A TRANSFORMATION-DEPENDENT DIFFERENCE IN THE HEPARAN SULFATE ASSOCIATED WITH THE CELL SURFACE

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SUMMARY: Glycosaminoglycans from the surface of cultured mouse cells (3T3, SV40-3T3, 3T6) were released by trypsin digestion and separated by ion-exchange chromatography into hyaluronic acid, heparan sulfate and chondroitin sulfate. Using a double label technique, the glycosaminoglycans from 3T3 cells were compared with those from SV40-3T3 and 3T6 cells. No differences were apparent in either the hyaluronic acid or chondroitin sulfate fractions, however, the heparan sulfate from 3T3 cells was found to elute from DEAE-cellulose at a higher ionic strength than that from transformed cells. This altered behavior implies a structural difference in the cell surface heparan sulfate which appears to be dependent upon transformation.

Normal and transformed cells grown in culture behave very differently with respect to saturation density, morphology, growth on agar and agglutination by lectins (for review, see 1,2). These characteristics may reflect differences in the composition of the cell surface. Indeed, there are many reports of alterations in both glycolipids and glycopeptides derived from the surface membranes of normal versus transformed cells. However, as yet no study has shown the dependence of transformation on the presence or absence of these molecules (for review, see 3).

Glycosaminoglycans have been found to be associated with the surface of a wide variety of cultured cells (4). While the physiological function of these molecules is unknown, their large size and high negative charge make these carbohydrates a likely candidate for regulating cell behavior. In this paper, we report a difference in the heparan sulfate from normal cells (3T3) compared to that from transformed cells (SV40-3T3, 3T6).

Experimental: The mouse cell lines 3T3 and 3T6 were obtained from ATCC, and SV40-3T3-101 was obtained from Dr. H. Green (M.I.T.). All cells were grown under a 10% CO_2 atmosphere in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin G (10^3 units/ml) and streptomycin (100 µg/ml). Cells were transferred once a week with 0.25% trypsin (Difco 1:250) and the medium was changed every 2 or 3 days thereafter. All cell stocks were routinely found to be mycoplasma-free when

tested by Dr. George Kenny (University of Washington, School of Public Health).

Unless otherwise stated, all cell labeling was performed as follows: Cells were grown on 150 mm plates (Falcon) for 2 days in regular medium, which was then replaced with fresh medium plus label ([^{14}C]glucosamine, 0.5 µCi/ml; [^{3}H]glucosamine, 1.0 µCi/ml; or [^{35}S]sulfate, 10 µCi/ml). In the case of normal cells, the fresh medium contained low amounts of glucose (1/20 normal) in order to increase the uptake of glucosamine. This decrease in medium glucose did not alter the cell growth or the chromatographic behavior of the cell trypsinate.

After two days of growth in the presence of a labeled precursor, the cell cultures were washed 3 times with phosphate buffered saline and then incubated with 5 ml of 1.0 mg/ml trypsin (IRL-Worthington) for 20 to 30 minutes at 37°. The resulting cell suspension was centrifuged (10,000 x 10 min) and the supernatant was digested for 5 days with 0.1 mg/ml pronase (Calbiochem), which was added fresh each day. The final material is referred to as cell trypsinate.

The cell trypsinate was diluted with distilled water until the conductivity was less than 1.25 mmho, and then was applied to a DEAE-cellulose column (1 x 20 cm). Elution was effected with a 200 ml linear gradient of from 0.01 to 1.0 M NaCl in 0.002 M Tris pH 7.5 and collected in 2 ml fractions. Aliquots (1 ml) were prepared for scintillation counting by mixing with 10 ml of a 1:2 solution of Triton X-100:toluene scintillation fluid (5).

