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Distribution of Glycoconjugates in Mouse Fibroblasts With Varying Degrees of Tumorigenicity

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Analysis of glucosamine labeled glycoconjugates in cultured cells has been made comparing 2 clones and the parent embryonic mouse cell line. Hyaluronic acid, heparan sulphate, and chondroitin sulphate as well as a complex mixture of glycopeptides were found in the medium, the trypsinate, and the trypsinized cells, although the distribution was not uniform. The 3 cell lines had very similar in vitro growth properties, including their plating efficiency in viscous medium. However, the tumorigenicity of the cells, determined in syngeneic mice, was found to differ. All 3 cell lines were found to have similar glycoconjugate distributions, although a slight relative increase in labeled hyaluronic acid was found in the more tumorigenic mass cell line than either of the clones. The possible significance of this increase is discussed.

Key words: glycoconjugate, glycosaminoglycan, hyaluronic acid, transformation, tumorigenicity

The glycoconjugate metabolism of transformed cells, especially that of material located at the surface, has frequently been reported to differ from that of control cells. A high-molecular-weight glycoprotein on the surface of many cells appears to be almost absent in most virally transformed cells (1). Examination of the molecular weight of glycopeptides obtained from the membranes of paired cell lines has established the presence of increased levels of the larger glycopeptides in transformed cells (2, 3). Glycolipids, particularly the gangliosides series, have been claimed to exist predominantly as the simpler molecules after transformation by RNA and DNA viruses (4). Changes have also been found in glycosaminoglycan metabolism. In SV40 transformed cells, chondroitin sulphate biosynthesis has been reported to be decreased (5–9), while hyaluronic acid synthesis in some cases has been found to increase after transformation (10, 11). Histological evidence also indicated an increase in acid mucopolysaccharides at the surface of transformed cells (12, 13). Exceptions to these generalizations have been reported, and in the case of GAG metabolism aa review describing the often contradictory reports of quantitative alterations and the effects of GAGs on cell growth and tumorigenicity has been published (14). The lack of general agreement is perhaps to be expected when the wide variety of cell types, transforming

Abbreviations: GAG – glycosaminoglycan; DEAE – diethylaminoethyl

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agents, and analytical methods are considered. The nonuniform use of the term "transformation" (15) only serves further to confuse the issue, especially since its relationship to tumorigenicity is uncertain (16).

In this study, a technique which allows a fairly complete quantitation of glycoconjugate production in the cultures, was applied to a family of closely related cell lines. The tumorigenicity of these cell lines was simultaneously determined in syngeneic mice. Basically the technique consists of growing a pair of cell lines in medium containing ^{3}H- or ^{14}C-labeled glucosamine, and after mixing and digesting with papain, analyzing the macromolecular products by ion exchange. Three culture compartments have been examined: the medium, material released from the cell surface by trypsin, and the trypsinized viable cells. In order to compare cells under as nearly identical conditions as possible the radioactive precursors in the medium were at the same concentration and the cultures were labeled when at the same cell density during exponential growth. Five flasks of each cell line were labeled separately and combined before analysis to reduce the effect of any variability in the cell growth.

In this report we present the results of a comparison between 2 clones and the more tumorigenic mass cell line from which they were isolated. In each experiment, a clone was labeled with [^{14}C] glucosamine and the parent cell line was labeled with [^{3}H] glucosamine. The corresponding compartments from the clone and the parent cells were combined at the earliest possible time in the analytical procedure. The advantage of this experimental design is that differences observed between the amounts of each isotope in any isolated fraction will be due only to differences in the metabolism of the 2 cell lines being compared, and not to artifacts (such as incomplete recovery of a certain component) incurred during the analysis.

