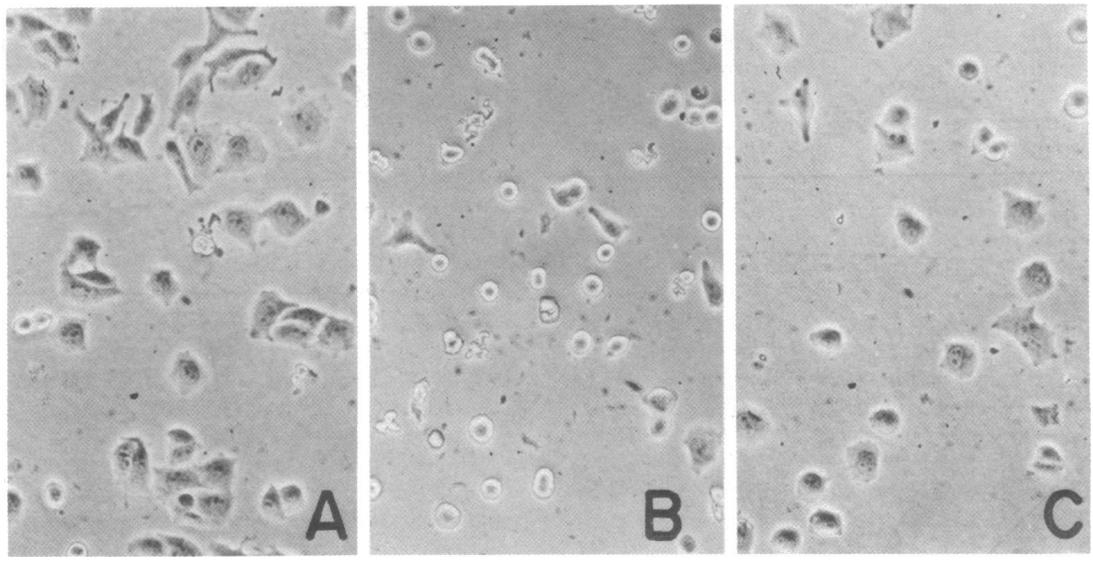


FIG. 2. The morphological appearance of B16 melanoma cells on uncoated (*A*), on collagen I-coated (*B*), and on collagen IV-coated (*C*) plastic dishes 4 hr after seeding. (×540.)

Because GAGs and collagen are components of the cellular adhesion site (33), we have sought information on the importance of the interaction of these macromolecules to cellular adhesion by measuring the susceptibility of the cells to EDTA-induced detachment from the various substrata. Cells were plated and permitted to develop attachment sites for 12 hr and then they were treated with 2 mM EDTA for 10 min at 37°C. Cells were less readily detached from collagen IV than from either uncoated plastic or from a collagen I substratum (Fig. 3). Thus, only 6% of the cells growing on collagen IV-coated flasks were detachable under the conditions of assay, compared to 28% and 29% of cells on collagen I-coated or uncoated plastic, respectively.

The effects of the collagen substrata on the production of GAGs were measured. As shown in Table 1, B16 melanoma cells grown on collagen I- or collagen IV-coated tissue culture dishes were similar in that they incorporated 20% and 40% less [$^3$H]glucosamine and [$^{35}$S]sulfate, respectively, into cetylpyridinium chloride-precipitable GAGs than cultures grown on plastic. The distribution of radiolabeled GAGs between the medium and cellular compartments was similar for all of the substrata employed, with 70% of [$^3$H]glucosamine-labeled and 90% of [$^{35}$S]sulfate-labeled GAGs being excreted into the medium. Because alterations in the degree of incorporation of precursors into GAGs by cells grown on collagen have previously been attributed to changes in the rate of degradation of the macromolecules (26, 27), the processing of GAGs synthesized by B16/YM1 cells was evaluated. Cells were labeled with [$^{35}$S]sulfate for 12 hr, the medium containing the radioactive precursor was removed, and the cultures were washed and chased in label-free medium. The total and cell-associated [$^{35}$S]GAGs in cultures grown on uncoated plastic, collagen I, or collagen IV decreased at similar rates (Fig. 4). The accumulation of [$^{35}$S]-GAGs in the medium fraction during the chase period was similar for all substrata, indicating that the rates of shedding of GAGs, as well as of degradation were equivalent.

