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Production of Mucopolysaccharides by Normal and Transformed Cells

(hyaluronic acid/transformed cells/hamster-embryo cultures)

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ABSTRACT The rate of hyaluronic acid and sulfated mucopolysaccharide production was measured for hamster embryo fibroblasts and for general oncogenic lines derived by virus transformation. A striking increase in both the rate of hyaluronic acid synthesis and the amount of cell-associated polymer was observed after transformation by herpes simplex type-2 or SV40 virus. Although no corresponding change was observed for the sulfated polysaccharides, the proportion of heparan sulfate increased significantly after transformation.

Recent reports from several laboratories have described effects on cell coat properties associated with virus transformation (1–3). In general, the morphologic observations have been attributed to changes in the content of those acid polysaccharides identified with the extracellular matrix—hyaluronic acid and a group of sulfated saccharides. Although differences in the rate of synthesis of cell coat have been observed for transformed cells (4), as a rule the polysaccharides involved have not been characterized.

The present study describes the production and characterization of mucopolysaccharides by normal and virus-transformed fibroblasts from hamster embryo under cell culture conditions. An attempt was made to differentiate material adhering to cell surfaces from that released to the culture medium, although only an operational distinction was used. In most of the experiments reported, [^{3}H]glucosamine was used as a radioactive precursor for both nonsulfated (hyaluronic acid) and sulfated (chondroitin sulfates, heparan sulfate, etc.) polysaccharides.

The identification of the polysaccharides was based on elution behavior from cetylpyridinium chloride precipitates (5), high-voltage electrophoresis, enzyme susceptibilities, and characterization of the amino sugar component after acid hydrolysis. Table 1 illustrates the results obtained for several polysaccharides under consideration.

MATERIALS AND METHODS

Syrian hamster-embryo fibroblasts cells (inbred LSH strain, Lakeview Hamster Colony, New Field, N.J.) were prepared by dispersion of 13-day-old embryos with 0.25% trypsin. The dispersed cells were grown in medium 199 supplemented with 10% tryptose phosphate broth, 10% fetal-bovine serum, and 0.075% $NaHCO_3$. Primary African green monkey kidney cells were also prepared by dispersion with 0.25% trypsin, and grown in Eagle's medium (BME, Flow Laboratories Inc.) with 10% fetal-calf serum and 0.075% $NaHCO_3$.

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The H-50 cell line was developed from a Syrian hamster tumor that was originally induced by SV40 (6). H-50 cells contain the SV40 T and transplantation-rejection antigens, and induce tumors when reinjected into weanling Syrian hamsters. They do not produce infectious virus. The cells were maintained in the identical manner as primary hamster embryo fibroblasts. The SV40-transformed BSC-1 cells are the results of the transformation of a stable line of African green monkey kidney cells (BSC-1) by SV40 (7). These cells synthesize the SV40-specific tumor antigen, the surface antigen, and the transplantation antigen; however, SV40 capsid antigen is not synthesized (8). BSC-1 (SV40) cells were grown in Eagle's medium (BME) supplemented as described above.

Hamster-embryo fibroblast cells were transformed (9, 10) by herpes simplex virus type-2. The 333-8-9 T no. 1 cells were maintained in medium 199 with the same supplements as used with normal fibroblast cells. Herpes virus-specific antigens are present in the 333-8-9 T no. 1 cells, which were developed from a tumor induced in a weanling Syrian hamster by the virus-transformed cells.

For polysaccharide analysis, cell cultures were maintained in medium 199 supplemented with 10% fetal-calf serum and 10% tryptose phosphate broth. The cells were passaged at 3- to 4-day intervals. Exposure to isotopically labeled glucosamine ([^{3}H]glucosamine, 10 μCi/ml final concentration, 3 Ci/mmol, New England Nuclear Corp.) was for 24 hr; 10^6 cells were used in the same medium with dialyzed serum. In a rate study, cells were labeled for 4 hr with 10 μCi/ml of [^{3}H]glucosamine, then the medium was removed and replaced with fresh medium containing an equivalent concentration of unlabeled glucosamine (1.6 pmol/ml). Cell and medium samples were collected at several intervals thereafter.

Medium was decanted from the cells and the cells were removed from the glass substrate by brief exposure to 0.1 mM EDTA or by mechanical means. The cells were collected by centrifugation and washed once with isosmotic phosphate-buffered saline (pH 7.3). The wash was combined with the decanted medium.