MATERIALS AND METHODS

The cell line 201 was an early frozen stock of the cells which gave rise to T AL/N (17). In these experiments it was used 27 subcultures after isolation from the AL/N mouse embryo. The mass cell line was cloned by placing 1 μl drops of a very dilute cell suspension in Falcon No. 3040 Microtest II trays. Medium was added only to wells which by microscopic determination contained single cells. In this way 2 clones were isolated from 201 cells after 26 (clone 210 C) and 23 (clone 216 C) subcultures; these were analyzed 28 and 3 subcultures, respectively, after cloning. Cells were cultured in 25cm^2 Falcon tissue culture flasks in the Dulbecco-Vogt modification of Eagle's medium supplemented with 10% heat-treated fetal calf serum, without antibiotics in an atmosphere of 95% air, 5% carbon dioxide. The cells were not contaminated by mycoplasma as determined by culture methods.

For each cell line 15 replicate cultures were prepared with 2×10^5 cells per flask. Ten flasks were used to establish a growth curve and the remaining 5 flasks used for the labeling experiment. One half of the medium was changed daily and the radioactive precursors were added when the paired cell lines were at approximately the same cell density in the mid-logarithmic phase of growth. Specific radioactivities of the precursors were adjusted to give a final concentration in the medium of approximately 5 μM for both isotopes. D-[6-^{3}H(N)] glucosamine HCl in the medium was at 2.5 μCi/ml and D-[1-^{14}C]-glucosamine HCl at 0.25 μCi/ml. After 24-h labeling the medium was removed and the cell sheet washed 4 times with phosphate-buffered saline, pH 7.4. Washes and media from the

5 flasks of the 2 cell lines to be compared were pooled. The cells were trypsinized in 0.9 ml of 0.01% (wt/vol) 3 times crystallized trypsin in Tris-buffered saline at pH 7.7 for 30 min at 37°C, after which the reaction was terminated by addition of 0.1 ml of 1 mg/ml soybean trypsin inhibitor. An aliquot of the suspension was taken to enumerate the cells using a Coulter counter. The 2 suspensions were mixed, centrifuged at 200 × g for 15 min and the cell pellet and the trypsinate were separated. From this point, the compartments were analyzed as doubly labeled mixtures. Gangliosides were isolated from the cell pellet as previously described (17), but using only a single extraction and removing the cell residue by filtration through Celite. The medium and trypsinate were equilibrated with papain digestion buffer (0.2 M sodium citrate, 0.5 M sodium chloride, 5 mM cysteine, 1 mM ethylenediaminetetraacetic acid, pH 5.5) by extensive dialysis, and the cell residue was suspended in the same buffer. All 3 culture compartments (medium, trypsinate, and cell residue) were digested with activated 2 times crystallized papain at 0.5 mg/ml for 4 h at 60°C. The reaction was terminated by cooling in ice and adding trichloroacetic acid to a final concentration of 10% (wt/vol). After 1 h at 4°C the precipitate was removed by filtration, the filtrate was neutralized with sodium hydroxide and dialyzed to equilibrium against 5 changes of 10 volumes of 10 mM Tris HCl, pH 8.4.

Each compartment was analyzed by anion exchange on a 1 × 4 cm column of Whatman DE52, equilibrated with 10 mM Tris HCl, pH 8.4. After applying the sample, the column was washed with 7 bed volumes of start buffer followed by a 300 ml linear gradient of 0–0.6 M sodium chloride. Fractions of 4 ml were collected at a flow rate of 36 ml hr^{-1} cm^{-1} and the conductivity was measured in every fourth fraction. Radioactivity in 0.5 ml aliquots was determined by liquid scintillation counting in Aquasol. Quench correction, double label evaluation, and graph plotting were performed by a computer program (LSCP).

Tumorigenicity was determined by intramuscular inoculation of tenfold dilutions of trypsinized cells into the hind leg of syngeneic AL/N mice. The mice were examined weekly for 18 weeks. Tumors occurred in 6–14 weeks. All tumors were lethal. Growth in semisolid medium was as described by Risser and Pollack (18). All enzymes were obtained from Worthington Biochemical Corporation (Freehold, New Jersey) and chemicals were analytical grade. Radiochemicals and scintillation fluid were obtained from New England Nuclear Corporation (Boston, Massachusetts).

