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Intercellular Glycosaminoglycans in Normal and Neoplastic Tissues[1]

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ABSTRACT

Intercellular glycosaminoglycans have been isolated from normal and neoplastic mammalian tissues. They have been characterized by cellulose acetate electrophoresis and by chemical and enzymatic degradation. The electrophoretic pattern of the intercellular glycosaminoglycans is tissue specific. Further, the electrophoretic patterns of all spontaneous neoplasias analyzed differ significantly from patterns obtained from the tissues of origin.

INTRODUCTION

Recent evidence favors the hypothesis that cell surface polysaccharides play a fundamental role in the regulation of eukaryotic cells. The relative amounts of hyaluronic acid and HS[2] of the cell coat vary widely following oncogenic virus transformation of rodent fibroblasts cultured *in vitro* (6, 10, 11), the differentiation of neuroblastoma cells in culture (2), and in the removal of density-dependent inhibition of growth in 3T3 cells (5). These observations led us to predict that HS was an inhibitor of growth and a promoter of cellular adhesiveness and differentiation, while hyaluronic acid increased cellular "slipperiness" and promoted growth (4). Indeed, Atherly *et al.* (1) recently showed that a detachment variant of Chinese hamster ovary cells has increased adhesiveness and a concomitant decrease of cell surface hyaluronic acid compared to normal cells.

Since neoplasia occurs at the level of tissues rather than as a simple cellular event, we believe that it is important to extend studies of *in vitro* cellular models to those of neoplastic tissues *in vivo*. Our goal is to gain an insight of the role of GSG in the homeostasis of tissues, cellular recognition, and maintenance of the differentiated state. This paper presents a study of the chemical properties of polysaccharides isolated from normal and neoplastic tissues after mild trypsin dissociation.

MATERIALS AND METHODS

Tissues were sliced with a razor blade (about 1 mm thick), and the slices were washed with 0.15 M NaCl to remove broken cells and intracellular materials. The slices were suspended in Dulbecco's medium containing trypsin (type XI; 10 μg/ml; Sigma Chemical Co., St. Louis, Mo.) and shaken at 37° for 10 min. The incubation mixture was immersed in an ice bath and then centrifuged at 1500 × rpm for 10 min at 0°. The sediment was observed under a microscope after incubation with trypan blue for 10 min at 37°. Neither the slices nor the dissociated cells were stained by the dye. The supernatant was centrifuged at 10,000 × *g* for 20 min. Pronase (Calbiochem, La Jolla, Calif.) was added to the supernatant to a final concentration of 30 μg/ml, 1 drop of toluene was added, and the mixture was incubated overnight at 37°. Trichloroacetic acid was added to a final concentration of 10%, and the precipitated proteins and nucleic acids were collected by centrifugation at 20,000 × *g* for 30 min. Ethanol (2.5 volumes) was added to the supernatant, and the mixture was stored overnight at −20°. The precipitated polysaccharides were collected by centrifugation and dissolved in distilled water. They were analyzed after enzymatic and chemical degradation as reported previously (2) and by electrophoresis on cellulose acetate strips (6 x 7.5 cm; Zip Zone; Titan Type III; Helena Laboratories). Cellulose acetate strips were presoaked overnight in 0.1 M barium acetate buffer (pH 8.1), dried with paper, and loaded with the polysaccharides. The electrophoresis was carried out in the same buffer with a current of 10 ma for 1 hr. The strips were stained with Alcian blue and destained in 0.1% acetic acid. The method was able to resolve authentic standards of hyaluronic acid, DS, and ChS A/C (Seikagaku Kogyo Co., Ltd., Nihombashi-Honcho, Tokyo, Japan) into 4 distinct bands. With tissue-extracted polysaccharides, hyaluronic acid and HS sometimes were not well separated, apparently because of their heterogeneous molecular sizes. It is possible, however, to resolve HS from hyaluronic acid at low pH (barium acetate buffer, pH 1.0), which strongly reduces hyaluronic acid migration. Barium acetate electrophoresis, which was the most suitable procedure in our hands, also compared with bidimensional techniques.