After the addition of 2.5 mg (each) of unlabeled hyaluronic acid and chondroitin 4-sulfate as carrier, the mucopolysaccharides were isolated by precipitation with excess cetylpyridinium chloride after 18 hr of digestion with 1 mg/ml of Pronase at pH 6.5 and 37°, 18 hr of exposure to 0.1 N NaOH, and dialysis against H_2O to remove peptides and glycopeptides. The cetylpyridinium-chloride precipitate was washed with 30 mM NaCl, and sequentially extracted with

TABLE 1. *Characterization profile of mucopolysaccharides*

	Cetylpyridinium chloride elution (M NaCl)	Enzyme susceptibility	Amino sugar
Haluronic acid	0.4	Hyaluronidase	Glucosamine
Chondroitin 4- and 6-sulfate*	1.2	Hyaluronidase: chondroitinase ABC	Galactosamine
Heparan sulfate†	1.2	Flavobacterium heparinase	Glucosamine
Heparin†	2.0	Flavobacterium heparinase	Glucosamine

* Differentiated by both chemical and enzymatic hydrolysis susceptibility of the sulfate ester grouping.

† Contains *N*-sulfate as well as *O*-sulfate.

0.4 M NaCl, 1.2 M NaCl, and, in some preliminary experiments, 2.0 M $MgCl_2$. The $MgCl_2$ extracts invariably contained insignificant radioactivity and were not studied further. The NaCl eluates were freed of cetylpyridinium chloride by dialysis before further analyses.

Electrophoresis was performed in pyridine–formic acid, (pH 3.0), on cellulose acetate strips for 30 min at a current of 0.22 mA/cm. Standards for hyaluronic acid and the chondroitin sulfates were prepared in this laboratory. The position of the standards was revealed by staining with Alcian Blue; the experimental strips were sectioned and assayed for radioactivity by scintillation counting. In all cases, the results obtained corresponded to those expected on the basis of the cetylpyridinium chloride elution profile. It should be noted that the sulfated polysaccharides (e.g., chondroitin 4- against 6-sulfate) are not distinguished by this procedure. We are indebted to Dr. Alfred Linker for an authentic sample of heparan sulfate.

Hyaluronidase susceptibility was observed by measurement of dialyzable radioactivity after 24 hr of digestion at pH 5.0 and 37° with 300 turbidity-reducing units of Worthington testicular hyaluronidase. Similar conditions were used for chondroitinase ABC and a flavobacterium heparinase obtained through the courtesy of Dr. Alfred Linker.

The amino sugar composition of the various fractions was measured after 24 hr of hydrolysis in an evacuated tube in 6 N HCl. Samples were resolved on a Beckman 120C aminoacid analyzer; a stream-splitting attachment was used. The radioactivity associated with the glucosamine or galactosamine peaks was determined by counting aliquots in a liquid scintillation counter in a toluene–Triton mixture as solvent and 2,5-diphenyl oxazole(PPO)–1,4-bis [2-(5-phenyl oxazole)]-benzene POPOP (40:1) as scintillators.

Estimates of molecular size are based on chromatography on porous glass beads with oligo- and polysaccharide samples of known degree of polymerization as standards. The glass beads were obtained from Corning Glass, Inc. The details of this technique will be published elsewhere.

RESULTS AND DISCUSSION

The effect of virus transformation on mucopolysaccharide production by hamster-embryo fibroblasts is notable in that there is a striking increase in label in the hyaluronic acid fraction without a corresponding effect on the sulfated polysaccharide components. Data are summarized in Table 2. Since incorporation of precursor into product under the conditions of this experiment must represent a composite of both synthesis and degradation, a time-course study was performed in an attempt to better define the nature of the observed effect. The data, shown in Table 3 and Fig. 1, clearly demonstrate an increase in the rate of glucosamine incorporation into hyaluronic acid of about tenfold that persists after 24 hr, and also suggest that greater amounts of the hyaluronic acid remain associated with the cells after viral transformation, although the nature of this association is unknown.

Although the amount of isotope incorporated into the sulfated fraction was not significantly different from that in control cells, and the characterization of this fraction is not complete, several facets are of interest. Examination of the data presented in Fig. 1 suggests a significant effect on the rate of sulfated polysaccharide synthesis during the 4-hr period after a medium change. In addition, there is an indication of some turnover of this fraction at extended time periods, a finding

TABLE 2. *Mucopolysaccharide production by normal and transformed cells*

Cell line	Hyaluronic acid		Sulfated polysaccharides	
	Cell	Medium	Cell	Medium
Hamster-embryo fibroblasts	43,800	262,700	111,600	683,200
333-8-9 T no. 1	463,000	8,578,900	107,000	1,042,000
H-50	96,300	655,800	13,200	918,500

The radioactivity incorporated into the identified fraction after exposure of 10^6 cells to 5 μCi/ml of [^{3}H]glucosamine for 48 hr under standard culture conditions. The sulfated-fraction radioactivity is based on high-voltage electrophoresis after elution from cetylpyridinium chloride. Both glucosamine and galactosamine were present, suggesting the presence of heparan sulfate, as well as a chondroitin sulfate (see Table 4). 333-8-9 T no. 1: hamster-embryo fibroblasts transformed *in vitro* by herpes simplex virus type 2, cell line recovered from tumor, carried in culture, 13th passage: H-50: hamster tumor induced by SV40, cell line recovered from tumor, carried in culture, 46th passage. Both transformed lines are oncogenic. The counts identified as hyaluronic acid represent those eluted from the cetylpyridinium chloride precipitate with 0.4 M NaCl. These fractions were completely (>95%) digested by hyaluronidase, and all radioactivity was associated with glucosamine; galactosamine was not present.