RESULTS

The growth properties of the 3 cell lines are shown in Table I. It can be seen that the clones are at least 100-fold less tumorigenic than the parent cell line 201. It should be noticed that there is very little difference between the cell lines in their in vitro growth properties, with the exception of 210 C which has a lower saturation density than the other 2 cell lines.

The fourth wash of the cells before trypsinization contained less than 0.005% of the radioactivity in the medium and less than 5% of the radioactivity subsequently released by trypsin. Under the trypsinization conditions chosen, the amount of macromolecular glucosamine label released reached a plateau after 30 min, while the plasma membrane remained sufficiently intact to exclude Trypan blue in greater than 90% of the cells. Therefore, in agreement with other reports (3, 9, 19, 20) the trypsinate represents material released from the surface of cells. Radioactivity in the medium is unlikely to be due to glucosamine spuriously bound to serum proteins (21), because in the absence of cells only

TABLE I. In Vitro and In Vivo Growth Properties of the Cell Lines

Cell line	Monolayer growth: Doubling time (h)	Monolayer growth: Saturation density (10^4 cells/cm^2)	Plating efficiency in methyl cellulose (%)	Tumorigenicity TD_{50}[a]
201	13	54	0.3	$10^{2.6}$
210 C	14	18	0.3	$10^{6.4}$
216 C	13	40	0.2	$10^{4.8}$

[a] TD_{50} represents the number of cells which produced tumors in 50% of the animals injected (10 animals at each dose). Plating efficiency in methyl cellulose represents the number of colonies greater than 0.1 mm (about 60 cells), as a percentage of the cells plated.

0.3% of the radioactivity was found in the high-molecular-weight fraction of papain digested medium. Also the majority of the radioactivity secreted by the cells was found to elute as hyaluronic acid from ion exchange columns (cf. Fig. 1a, b).

Elution profiles from the ion exchange column generally showed 7 radioactive peaks which eluted at highly reproducible sodium chloride concentrations. The first 4 peaks were considered to be glycopeptides due to their charge, molecular weight, and carbohydrate composition (22). The fifth peak comigrated with a commercial preparation of hyaluronic acid, was not labeled by radioactive sulphate, and was depolymerized by testicular hyaluronidase (22). This peak was therefore designated hyaluronic acid. Peaks 6 and 7 were both labeled by radioactive sulphate, while only peak 7 was degraded by testicular hyaluronidase. The principal amino sugars in these 2 peaks were glucosamine and galactosamine, respectively (22). These results are consistent with the designations of heparin sulphate (peak 6) and chondroitin sulphate (peak 7), results which have been confirmed by chondroitinase ABC and AC digestion and by nitrous acid degradation (manuscript in preparation).

The majority of the radioactivity in the medium was due to hyaluronic acid for all 3 cell lines (Fig. 1a, b). In this compartment the fourth glycopeptide peak could not readily be identified. Radioactivity in the trypsinate was more uniformly distributed and all 7 peaks could be identified (Fig. 2a, b). Minor changes in the elution positions of peaks 6 and 7 (most noticeable in Fig. 2a) could be detected between the clones and the mass cell line, with the latter consistently showing a greater overlap between the 2 peaks (Fig. 2a, b). These changes possibly reflect slight differences in degrees of sulphation. In contrast to the other 2 compartments the trypsinized cells contained little radioactivity in the GAGs, the majority eluting in the second glycopeptide peak immediately after the sodium chloride gradient was started (Fig. 3a, b).

The similarity of the profiles of the cell lines is apparent from the figures. To determine if any quantitative differences existed, the amount of radioactivity in the peaks was summed and then expressed as the percentage of radioactivity applied to the cultures incorporated into the peak per 10^6 cells. The most prominent quantitative differences occurred between the clone 210 C and its parent, 201 (Table II). This clone incorporated substantially more radioactivity per cell into all fractions except hyaluronic acid throughout the culture. The other clone (216 C) incorporated very similar amounts of radioactivity to the parent cell line in all fractions, except into hyaluronic acid, in which the incorporation was reduced. Although some peaks were not well resolved and therefore the summa-