For the measurements of RNA release, two sets of cells were cultured with [^{3}H]uridine (0.025 µCi/ml) for three days. One set of cells was harvested and centrifuged as previously described, the other set was subjected to brief sonication at low power before centrifugation. Carrier RNA was added to both supernatant and pellet fractions. An equal volume of 20% trichloroacetic acid was then added and the resulting precipitates were washed one time with 10% trichloroacetic acid. The final pellet was dissolved in 0.1 N NaOH, neutralized and prepared for scintillation counting as described above.

The identification of the hexosamines present in the isolated glycosaminoglycans was performed by the method of Gardell (6) on samples hydrolyzed *in vacuo* with 6 N HCl at 100° for 6 hr. The resulting fractions were assayed for both radioactivity and hexosamine (7).

The nitrous acid degradation of heparan sulfate was performed according to the procedure described by Kraemer (8). The degradation products were separated on a Sephadex G-100 column (1 x 55 cm) in phosphate buffered saline.

RESULTS AND DISCUSSION: Extracellular Origin of the Cell Trypsinate. In order to establish the extracellular nature of the cell trypsinate, the amount of cell leakage was ascertained by measuring the proportion of RNA released during the preparation of the trypsinate. As shown in Table I, only a small fraction (3-6%) of the total TCA-precipitate RNA was released. However, when the cells were lysed by brief sonication, most (90-95%) of the RNA was found in the supernatant. If the release of RNA is equated with the number of damaged cells, then in the process of isolating cell trypsinate cell breakage

Table I. Release of RNA and Glucosamine into Cell Trypsinate[a]

	Relative Release (%)[b]		
	RNA		Glucosamine
Cell Line	trypsin-treated	sonicated	trypsin-treated
3T3	5.4	91.9	35.2
SV40-3T3	3.0	94.4	19.7
3T6	3.1	94.5	19.9

[a]The cells were labeled and harvested as described in Materials and Methods. The results are the average of two or three experimental points and represent the percent of total incorporated radioactivity released by the trypsin treatment as described in Materials and Methods.
[b][CPM supernatant]/[CPM supernatant + CPM pellet] x 100.

is on the order of 3-6%. This amount of cell lysis does not contribute significantly to the cell trypsinate, since 20-35% of the total glucosamine label was contained in the cell trypsinate (Table I). Thus, not more than 20% of the glucosamine label in the cell trypsinate could be due to the release of intracellular molecules, the remainder must be extracellular in origin.

These extracellular molecules, however could be material bound either to the cell surface or to the culture dish. We have attempted to distinguish between these possibilities by an analysis of the trypsinate from cells which were removed from the plate with 0.02% EDTA. This cell trypsinate contained all of the glycosaminoglycan species present in the regular preparation of cell trypsinate. On this basis, we conclude that we are examining molecules derived from the cell surface.

Identification of Heparan Sulfate. The cell trypsinate from 3T3 cells was resolved into four major fractions by DEAE-cellulose chromatography (Fig. 1). Fractions 1 and 2 were identified as low molecular weight glycopeptides and fraction 3 was found to be hyaluronic acid (Underhill and Keller, unpublished). The major portion (75-80%) of fraction 4 was characterized as heparan sulfate on the basis of the following evidence. First, this fraction has a high molecular weight since it was excluded from Sephadex G-100 (data

