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DIFFERENCES IN THE SULFATED MACROMOLECULES SYNTHESIZED BY NORMAL AND TRANSFORMED HAMSTER FIBROBLASTS

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Summary

Analysis of the $^{35}SO_4$-labelled macromolecules synthesized by cultures of normal (NIL8) and transformed (NIL8-HSV) hamster fibroblasts has revealed the following differences between the two cell lines:

(1) The proportion of sulfate incorporated into cell-associated macromolecules is three times higher in normal than in transformed cells. In addition, normal fibroblasts incorporate more sulfate into extracellular, middle and low molecular weight species than do transformed cells. Transformed cells, however, incorporate more sulfate into extracellular, very high molecular weight species than do normal cells.

(2) Normal fibroblasts, which synthesize much more extracellular dermatan sulfate than do transformed cells, produce a class of extracellular heterogeneous sulfated proteoglycans absent from transformed cultures. This macromolecular species consists largely of dermatan sulfate. The transformed cells instead release a lower molecular weight class of proteoglycans which consist of chondroitin sulfates A and C.

(3) The large, external, transformation-sensitive glycoprotein is sulfated in NIL8 cultures. This macromolecular species is present on the surface membrane of normal cells, but absent from transformed cells. Sulfated large, external transformation-sensitive protein is also present in the conditioned medium from normal cultures. A similar species is present in the conditioned medium from transformed cultures, but has a slightly higher apparent molecular weight and differs in other properties from the large, external, transformation-sensitive protein of normal cells.

Introduction

The surface membrane of malignant cells in vivo and transformed cells in culture is strikingly different from that of normal and untransformed cells. One of

the most prominent changes accompanying viral transformation of cultured fibroblasts is an alteration in the array of surface proteins accessible to labelling by lactoperoxidase-catalyzed iodination [1]. The major iodinatable species, a glycoprotein with a molecular weight of approximately 250 000, is absent or much reduced on the surfaces of virally transformed hamster cells [2—4], chicken embryo fibroblasts [5—7] and other cells [1]. These data and experiments with rat fibroblasts transformed by adenovirus and tested for malignancy in vivo [8] seem to indicate that the absence of this large, external, transformation-sensitive (LETS) glycoprotein is intimately associated with transformation and malignancy.

Differences in the proteoglycans synthesized by normal and transformed cells have also been reported. For example, a number of studies have found that SV40-transformed 3T3 cells produce less sulfated proteoglycan than do untransformed 3T3 cells [9—12]. These studies have demonstrated that 3T3 cells incorporate from 2 to 5 times as much radioactive sulfate into cell-bound proteoglycans as do SV3T3 cells, while the amount of labelled proteoglycans in the medium remains approximately constant. Studies with other SV40-transformed cell lines, however, have yielded inconsistent results. Increased proteoglycan production was reported to follow SV40 transformation of monkey kidney cells [13,14], hamster embryo cells [14], and human fibroblasts [15]. The general trend, however, seems to indicate that transformed cells produce less cell-associated sulfated proteoglycan than do untransformed cells (see also ref. 16).

Other experiments have been performed to test the role of proteoglycans of the cell periphery directly. For example, addition of crude proteoglycans obtained from normal liver to cultured hepatoma cells was found to result in a return to density-dependent inhibition of growth [17]. Exogenous dextran sulfate has also been shown to have similar effects [18]. A solution of 2 μg/ml dextran sulfate was capable of lowering the saturation density of 3T6 cells. In addition, the morphology of the treated cells became flattened. These and other results [19,20] suggest that at least some highly sulfated polyanions, when at the cell surface, can cause morphological and growth control changes.

In this report, the nature and distribution of the sulfated macromolecules synthesized by cultures of normal and hamster sarcoma virus (HSV)-transformed NIL8 hamster cells is examined. Evidence is presented showing both quantitative and qualitative differences in the proteoglycans synthesized by these two cell lines, and the sulfated character of the LETS glycoprotein in these cells.

Materials and Methods

Cell culture

Cells were grown in 35- and 60-mm plastic tissue culture flasks (Falcon), and plastic (Linbro) dishes containing 24 16-mm wells. Culture medium was Dulbecco's modified Eagle's (DME) high glucose medium containing 5% fetal calf serum, penicillin, streptomycin, and antibiotic-antimycotic (GIBCO).