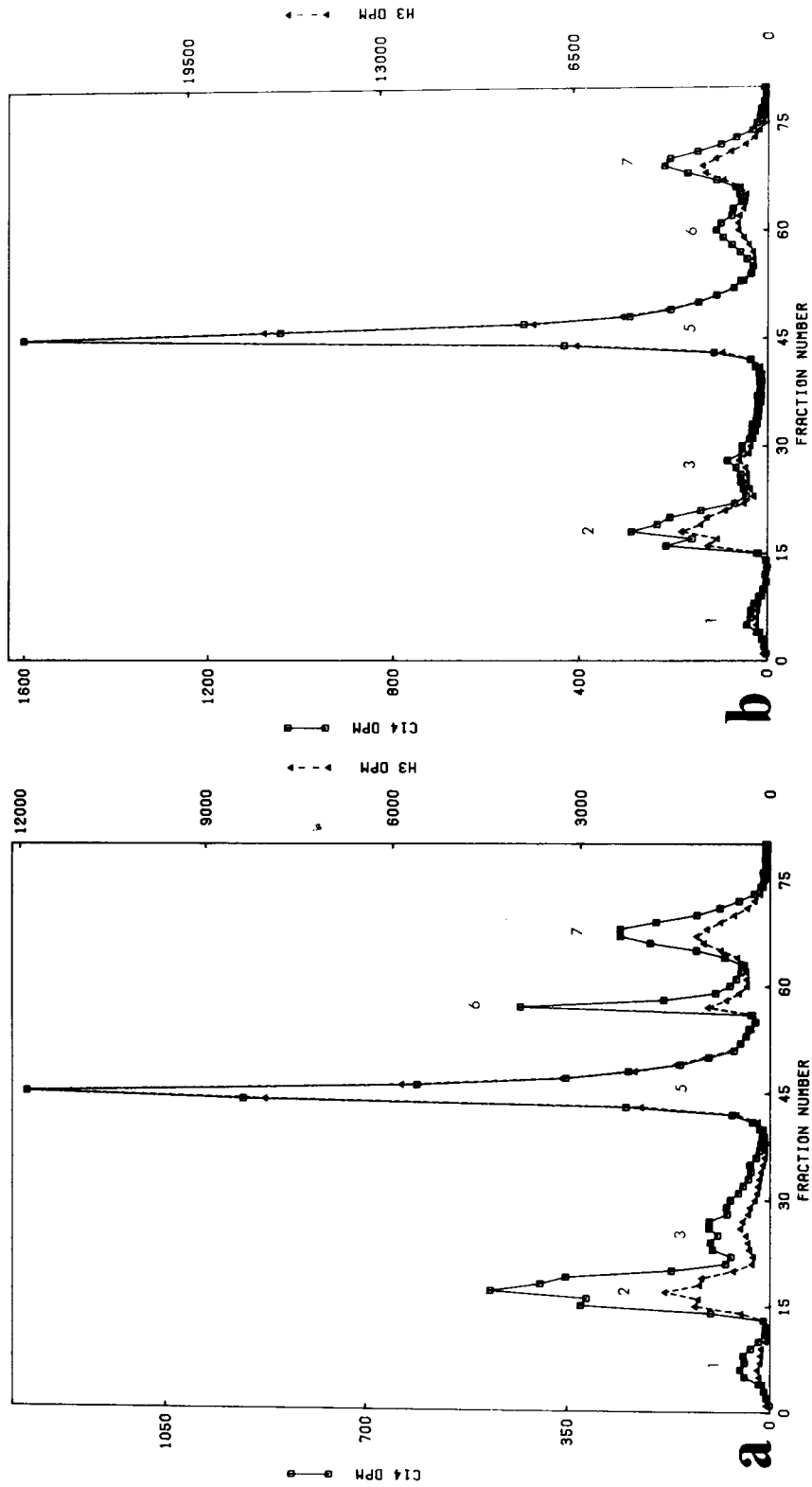

Fig. 1. Anion exchange elution profiles of papain digested media. a) Comparison of media from 210 C cells (^{14}C DPM) and 201 cells (^{3}H DPM). b) Comparison of media from 216 C cells (^{14}C DPM) and 201 cells (^{3}H DPM). Media from cells grown separately in the presence of radioactive glucosamine were pooled, digested with papain, and the high-molecular-weight material analyzed on a column of DEAE-cellulose. The gradient started at fraction 12 in a) and 13 in b). In all figures the data is plotted with the maximum DPM value at the same height for both isotopes. Recovery of applied radioactivity in the 6 elutions shown was 98.5 ± 4.1%.