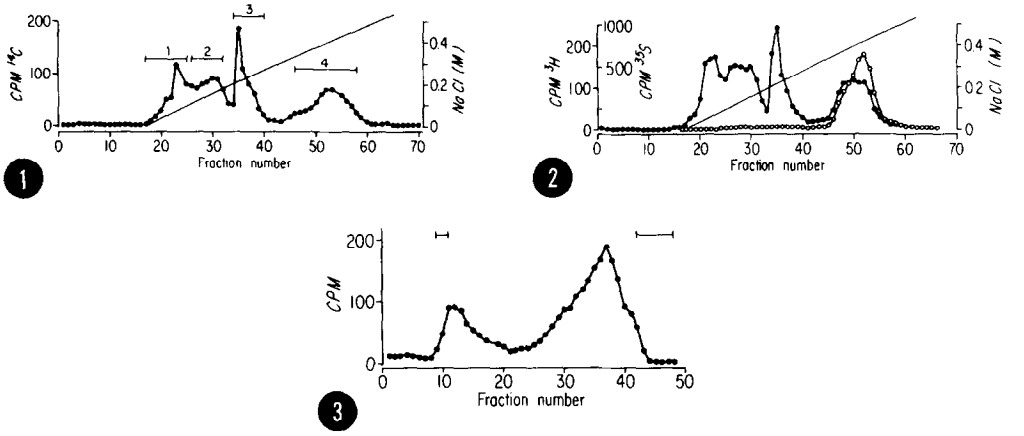

Figure 1. Fractionation of trypsinate from subconfluent 3T3 cells by ion-exchange chromatography on DEAE-cellulose. Cells were grown with [^{14}C] glucosamine as described in Materials and Methods.

Figure 2. The identification of a single sulfate-containing fraction in the trypsinate from subconfluent 3T3 cells by chromatography on DEAE-cellulose. Cell trypsinate was prepared from cells grown in the presence of either [^{35}S]sulfate or [^{3}H]glucosamine and subsequently dialyzed against saline in order to remove free sulfate. The trypsinates were then mixed and subjected to ion-exchange chromatography. 0—0, [^{35}S]sulfate-labeled cell trypsinate; •—•, [^{3}H]glucosamine-labeled cell trypsinate.

Figure 3. The effect of nitrous acid on the gel filtration pattern of fraction 4 material (see Fig. 1). The sample was incubated with nitrous acid at room temperature for 90 minutes. Blue dextran (fractions 10-12) and phenol red (fractions 43-49) were added as high and low molecular weight markers.

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not shown). Second, this fraction contained the bulk of the covalently bound sulfate present in the cell trypsinate (Fig. 2). Third, upon treatment with nitrous acid approximately 80% of fraction 4 was converted to a lower molecular weight material as judged by Sephadex G-100 chromatography (Fig. 3). This behavior is unique to polymers, such as heparan sulfate which contain N-sulfate substituted hexosamines (9). Fourth, 77% of the label in fraction 4 was glucosamine (Table II), the hexosamine present in heparan sulfate. And finally, the bulk of fraction 4 was degraded by a crude heparinase preparation from Flavobacterium heparinum (Underhill and Keller, unpublished).

In addition to heparan sulfate, fraction 4 also contained a minor

Table II. Relative Amounts of Hexosamine (%)[a]

Fraction	Glucosamine	Galactosamine
4 (3T3)	76.6	23.4
4a(3T6)	92.5	7.5
4b(3T6)	19	81

[a]Samples were isolated by DEAE-cellulose chromatography of trypsinate from cells grown in the presence of labeled glucosamine. The samples were hydrolyzed and analyzed for hexosamine as described in Materials and Methods. The values in the table refer to the percentage of recovered radioactivity.

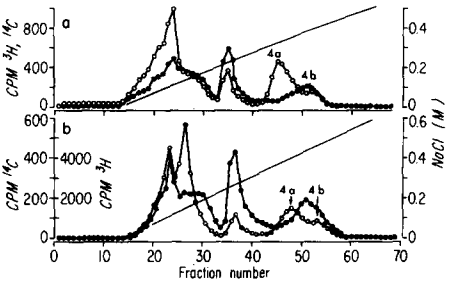

Figure 4. Comparison of trypsinates from normal (3T3) and transformed (SV40-3T3 and 3T6) cells with respect to their chromatographic behavior on DEAE-cellulose. All trypsinates were prepared from low density cultures grown in the presence of [^{3}H] or [^{14}C]glucosamine. a. O—O, [^{14}C]glucosamine-labeled cell trypsinate from SV40-3T3; ●—●, [^{3}H]glucosamine-labeled cell trypsinate from 3T3. b. ●—●, [^{14}C]glucosamine-labeled cell trypsinate from 3T3; O—O, [^{3}]glucosamine-labeled cell trypsinate from 3T6.

portion (20-25%) of chondroitin sulfate. This conclusion was suggested by the observation that 23% of the labeled hexosamine in fraction 4 was recovered as galactosamine (Table II), a component of chondroitin sulfate. Furthermore, standard chondroitin sulfate coeluted with fraction 4 from the DEAE-cellulose column. The presence of this glycosaminoglycan would also account for the 20% of fraction 4 which was resistent to degradation by nitrous acid (Fig. 3).