Radioactive labelling of cell cultures

After cells had attained confluency, the culture medium was removed and

replaced with fresh medium containing $Na_2{}^{35}SO_4$ (carrier-free, New England Nuclear, Boston, Ma.) at 100 μCi/ml. ^{125}I-labelled conditioned medium was prepared by removing the culture medium and labelling externally accessible proteins and glycoproteins by the technique of lactoperoxidase-catalyzed iodination [2]. Fresh culture medium was subsequently added and labelled macromolecules were allowed to chase into the medium with time.

Preparation of $^{35}SO_4$-labelled and ^{125}I-labelled samples

At the end of the labelling period, the conditioned medium was removed and centrifuged at approximately 1000 $\times g$ for 10 min. Cells and pellet (containing any floating cells) were washed twice with Dulbecco's phosphate-buffered saline Solution A and dissolved with either 2% sodium dodecyl sulfate (SDS) (BioRad) in water or 0.1 M Tris · HCl (pH 6.8) containing 15% glycerol (Fisher) and 2% SDS. Cells removed with 2% SDS in water were lyophilized to dryness and resuspended in 0.05 M Tris · HCl (pH 6.8) containing 10% glycerol and 0.1 M dithiothreitol (Sigma). All samples (cells, conditioned medium, column fractions, etc.) contained approximately 2% SDS, 0.05 M Tris · HCl (pH 6.8), 10% glycerol, and 0.002 M phenyl methyl sulfonyl fluoride (PMSF, Sigma) before application to SDS polyacrylamide gels. Reduced samples contained 0.1 M dithiothreitol, non-reduced samples did not. All samples were boiled for 2 min before application to the SDS polyacrylamide gels.

Quantitation of $^{35}SO_4$-labelled and ^{125}I-labelled proteoglycans

Aliquots of conditioned medium, cell lysate, etc., were spotted on 24 mm circular paper filters (Schleicher and Schuell). Cell lysates were boiled for 2 min before aliquoting in order to denature cellular DNA and reduce viscosity. Proteoglycans (and other macromolecules) were precipitated at 4°C in 10% trichloroacetic acid (Fisher) for 10 min followed by 5% trichloroacetic acid for 10 min and two rinses in ethanol at 4°C. ^{125}I filters were counted in a gamma radiation counter. Dried ^{35}S filters were placed in vials containing 5 ml toluene scintillant and counted in a liquid scintillation counter. Lowry protein determinations [21] were performed on cell lysates in order to normalize counts from cultures grown in different dishes.

In preliminary experiments samples were also precipitated in a solution of 5 mg/ml cetyl pyridinium bromide (Sigma) in water for 2 h at 37°C. Precipitates were collected by suction filtration on Millipore filters. The latter method, however, was only used in order to determine the most efficient method for recovering labelled material; the trichloroacetic acid method was used primarily throughout the investigation.

Autoradiograms were quantitated by a soft laser scanning densitometer (Zeineh). Areas under peaks were measured with the automatic integrator.

Column chromatography

Conditioned medium samples containing approximately 10% glycerol were added to 120 ml of packed A5m agarose beads (Biogel, 100—200 mesh) in a column (2 × 90 cm) at 4°C. Running buffer was 50 mM Tris · HCl (pH 7.4) (Sigma) and the flow rate was 15 ml/h. Eluate was collected in 1—2.5-ml fractions. Selected column fractions were concentrated by lyophilizing to dryness

and redissolving in water. Concentrated fractions were applied to SDS polyacrylamide gels.

Gel electrophoresis and autoradiography

Electrophoresis was performed in a vertical slab gel apparatus using three types of SDS polyacrylamide slab gels: a 1% stacking gel with 3% running gel (both containing 0.5% agarose), a 3% stacking gel with 5% running gel, and a 4% stacking gel with 6% running gel [22]. All gel materials were purchased from BioRad. The slab gels were subsequently stained and fixed with Coomassie Brilliant Blue (BioRad) in 1 : 1 methanol/H_2O containing 7% acetic acid, and destained in the same solution without Coomassie Blue. 2,5-diphenyloxazole (PPO) was then absorbed into the gel, in order to increase the sensitivity for the subsequent autoradiography [23] by initially removing water from the gels with 2 incubations in dimethyl sulfoxide (Me_2SO) and then incubating the gels in a solution of 40 g PPO in 180 ml Me_2SO for 3 h. The Me_2SO/PPO was then removed and H_2O added in order to precipitate the PPO in the gel. Gels were dried on filter paper and autoradiographed with RP/R2 medical X-ray film (Kodak).