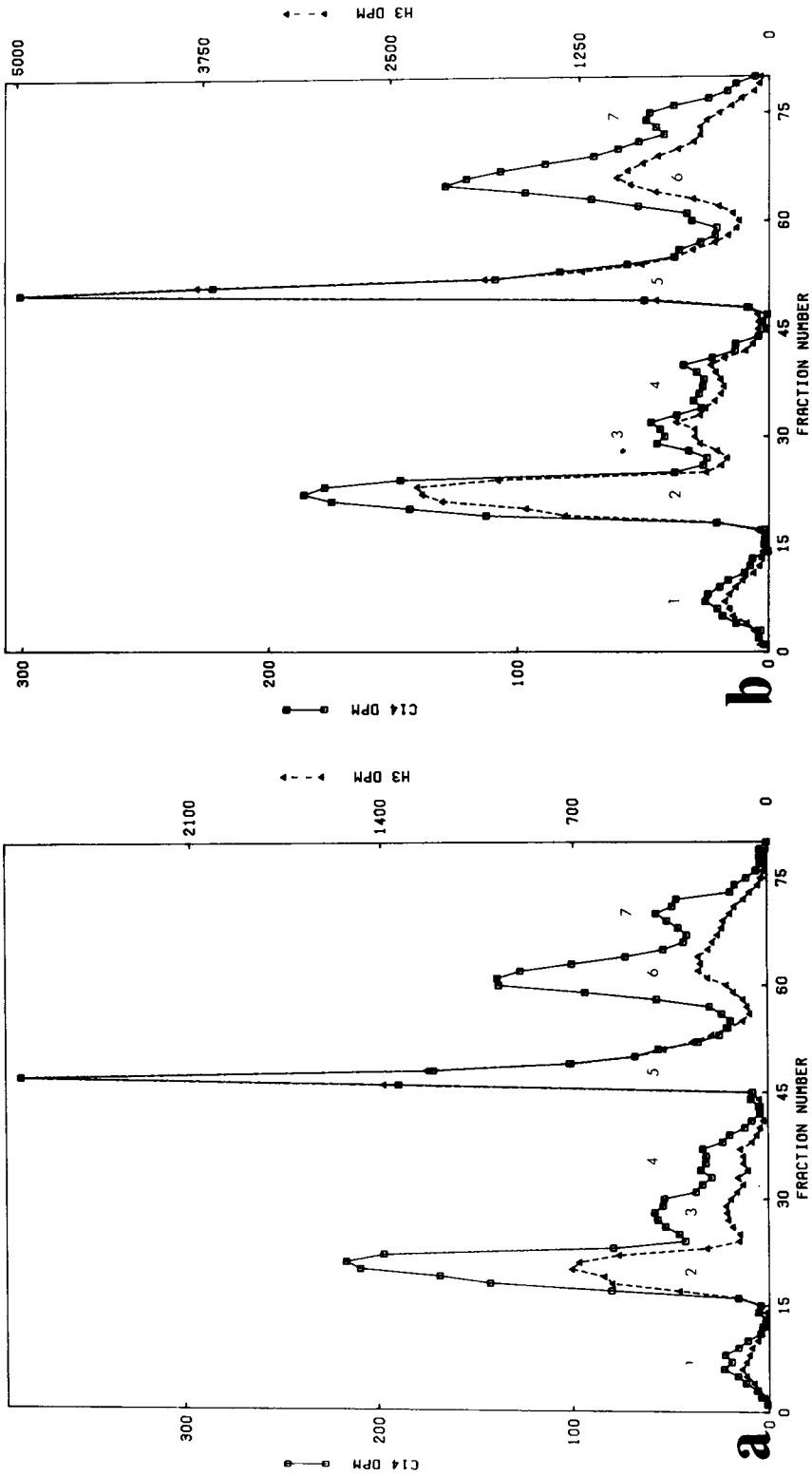

Fig. 2. Anion exchange elution profiles of papain digested trypsinates. a) Comparison of trypsinates from 210 C cells (^{14}C DPM) and 201 cells (^{3}H DPM). b) Comparison of trypsinates from 216 C cells (^{14}C DPM) and 201 cells (^{3}H DPM). Glucosamine labeled material released from the cell surface by trypsin was pooled, digested with papain, and the high-molecular-weight material analyzed on a column of DEAE-cellulose. The gradient started at fraction 15 in a) and 17 in b).