In some experiments GSG of mouse tissues were labeled by giving mice injections of 1 mCi of ^{35}S-labeled inorganic sulfate (268 Ci/mmol; Radiochemical Centre, Amersham, England) for 16 hr. GSG of mastocytoma cells were labeled by incubation of cultures with ^{35}S-labeled inorganic sulfate (20 μCi/ml) and of [^{3}H]glucosamine (1 μC/ml; 12 Ci/mmol; Radiochemical Centre) for 24 hr. Labeled GSG were separated by 2 different procedures. After electrophoresis the stained bands were cut with scissors, transferred to scintillation vials, dissolved with NCS (Nuclear Chicago, Chicago, Ill.), and counted with standard toluene scintillation mixture. Alternatively, chromatography was carried out on AG1-X2 anion exchange resin (Bio-Rad Laboratories, Richmond, Calif.) as reported previously (11), and fractions were assayed by liquid scintillation counting. In the experiments on mastocytoma tumors (The Jackson Laboratory, Brookhaven, N. Y.), cells were trypsinized as previously described (11). Both the trypsinates and homogenized cells were analyzed for GSG as described above. For inhibition of trypsin-stimulated secretion of intracellular heparin,

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[2] The abbreviations used are: HS, heparan sulfate; GSG, glycosaminoglycans; DS, dermatan sulfate; ChS A/C, chondroitin sulfate A/C; ChS C, chondroitin sulfate C.

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mastocytoma tumors were treated with cytochalasin B (3 μg/ml; Calbiochem) for 30 min at 37° before trypsinization.

Mastocytoma cells appear viable after the trypsin treatment as revealed by the trypan blue assay, but some RNA was released in the supernatant and revealed in the trichloroacetic acid precipitate. This RNA release was observed also during tissue dissociation (liver, brain) and most probably indicates initial cellular lysis.

RESULTS AND DISCUSSION

Table 1 summarizes experiments carried out for the identification of the 4 bands of tissue GSG resolved by cellulose acetate electrophoresis in barium acetate buffer. It is particularly significant that the slowest band (indicated as Band 1) disappeared upon treatment with *Streptomyces* hyaluronidase (Amano Chemical Co., Japan) and Band 2 disappeared with nitrous acid treatment; these agents specifically hydrolyze hyaluronic acid and HS, respectively. The other 2 faster bands have been identified as DS and ChS A/C on the basis of electrophoretic mobility, metachromatic staining with toluidine, and selective sensitivity to specific chondroitinases. We found that identification of an unknown GSG by comparison of its electrophoretic mobility to an authentic standard is sometimes misleading because of differences in molecular size. This is particularly true for hyaluronic acid and HS of eukaryotic cells, which are very heterogeneous in size. Acidification of the electrophoresis buffer to pH 2.0 with 1 HCl specifically impairs the migration of hyaluronic acid and is useful in discriminating hyaluronic acid from HS or DS. Sialylated oligosaccharides also overlap hyaluronic acid but can be eliminated with a neuraminidase treatment.

Chart 1 illustrates the electrophoretic patterns of GSG from mouse organs after *in vivo* labeling with [^{3}H]glucosamine and $^{35}SO_4$. Fig. 1 shows the electrophoretic distribution of unlabeled intracellular polysaccharides from guinea pig organs as detected with Alcian blue. The tissue-specific distributions differ strongly from one organ to another, and the differences are reproducible. For example, chondroitin is high in the brain but low in both liver and kidney, while the opposite is observed for HS. The amount of hyaluronic acid (Fig. 1, first column from left labeled only by glucosamine) is generally a minor fraction of the GSG of brain, liver, and kidney but is present in a higher proportion in other tissues.