Antibody precipitation

10 μl of rabbit antiserum raised against SDS gel-purified LETS protein was added to 1 ml of conditioned medium and allowed to incubate for 24 h at 4°C. 10 μl of preimmune serum was used in place of the antiserum in the control samples. All incubation mixtures contained 2 mM EDTA (Matheson, Coleman and Bell) and 2 mM PMSF to inhibit any proteases. 100 μl of goat anti-rabbit antiserum was subsequently added to the mixtures which were then allowed to incubate for 4 h at 4°C. Samples were centrifuged at 1000 $\times g$ for 10 min at 4°C and the precipitates collected. Pellets were washed with Dulbecco's phosphate-buffered saline Solution A 3 times (resuspended and centrifuged) and dissolved for application to SDS polyacrylamide gels. Aliquots were taken for trichloroacetic acid precipitations and scintillation counting.

Ammonium sulfate precipitation of LETS glycoprotein

Conditioned medium samples were pre-pelleted by centrifugation for 10 min at approximately 12 000 $\times g$. Ammonium sulfate (ultrapure, Schwartz-Mann) was added slowly with stirring at room temperature to a final concentration of 30% (w/v). Samples were stirred for approximately 3—4 h after all the ammonium sulfate had been added. Precipitates were collected by centrifugation for 10 min at approximately 12 000 $\times g$. Resulting pellets were dissolved in a solution of 0.1 M Tris · HCl (pH 6.8), 0.01 M sodium azide (Sigma), and 2 mM PMSF.

Enzymatic digestions

The following enzymatic treatments were performed:

(1) Trypsin (Sigma): 10 min, 10 μg/ml, room temperature. Trypsin digestion was stopped with a two-fold excess of soybean trypsin inhibitor.

(2) Pronase (Calbiochem): 4—20 h, 100 μg/ml, 37°C. The pronase stock was incubated at 37°C for 0.5 h before using in order to eliminate contaminating enzymatic activities.

(3) Hyaluronidase (Sigma): 1—20 h, 100 μg/ml, 37°C.

(4) Hyaluronidase: 1—20 h, 100 μg/ml, 37°C, followed by trypsin: 10 min 10 μg/ml, room temperature..

(5) Chondroitinase abc (Miles): 1—20 h, 0.5 U/ml, 37°C.

(6) Chondroitinase ac (Miles): 1—20 h, 0.5 U/ml, 37°C followed by trypsin: 10 minutes, 10 μg/ml, room temperature.

(8) Dulbecco's phosphate-buffered saline solution (control): 0—20 h, room temperature, 37°C. Reactions were stopped by the addition of 1/3 vol of a solution containing 33% glycerol, 6.7% SDS, and 0.33 M dithiothreitol (in preparation for application to SDS polyacrylamide gels). Aliquots were taken for trichloroacetic acid precipitation and subsequent quantitation of label. Pronase treatments degrade LETS glycoprotein [1,3]. Chondroitinase abc degrades chondroitin sulfate and dermatan sulfate; hyaluronidase and chondroitinase ac degrade only chondroitin sulfate.

Results

I. Control experiments

Initial experiments were designed to determine the most appropriate methods for collecting and analyzing the sulfated proteoglycans synthesized by NIL8 and NIL8-HSV cultures. Two precipitation procedures were employed: 10% trichloroacetic acid, and 5 mg/ml cetylpyridinium bromide (see Materials and Methods). The trichloroacetic acid procedure is usually effective for most macromolecules, while the cetylpyridinium bromide method is designed specifically for polysaccharide-containing macromolecules: polyanions form water insoluble salts with detergent cations such as pyridinium [24]. Both methods were found to precipitate approximately the same number of counts from $^{35}SO_4$-labelled NIL8-conditioned medium. In addition, cetylpyridinium bromide treatment following pronase digestion (100 μg/ml, 13 h, 37°C) precipitated approximately 60% of the counts precipitated by cetylpyridinium bromide or trichloroacetic acid before pronase, while trichloroacetic acid treatment following pronase digestion precipitated only a small fraction (<5%) of the presumably protein-free labelled material. However, since the trichloroacetic acid method could be conveniently generalized to precipitation on paper filters (see Materials and Methods), this procedure was used subsequently throughout the investigation.