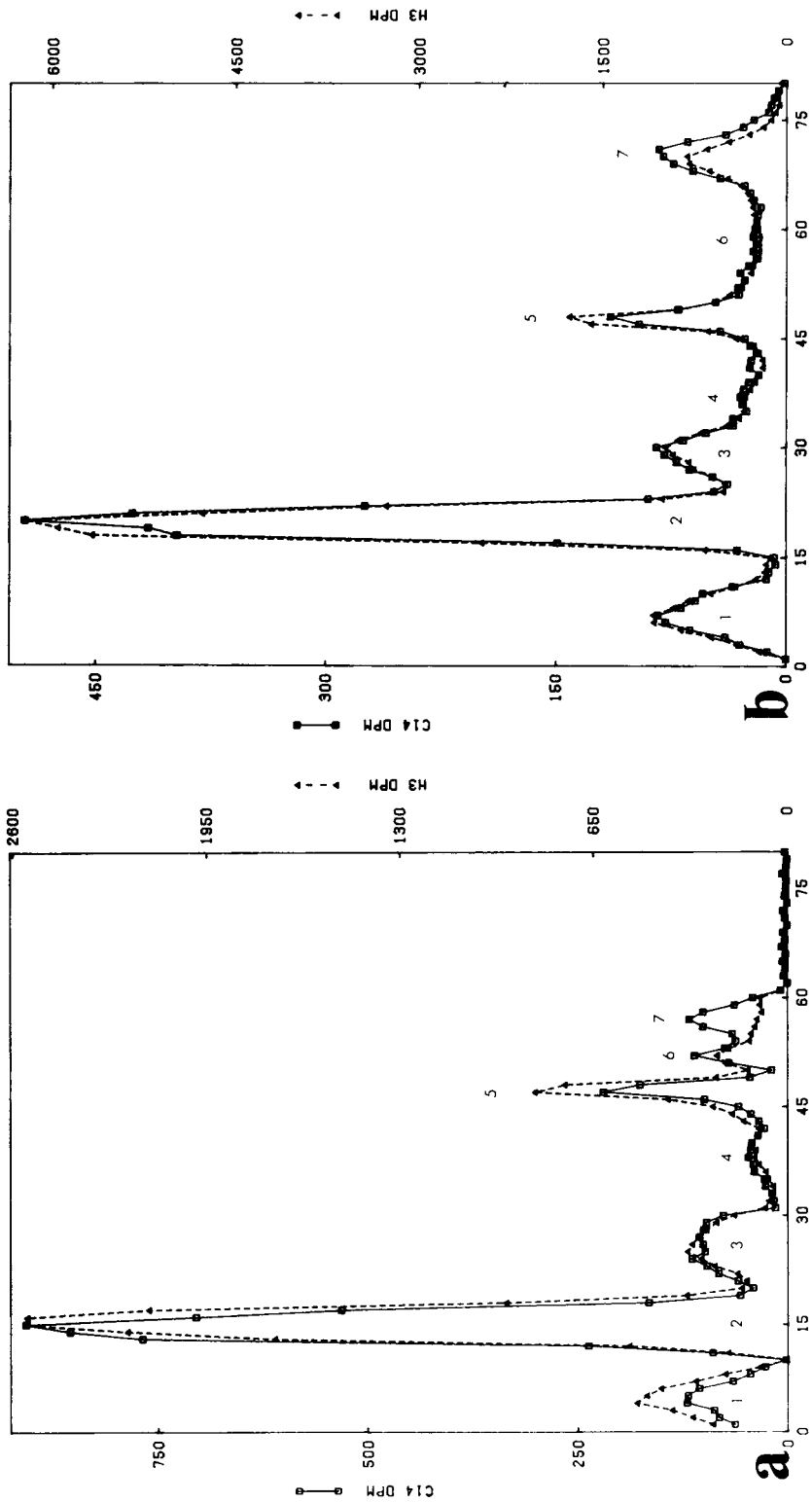

Fig. 3. Anion exchange elution profiles of papain digested cell residues. a) Comparison of 210 C cell residue (^{14}C DPM) and 201 cell residue (^{3}H DPM). b) Comparison of 216 C cell residue (^{14}C DPM) and 201 cell residue (^{3}H DPM). Cells labeled separately with glucosamine were trypsinized and then mixed. The cells, collected by centrifugation, were extracted with chloroform and methanol, and the insoluble residue was digested with papain. High-molecular-weight material was analyzed on a column of DEAE-cellulose. The gradient started at fraction 12 in a) and 16 in b).

TABLE II. Distribution of the Radioactive Glycoconjugates in the 3 Cell Lines*

Culture compartment	Cell line	Radioactivity incorporated per fraction			
		Glycopepties	Hyaluronic acid	Heparin sulphate	Chondroitin sulphate
Medium	210 C	66	77	18	27
	201	37	110	11	20
Trypsinate	210 C	37	21	17	7
	201	17	22	5	3
Cell	210 C	120	10	6	8
	201	59	7	3	2
Medium	216 C	32	66	12	17
	201	29	92	11	15
Trypsinate	216 C	25	13	13	4
	201	26	19	10	3
Cell	216 C	52	7	3	8
	201	61	9	3	7

*Data from the figures are expressed as 10^{-3} × % of applied radioactivity incorporated per 10^6 cells. The glucosamine precursors were present at 5 μM in all cases and at the end of the labeling period the cell densities were respectively 4 × 10^4 and 2 × 10^4 cells/cm^2 for 210 C and 201 and in the second experiment, 5 × 10^4 and 4 × 10^4 cells/cm^2 for 216 C and 201.