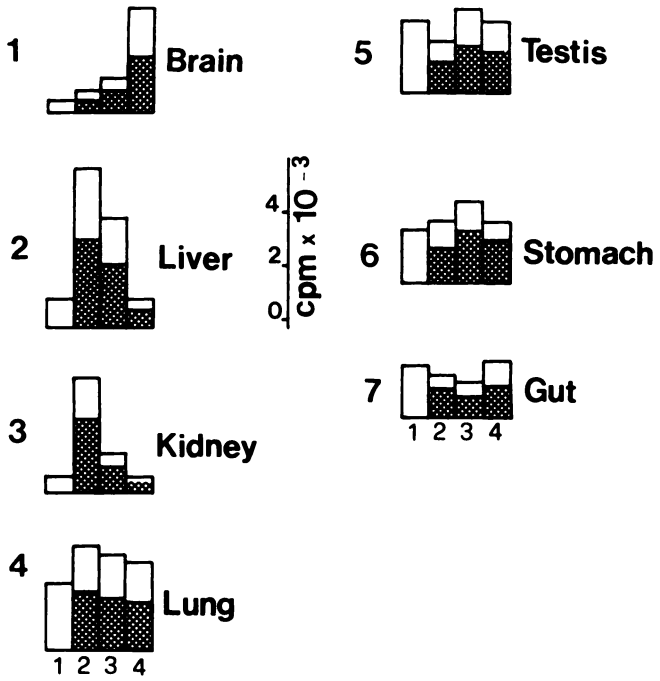

Chart 1. Electrophoretic pattern of labeled GSG from mouse organs. GSG, removed by trypsin, was purified and resolved by cellulose acetate electrophoresis as described under "Materials and Methods." *White columns*, ^{3}H cpm; *dotted columns*, ^{35}S cpm. The direction of migration is from *left* to *right*. *Columns 1, 2, 3,* and *4* correspond to Bands 1, 2, 3, and 4, respectively, of Table 1. The origin of migration is about 1 cm on the *left* of *Column 1*.

Table 1

GSG sensitivity to enzymatic or chemical treatments and staining with polycation dyes

GSG	*Proteus* chondroitinase ABC	*Proteus* chondroitinase AC	*Streptomyces* hyaluronidase	Testicular hyaluronidase	*Flavobacterium* heparitinase	Nitrous acid	Toluidine staining	Alcian blue staining	Impairment of migration at pH 2
Standard hyaluronic acid	+[a]	−	+	+	−	−	±[b]	+	+
Standard HS	−	−	−	−	+	+	+	+	−
Standard DS	+	−	−	+	−	−	+	+	−
Standard Chs A/C	+	+	−	+−	−	−	+[c]	+	−
Band 1	+	−	+	+	−	−	−	+	+
Band 2	−	−	−	−	+	+	+	+	−
Band 3	+	−	−	+	−	−	+	+	−
Band 4	+	+	−	+−	−	−	+	+	−

[a] +, disappearance of the band after the treatment or staining with the indicated dye and impairment of migration; −, insensitivity to the indicated treatments.

[b] Hyaluronic acid forms a rapid complex with toluidine, but the color disappears during the destaining. Hyaluronic acid affinity to toluidine is 25 to 30 times lower as compared with sulfated GSG.

[c] Usually chondroitinases A and C migrate together; however, by increasing the concentration of the buffer to 0.2 M and the current to 40 ma (under refrigeration), it is possible to separate those 2 compounds, since ChS C migrates much faster under these conditions.

Similar tissue-specific proportions of GSG are observed by analysis of mouse labeled GSG (Chart 1) or from patterns of unlabeled polysaccharides obtained from guinea pig organs (Fig. 1). It seems that similar tissue-specific patterns occur among the mammals thus far examined (mouse, rat, guinea pig, humans, or swine) (V. P. Chiarugi *et al.*, unpublished observations).

When normal and neoplastic tissues have been compared, striking differences have been observed. Fig. 2 shows an example of the relative increases of ChS A/C in lung cancers and of hyaluronic acid in an uterine carcinoma. Examining a number of spontaneous human neoplasias (about 100 cases) (Table 2), we failed to find a single malignant tumor without variations from the GSG pattern characteristic of the normal tissue. In contrast, noninvasive neoplasias showed only slight variations. The most frequent finding for malignant neoplasias is a relative increase of ChS A/C or of hyaluronic acid and a decrease of hyaluronic acid or DS. Alterations of the electrophoretic mobility and of the shape of single bands are also observed, suggesting changes in the molecular size or in the 3-dimensional conformation of the GSG molecules.

The data presented in this paper are in general agreement with the findings of Dietrich *et al.* (7) and Takeuchi (9) and suggest a regulative role for surface polysaccharides of eukaryotic cells (4, 8) in cellular recognition and tissue homeostasis (3).

The data of Dietrich *et al.* (7), however, are concerned with analysis on GSG obtained from total organ homogenates. We present evidence that the total GSG composition of a given cell is not necessarily representative of its cell coat GSG composition. Fig. 3 shows that the electrophoretic pattern of GSG obtained by trypsinization of a mouse mastocytoma is very different from the pattern of the GSG obtained from the cells. The cell trypsinate is rich in hyaluronic acid, while the trypsinized cell contains relatively little hyaluronic acid but instead mostly heparin. This finding has been confirmed by ion-exchange chromatography of double-labeled GSG from mouse mastocytoma (Chart 2). These results are consistent with our hypothesis because the neoplastic behavior of mastocytoma cells (a rounded shape and a very low adhesion to the substrate *in vitro* and growth in ascitic form *in vivo*) correlates with a low exposure of HS and a high exposure of hyaluronic acid at the cell surface. As suggested this appears to be a general phenomenon accompanying transformation. However, we are aware that the apparent difference between the GSG composition of the cell trypsinate and the total cell could be due to the differential release of certain polysaccharides by trypsin, as seems to be the case for HeLa cells (C. P. Dietrich, personal communication). Nevertheless, we have consistently observed differences between cell surface and endocellular GSG in a number of normal and neoplastic cells (established cell lines, peritoneal macrophages, circulating granulocytes, and lymphocytes) (V. P. Chiarugi *et al.*, unpublished results).