Effects of Transformation. The DEAE-cellulose chromatographic patterns of the trypsinates from 3T3, 3T6 and SV40-3T3 cells are shown in Figure 4. Differences between normal and transformed cells were apparent with respect to fraction 4 (sulfated glycosaminoglycans). In the column profile from

transformed cells, two peaks were resolved in the region of fraction 4, labeled 4a and 4b. These fractions (3T6) were isolated and their hexosamine contents analyzed; 93% of the label in fraction 4a was glucosamine, whereas 81% of the label in fraction 4b was galactosamine (Table II). On this basis, we identified fraction 4a as heparan sulfate and fraction 4b as chondroitin sulfate.

The difference in the elution behavior of heparan sulfate appears to be a characteristic of the transformed state. It was unaltered by reversing the labels or by varying the density of the cells from which the heparan sulfate was derived. Furthermore, this alteration was also observed in the heparan sulfate from both PY-3T3 and SV40-PY-3T3 cells (Underhill and Keller, unpublished). However, many more cell lines must be tested before the significance of this change can be evaluated.

The fact that the heparan sulfate from transformed cells elutes from DEAE-cellulose at a lower ionic strength than that from normal cells, suggests that there is a structural difference between these carbohydrates with respect to either molecular weight or degree of sulfation (10). Indeed, standard heparan sulfate is composed of a wide spectrum of polymers, varying in size, degree of sulfation (11,12) and iduronic acid content (13). We are currently performing structural studies on the heparan sulfates from normal and transformed cells in order to distinguish between the various possibilities. Preliminary evidence suggests that there is no significant difference in the molecular weights of these heparan sulfates (Underhill, Bowe and Keller, unpublished).

While the significance of this alteration in heparan sulfate is unclear, it is possible that it is directly related to cell behavior. Heparan sulfate is a highly charged molecule on the outer portion of the cell surface. Since this is the area of first contact between a cell and its surroundings, alterations in the shape and/or charge of this molecule could have an influence on the response of a cell to its environment.

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REFERENCES

1. Dulbecco, R. (1969) Science 166, 962-968
2. Burger, M. M. (1973) Fed. Proc. 32, 91-101
3. Hakomori, S. (1973) Adv. Cancer Res. 18, 265-315
4. Kraemer, P. M. (1971) Biochemistry 10, 1445-1451
5. Patterson, M. S. and Green, R. C. (1965) Anal. Chem. 37, 854-857
6. Gardell, S. (1953) Acta Chem. Scand. 7, 207-215.
7. Gatt, R. and Berman, E. R. (1966) Anal. Biochem. 15, 167-171
8. Kraemer, P. M. (1971) Biochemistry 10, 1437-1445
9. Cifonelli, J. A. (1968) The Chemical Physiology of Mucopolysaccharides (Qunitarelli, G., ed.) pp. 91-105, Little, Brown and Co., Boston
10. Hallen, A. (1972) J. Chromatogr. 71, 83-91
11. Dietrich, C. P., Nader, H. B., Britto, L. R. G. and Silva, M. E. (1971) Biochim. Biophys. Acta 237, 430-441
12. Silva, M. E. and Dietrich, P. (1974) Biochem. Biophys. Res. Commun. 56, 965-972
13. Taylor, R. L., Shively, J. E., Conrad, H. E. and Cifonelli, J. A. (1973) Biochemistry 12, 3633-3637