TABLE III. Ratio of the Radioactivity Incorporated by the Clones Compared to the Parent Cell Line*

Culture compartment	Ratio	Ratio of radioactivity			
		Glycopeptides	Hyaluronic acid	Heparin sulphate	Chondroitin sulphate
Medium	210 C:201	1.8	0.7	1.6	1.4
Trypsinate	210 C:201	2.2	1.0	3.4	2.3
Cell	210 C:201	2.0	1.4	2.0	4.0
Whole culture	210 C:201	2.0	0.8	2.2	1.7
Medium	216 C:201	1.1	0.7	1.1	1.1
Trypsinate	216 C:201	1.0	0.7	1.3	1.3
Cell	216 C:201	0.9	0.8	1.0	1.1
Whole culture	216 C:201	0.9	0.7	1.2	1.2

*The ratio seen in the whole culture is not a mean of the other ratios, due to the nonuniform distribution of radioactivity through the culture. The differences between the ratios for hyaluronic acid and those in the rest of the culture is significant ($P < 0.025$ for 210 C:201 and $P < 0.0025$ for 216 C:201, by Student's t-test).

tion may be subject to error, yet the reproducibility of these results is satisfactory as can be seen by comparing the 2 sets of values for 201 in Table II.

When the data was expressed as a ratio of the radioactivity incorporated a statistically significant decrease in the amount of radioactive hyaluronic acid relative to all the other components was found between both clones and the parent cell line (Table III).