TABLE 3. *Rate of hyaluronic acid synthesis*

	Cell		Medium	
	0 Hr	4 Hr	0 Hr	4 Hr
HEF	5,300	9,000	8,100	38,900
333-8-9 T no. 1	82,400	95,600	95,600	98,300

Rate of hyaluronic acid synthesis by normal and transformed fibroblasts. 10^6 Cells were labeled for 4 hr by exposure to 10 μCi/ml of [^{3}H]glucosamine. At "zero time," fresh medium containing a comparable concentration of unlabeled glucosamine was added, and the cells were incubated for an additional 4 hr. 333-8-9 T no. 1 cells are herpes-transformed cells (see Table 2).

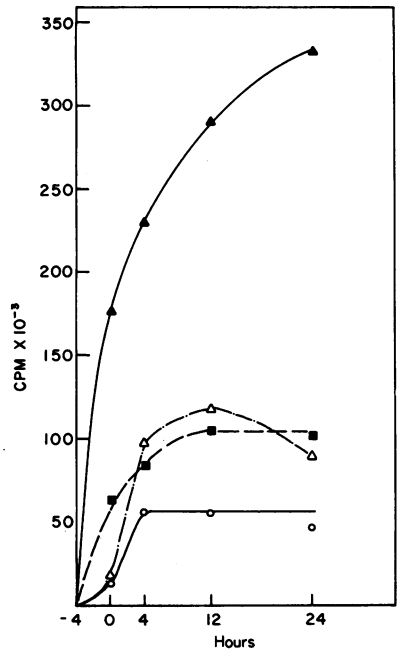

FIG. 1. Rate of hyaluronic acid and sulfated mucopolysaccharide production by hamster-embryo fibroblasts and a cell line transformed by herpes virus type-2, 333-8-9 T no. 1. O——O, [^{3}H]glucosamine incorporation into total hyaluronic acid by control cells: ▲——▲, [^{3}H]glucosamine incorporation into total hyaluronic acid by transformed line 333-8-9 T no. 1; Δ——Δ, [^{3}H]glucosamine incorporation into total sulfated polysaccharides by control lines; ■——■, [^{3}H]glucosamine incorporation into total sulfated polysaccharides by 333-8-9 T no. 1 cells.

that was not observed in several experiments with transformed cell lines. An apparent result of *in vitro* SV40 transformation on a line of monkey kidney cells was to cause a change in the average molecular size of the sulfated polysaccharide fraction and, concomitantly, to cause a marked increase in the proportion of heparan sulfate in this fraction. A similar effect was observed for herpes-transformed hamster-embryo fibroblasts. Data are summarized in Table 4.

Although accurate molecular weights cannot be determined for most of the isolated samples due to material limitations, size estimates were made based on behavior of the polysaccharide samples on columns of porous glass beads. In general, both the hyaluronic acid and the sulfated polysaccharides appeared altered in molecular size after viral transformation. The possibility of a degradative activity associated with untransformed lines cannot be excluded, either by these observations or by the rate studies. In addition, since this study was focused on the saccharide moieties, we have no information regarding effects on covalently associated protein, a normal constituent of at least the sulfated proteoglycans.

Several laboratories have observed alterations in cell-surface polysaccharides coincident with a transforming event. Changes in cell-coat thickness and rates of formation (4) have been reported, although the identification of the associated

TABLE 4. *Properties of sulfated polysaccharide fraction*

	% Low molecular weight	% Heparan sulfate
Green monkey kidney	24	2.7
BSC-1 (SV40)	45.7	16.0
Hamster embryo fibroblast	46	4.4
333-8-9 T no. 1	35	23.1

% Low molecular weight is based on the fraction of radioactively labeled material eluting after standard chondroitin 4-sulfate (molecular weight = 14,500) on a 0.9 × 50 cm column of CPG-240 porous glass beads with 0.5 M $CaCl_2$ as eluting solvent. If the profiles overlapped completely, the percentage of low molecular weight material is assumed to be 50. Heparan sulfate content is an estimate based on hyaluronidase-resistant, heparinase-sensitive material remaining after dialysis. BSC-1 (SV40), a stable line of monkey kidney cells (BSC-1) transformed by SV40 virus; 333-8-9 T no. 1 cells are described in Table 2.

macromolecule was limited to those able to bind electron-dense stain (ruthenium red) (11) or to purely physical observations (4). Similarly, the presence of heparan sulfate in several tumor cell lines has been reported, but direct comparison with control cells has not been reported (12, 13).

The physiological role of these mucopolysaccharides remains unknown, as does the nature of their association with the cell, but it is suggestive that selective differences in expression are found in the transformed lines. The possible association between the marked change in hyaluronic acid content and phenomena such as contact inhibition needs further study.

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