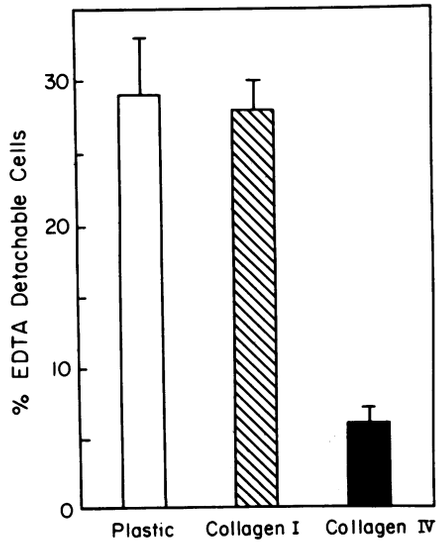


FIG. 3. Percentage of cells detached from plastic, collagen I, and collagen IV by treatment with EDTA. Detachment was measured 12 hr after plating of cells on substrata. Each bar is the mean ± SEM of four to seven determinations.

To measure the species of GAGs produced by cells grown on collagen I, collagen IV, or uncoated plastic substrata, the medium and cell-associated fractions were pooled and untreated, hyaluronidase-, chondroitin ABC lyase-, or nitrous acid-treated samples were analyzed by gel permeation chromatography. No hyaluronic acid was present in any of the cultures. In all cultures, ≈60% of the [$^3$H]glucosamine-labeled GAGs were chondroitin ABC lyase sensitive, and the remaining 40% were sensitive to nitrous acid degradation, with deviation between identical samples being <10%. However, although the degree of sulfation of the chondroitin ABC lyase-sensitive material was similar for cells grown on either collagen I, collagen IV, or un-

Table 1. Incorporation of [$^3$H]glucosamine and [$^{35}$S]sulfate into GAGs of B16 melanoma cells

| Substrate | Radioactivity, dpm × $10^{-4}$ per well | |
|---|---|---|
| | [$^3$H]GAG | [$^{35}$S]GAG |
| Plastic | 4.0 | 1.7 |
| Collagen I | 3.0 | 0.9 |
| Collagen IV | 3.4 | 1.1 |

Each number is the mean determination of four cultures grown on plastic and eight cultures on each collagen substratum. For each substratum, deviation between flasks was <10%.

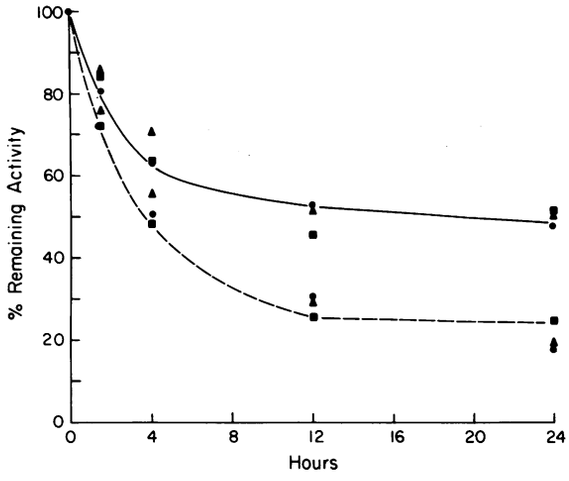


FIG. 4. Percentage of [$^{35}$S]sulfate-labeled cellular GAGs remaining during chase in label-free medium after 12 hr of exposure to radioactive precursor. The cell-associated incorporation was 1,100 cpm per well. Each point is the average of four cultures, with deviation between replicate cultures being <10%. Plastic (■), collagen I (●), and collagen IV (▲). Cell-associated (----) and total (——) GAGs.

coated plastic, the chondroitin ABC lyase-resistant macromolecular material was undersulfated in cultures grown on either type of collagen. Thus, the ratio of glucosamine to sulfate radiolabel in GAGs in the undigested macromolecular peak after chondroitin ABC lyase treatment was 3-fold greater in cultures grown on collagen, suggesting an ≈70% reduction in sulfation of heparin or heparan sulfate molecules. This reduction in the degree of sulfation was consistent between experiments, with a minimum of four cultures of each substratum being employed per experiment and <1% deviation in results being observed.

Macromolecular material remaining after neuraminidase and chondroitin ABC lyase treatment was sensitive to nitrous acid degradation and therefore was considered to be heparin or heparan sulfate. Aliquots of the [$^{3}$H]glucosamine-labeled material were subjected to ion-exchange chromatography by using a LiCl linear gradient to further define the changes in the degree of sulfation of GAGs suggested by gel chromatography. The heparin or heparan sulfate molecules from cultures grown on collagen eluted earlier than material isolated from cultures grown on plastic, suggesting a decrease in the degree of sulfation of these macromolecules (Fig. 5). Thus, the nature of the heparin or heparan sulfate molecules synthesized by B16 melanoma cells was altered by the presence of a collagen substratum.