The distribution of glycoconjugates throughout the culture can be seen in Table II. This was very similar for all 3 cell lines. Chondroitin sulphate, in agreement with other

studies (5, 6, 8, 11), was found predominantly in the medium. A substantial portion of the heparin sulphate was associated with the cell surface, a location first reported by Kraemer (19). Hyaluronic acid was mostly secreted in agreement with earlier reports (11, 23), and the cells retained a major portion of the glycopeptides even after trypsinization.

DISCUSSION

The difficulty in obtaining a biological system for studying the biochemical events which may lead to tumor induction in vivo, and the lack of a general correlation between culture growth properties and tumorigenicity has recently been reviewed (16). In the cells described here there is no correlation between tumorigenicity and plating efficiency in viscous medium, in fact the highly tumorigenic cell line 201 has a very low plating efficiency (Table I), under conditions where other related cells, such as an SV 40 transformed analog of the T AL/N cells (SV AL/N) gave a minimum of 10% plating efficiency (24).

The data presented here show marked similarity in the ion exchange elution profiles of papain digested glycoconjugates from 3 related cell lines that differ in their tumorigenicity. In fact the only detected difference that correlates with higher tumorigenicity is a slightly higher labeling of hyaluronic acid relative to the other glycoconjugates. This difference is most noticeable between 210 C and 201, the cells whose tumorigenicity differs by the largest amount.

Slightly increased quantities of hyaluronic acid were found in the medium of Swiss 3T6 and 3T3 (23). Although the tumorigenicity of these cells is not known, cells isolated in a similar way from Balb/c mice showed 3T3 to have the lowest tumorigenicity (25). After transformation of 3T3 cells by DNA viruses, the amount of hyaluronic acid in the medium was greatly reduced (23). Results conflicting with this early observation have been obtained. After SV40 transformation, 3T3 cells produced larger quantities of an aggregation factor, shown to be hyaluronic acid (26). Also the radioactivity incorporated into hyaluronic acid from glucose represented a greater proportion of the total GAG label in SV3T3 than in 3T3, when the cells were at high densities (9). However, this report of a relative increase in hyaluronic acid may be due to the decreased synthesis of sulphated GAGs (5, 6, 8). Increased hyaluronic acid production after transformation by SV40 has been reported for monkey (7) and hamster (11) cells and we have unpublished data for a T-antigen positive subclone of 210 C.

Increased hyaluronic acid synthesis seems to correlate well with Rous sarcoma virus (RSV) transformation of avian cells (10, 27) and tumors induced in chicken by this virus are known to synthesize large quantities of hyaluronic acid (28). Rakusanova (29) confirmed these results with avian cells, but found that mammalian cells transformed by RSV either showed no change or produced less hyaluronic acid than normal or spontaneously transformed fibroblasts. Decreased hyaluronic acid synthesis was found in a melanoma when compared to iris melanocytes (30) and no change was noticed when comparing normal and malignant human mesothelial cells (31).

The mechanism by which increased secretion of hyaluronic acid could benefit tumor growth is not clear. The possibility that mucopolysaccharides can mask antigens has been discussed (14, 32, 33) and the exposure of lectin-binding sites by hyaluronidase was reported (34); furthermore, hyaluronic acid has been found to block in vitro stimulation of lymphocytes by phytohemagglutinin (35). Another mechanism could involve the production of an appropriate extracellular matrix in which the cells can proliferate (16). Boone et al. (36) found that Balb/c 3T3 cells readily produced tumors when implanted attached to

a solid substrate. Hyaluronic acid has been shown to be a major component of the substrate-attached material thought to be involved in cell-to-substrate and cell-to-cell adhesion (37), and an increase in its synthesis was found to be an early cellular response to stimulation by epidermal growth factor (38).

Whether the increased production of hyaluronic acid by more tumorigenic cells is a general phenomenon, is being explored in other matched cell lines from this and similar series. Changes in molecular weight, both of hyaluronic acid and the other glycoconjugates, have not been excluded and we are examining this possibility also.

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