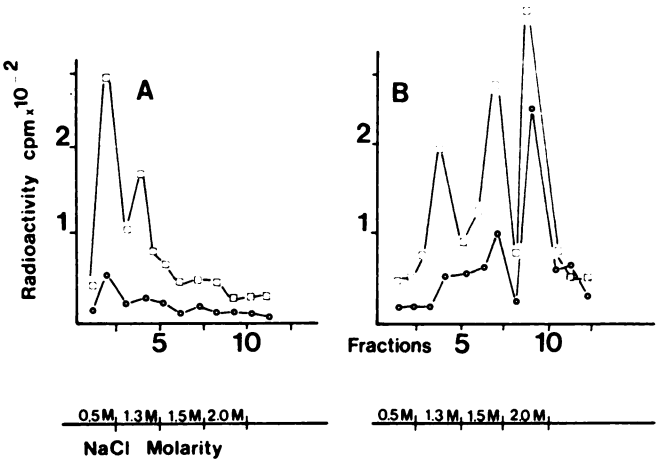

Chart 2. Stepwise elution from AG1-X2 anion-exchange resin of double-labeled GSG from mouse mastocytoma. AG1-X2 chromatography was carried out as previously described (11). *A*, trypsinate; *B*, cells. Hyaluronic acid and heparin standards eluted at 0.5 and 2.0 M NaCl, respectively. ○, [^{35}S]sulfate; □, [^{3}H]glucosamine.

The emerging picture is that the polysaccharide composition of the cell coat is strongly regulated and does not necessarily reflect the endocellular GSG content. The data reported by Dietrich *et al.* (8) stress the increase of ChS C as a general phenomenon in malignant neoplasia. Our interpretation is that this is not the only mechanism that releases the cell from the homeostatic relationships of the tissue but that any variation in the GSG composition from the normal specific pattern may do it. We believe that HS and DS are positive elements for cell adhesion and tissue homeostasis while ChS C and hyaluronic acid could interfere in mechanisms disrupting recognition sites; the relative amounts of these compounds on the cell surface may be of crucial importance for maintaining cellular relationships in a solid tissue. Since cellular transformation and especially malignant growth are not all-or-none events, an initial variation of the relative amounts of GSG of the tissue could impair cellular recognition and produce non-infiltrative tumors. A decrease in the relative amounts of GSG with high affinities for calcium or an increase of GSG with low affinities for calcium may be required for infiltrative growth and metastasis.

Although the role of polysaccharides in tissue homeostasis is far from being known, our observations encourage further analysis of the changes in GSG associated with neoplasia.

Table 2

GSG variations in spontaneous neoplasias

The percentage has been calculated for 94 cases of malignant neoplasias and 28 cases of benign tumors with clear clinical and histological diagnosis. The malignant neoplasias examined were concerned with various tissues including lung, liver, brain, kidney, breast, etc., while benign tumors were prevalently uterine and breast fibromas. The increase or decrease as well as the variations of the shape of a given band have been determined either by visual examination or by densitometric tracing.

GSG variation	Malignant infiltrative (%)	Benign non-infiltrative (%)
Hyaluronic acid increase	3.1	
ChS C increase	17.0	
Hyaluronic acid and ChS C increase	29.0	
ChS C increase together with other variations	41.0	20.0
Various minor alterations	9.8	40.0
No variations		40.0