The staining-destaining of gels and the methods used to increase the sensitivity of the autoradiography (see Materials and Methods) were also examined in order to determine if these techniques, normally employed in the characterization of proteins, were appropriate for the study of sulfated proteoglycans and glycoproteins. Autoradiographs of untreated gels and stained, destained and Me_2SO-treated gels were qualitatively identical in pattern. Densitometric quantitation showed some losses of radioactivity from molecular weight regions less than 250 000 but none from higher molecular weight regions of the gels. Material from both NIL8 and NIL8-HSV cultures responded similarly to the treatments, indicating that valid comparisons could be made between the two cell lines.

II. Distribution of $^{35}SO_4$-labelled macromolecules

Kinetic analysis showed that for the first 4—6 h of labelling, incorporation of

sulfate into cell-associated and released material was approximately equivalent. Sulfate-labelled macromolecules accumulated in the culture medium linearly with time for at least 52 h. In contrast, the amount of cell-associated labelled material reached a plateau value after approximately 12—14 h. When cells were labelled for 6 hours and then placed in fresh medium, approximately 50% of the cell-bound label was released into the medium after 24 h. Only minor differences were observed in these kinetics of $^{35}SO_4$ incorporation between normal and transformed cells. Buonassissi [25] has reported similar $^{35}SO_4$ incorporation kinetics for endothelial cell cultures.

The distribution of incorporated $^{35}SO_4$ after 48 h of labelling is shown in Table I. While the transformed cells incorporated more radioactivity overall, they incorporated a smaller proportion into cell-associated material than did normal cells (6% vs. 18—20%). The majority of the sulfated macromolecules synthesized over a period of 48 h were released into the medium in both cases.

SDS-polyacrylamide gel analysis of $^{35}SO_4$-labelled samples of cells and culture medium are shown in Fig. 1. Both normal and transformed cells showed a heterogeneous population of high molecular weight material trapped at the top of the gels and extending down into the running gel (tracks A and B). In addition, the normal (NIL8) cells showed labelling of two discrete bands, one of which comigrated with iodinated LETS glycoprotein (arrow on track A). These were not observed on the NIL8-HSV cells (track B). The distributions of radioactivity in the conditioned medium samples differed from those of the cells and showed greater differences between normal and transformed cultures (tracks C and D). Most sulfate-labelled macromolecules did not migrate as discrete bands on the SDS-polyacrylamide gels although the proteins detected by Coomassie Blue staining of the same gels showed sharp bands. This presumably reflects the heterogeneity of proteoglycans. Both NIL8 and NIL8-HSV released high molecular weight material which did not enter the running gel. In addition, the NIL8 conditioned medium contained a discrete labelled band migrating at about 250 000 daltons and a broad heterogeneous band of labelled material migrating ahead of it (approximate apparent molecular weights 150 000—250 000 relative to marker proteins). The NIL8-HSV-conditioned medium lacked this broad band and it was replaced by a broad heterogeneous band of lower molecular weight (100 000—180 000). Pronase treatment greatly increased the mobility in the gels of all the labelled macromolecules indicating that they contain protein.

TABLE I

DISTRIBUTION OF TRICHLOROACETIC ACID-PRECIPITABLE SULFATED MACROMOLECULES IN CULTURES OF NORMAL (NIL8) AND TRANSFORMED (NIL8.HSV) CELLS

48-h label.