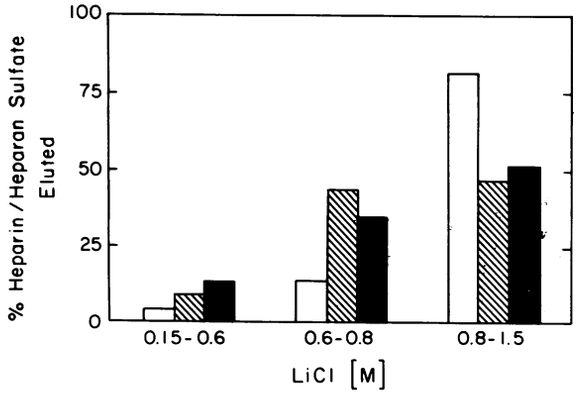


FIG. 5. Ion-exchange chromatography of heparin or heparan sulfate isolated from B16 melanoma cells grown on plastic (□), collagen I (▨), or collagen IV (■). Elution was conducted by using a 0.15–1.5 M LiCl gradient.

## DISCUSSION

B16 melanoma cells cultured on matrices of collagen I or collagen IV differed from many other cell lines (37, 38) in that they did not exhibit a significant change in their rate of cellular proliferation. However, although cellular replication was not affected by the presence of a surface of collagen I or collagen IV, cells grown on these substrata did exhibit morphological differences from their counterparts propagated on uncoated plastic. This influence of substratum on cell spreading has been reported for other cell lines (39, 40).

GAGs have been implicated in maintaining morphologic integrity (7). Furthermore, a decrease in sulfated proteoglycans has previously been associated with a more rounded cellular morphology in chicken embryo fibroblasts treated with tunicamycin (41). Concomitant changes in morphology and proteoglycan synthesis may be related to changes in the distribution of the microfibrillar system (42). Although a similar reduction in sulfated GAGs of B16/YM1 cells grown on either type of collagen was observed in the present experiments, the less prominent morphologic changes in cells on collagen IV suggests that other molecules of importance to adhesion sites, such as laminin, may be involved in promoting cellular spreading. The reduction in EDTA-susceptible detachment of cells grown on collagen IV is also most likely due to the preference of laminin for collagen IV (43).

David and Bernfield (26, 27, 29) have shown that mammary epithelial cells grown on collagen I display a decreased rate of degradation of basal lamina proteoglycan, whereas neoplastic transformation of these cells interferes with the capacity of collagen to decrease the rate of degradation of these macromolecules. Our observation of similar [$^{35}$S]GAG turnover in cultures of the malignant melanoma line grown on collagen or uncoated plastic is in agreement with these findings and suggests that neoplastic cells might well form a less complete basal lamina, thereby facilitating local invasion and metastasis (29).

Because the total incorporation of radioactive precursors of GAGs into these macromolecules was decreased in cultures grown on collagen I and collagen IV substrata, whereas (*i*) relative precursor pool sizes were equal and (*ii*) rates of degradation of GAGs were similar, it follows that an alteration in GAG biosynthesis occurred in cells grown on the collagen substrata. This finding contrasts with previous studies that have not shown a decrease in the incorporation of [$^{35}$S]sulfate into GAGs of cells grown on collagen substrata (24–29). These differences may be related to differences in cell type, cell density, collagen preparations, or the malignant potential of the cells employed. Our experiments were performed on exponentially growing cells, which may more closely approximate the *in vivo* situation during spontaneous formation of a metastatic foci than that of the high cell density situation occurring in a relatively large primary tumor. When cells were seeded at a higher density, total radioactivity from sulfate and glucosamine incorporated into GAGs of cells propagated on all substrata was similar (data not shown). This result is in agreement with the reported cellular density dependency of GAGs synthesis (44).

The ability of collagen substrata to alter the degree of sulfation of heparin or heparan sulfate molecules demonstrated in these experiments was previously suggested by Gallagher *et al.* (28), who reported a change in the DEAE-cellulose chromatography of heparan sulfate from cultures of fibroblasts grown on collagen gels. The experiments described in this report demonstrate that the morphology of metastatic B16 melanoma cells is altered by growth on collagen I or IV substrata. These differences may, in part, be related to the change in the degree of sulfation of heparin or heparan sulfate, for previous studies

have shown that heparin or heparan sulfate binds to laminin (45) and fibronectin (46–48) and stabilizes the interaction of collagen and fibronectin (49). This interaction is very specific in that heparin or heparan sulfate with a low or high sulfate content each has a markedly different affinity for fibronectin (49).

It has previously been suggested that malignant cells may be able to alter their environment by degradation of extracellular matrix macromolecules (50, 51). Our observations would suggest that in addition, in response to a component of their environment (i.e., collagen), malignant cells are able to regulate the quantity and nature of the GAGs being produced. These alterations in the configurations of GAGs may play an important role in the regulation of tumor cell migration by providing hydrated matrices to act as avenues for cell movement (52, 53). The mechanism by which environmental information is transmitted to those processes regulating the formation of specific types of GAGs and the impact of this phenomenon on the malignant process remain to be elucidated.

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