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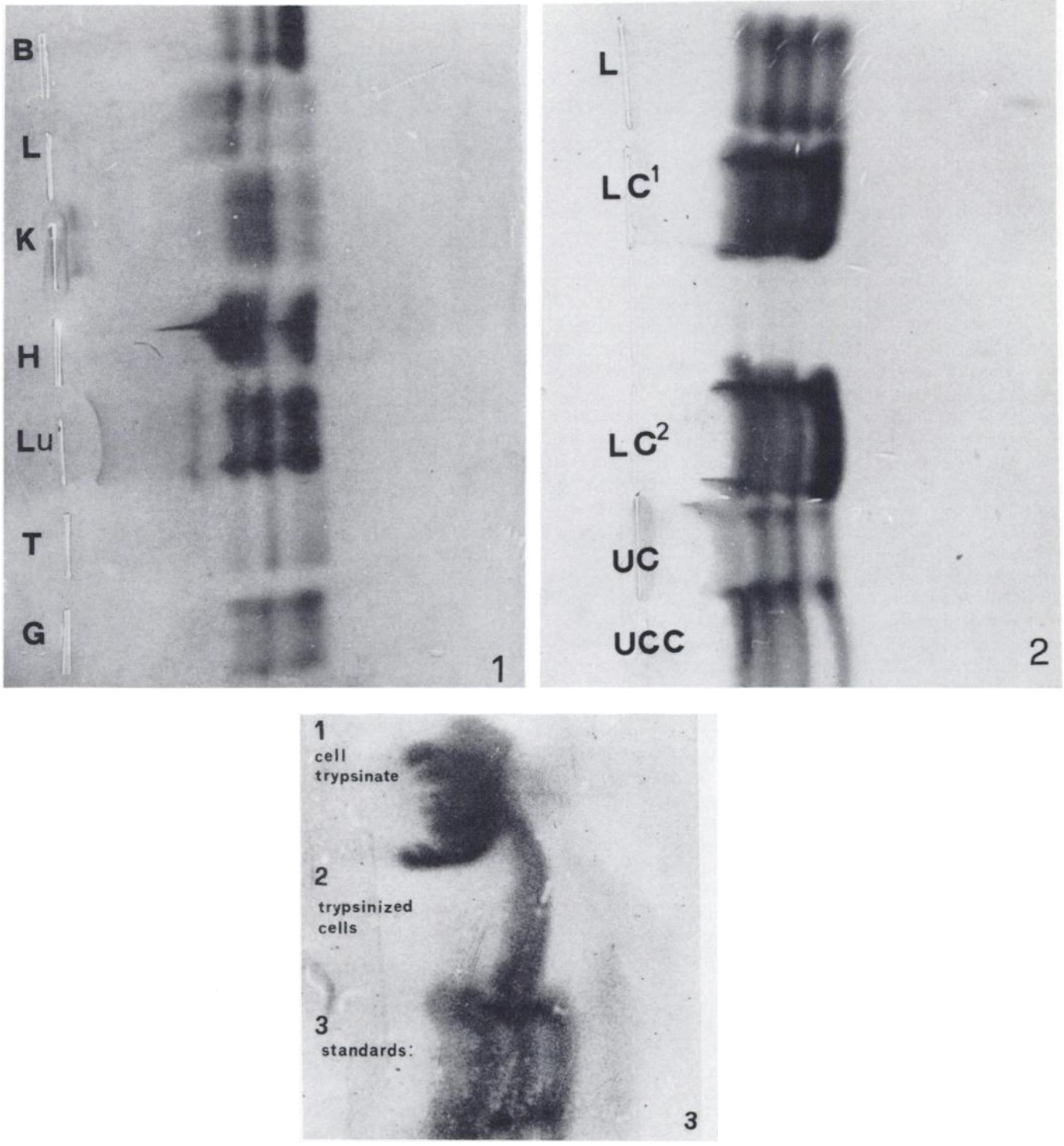

Fig. 1. Cellulose acetate strip of intercellular GSG from guinea pig tissues. *B*, *L*, *K*, *H*, *Lu*, *T*, *G*, and brain, liver, kidney, heart, lung, testes, and gut, respectively. The direction of anodic migration is from *left* to *right*. For the identification of the bands, see Table 1. The bands are progressively numbered from *left* to *right*. Alcian blue, ×2.

Fig. 2. Cellulose acetate strip. Intercellular GSG are run and stained with Alcian blue. GSG are from normal and neoplastic human tissues. *L*, *LC*^{1}, *LC*^{2}, *UC*, and *UCC*, lung, lung carcinoma 1, lung carcinoma 2, uterine cervix, and uterine cervix carcinoma, respectively. For the identification of the bands, see Table 1. The bands are progressively numbered from *left* to *right*. ×2.

Fig. 3. Cellulose acetate strip. GSG from a mouse mastocytoma are compared with reference standards. *1*, cell trypsinate; *2*, trypsinized cells; *3*, standards hyaluronic acid, HS, and DS (*left* to *right*). GSG is stained with Alcian blue. ×4.