Culture	cpm incorporated per μg cell protein			% of total cpm incorporated	
	Cells	Medium	Total	Cells	Medium
NIL8 A	20.3	80.6	100.9	20	80
NIL8 B	21.7	101.1	122.8	18	82
NIL8.HSV A	10.2	151.7	161.9	6	94
NIL8.HSV B	9.5	154.6	164.1	6	94

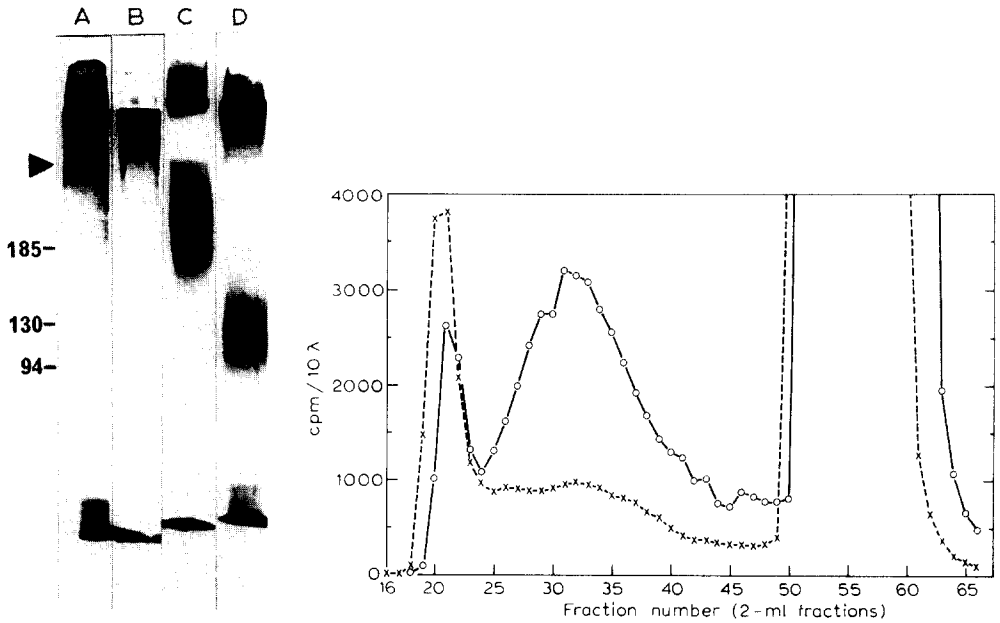

Fig. 1. SDS polyacrylamide gel analysis of $^{35}SO_4$-labelled cell and medium samples. Autoradiogram of 5% slab gels. A and B are from a different gel from C and D. A, NIL8 cells (6-h label). B, NIL8-HSV cells (6-h label). C, NIL8 conditioned medium 48-h label). D, NIL8-HSV conditioned medium (48-h label). Arrow marks band comigrating with iodinated LETS protein. Molecular weight scale indicates migration position of marker proteins; LETS protein (230 000); α2-macroglobulin (185 000); β-galactosidase (130 000); phosphorylase A (94 000).

Fig. 2. Chromatography of $^{35}SO_4$-labelled conditioned medium samples on an A5m column. Samples of culture medium derived from equal quantities (protein) of NIL8 (○———○) and NIL8-HSV (×———×) cells were applied to the column. All radioactivity before Fraction 50′ was trichloroacetic acid-precipitable.

Thus, NIL8 cultures contain several types of sulfated macromolecules: distinct, resolved species one of which co-migrated with LETS glycoprotein and which is found both on the cells and in the medium, and heterogeneous, largely extracellular material which migrates ahead of LETS glycoprotein. NIL8-HSV cells appear to lack the discrete species, although a similar band having a slightly higher apparent molecular weight is present in the conditioned medium (Fig. 1, track D). The heterogeneous extracellular material is replaced by heterogeneous material of lower molecular weight.

III. Characterization of sulfated macromolecules

$^{35}SO_4$-labelled material from conditioned media was also separated on A-5m agarose gel columns (Fig. 2) in order to quantitate the incorporation into various components. NIL8 culture medium contained more radioactivity in material included in the column beads, whereas NIL8-HSV medium was enriched for very high molecular weight material excluded from the column. Analysis of A5m column fractions on gels shows that the sulfated species included in the column are those described earlier. The only anomaly is that the heterogeneous molecules in the NIL8-HSV samples comigrate with those from NIL8 on the column but migrate with greater mobility on SDS-polyacrylamide gels (Fig. 3).

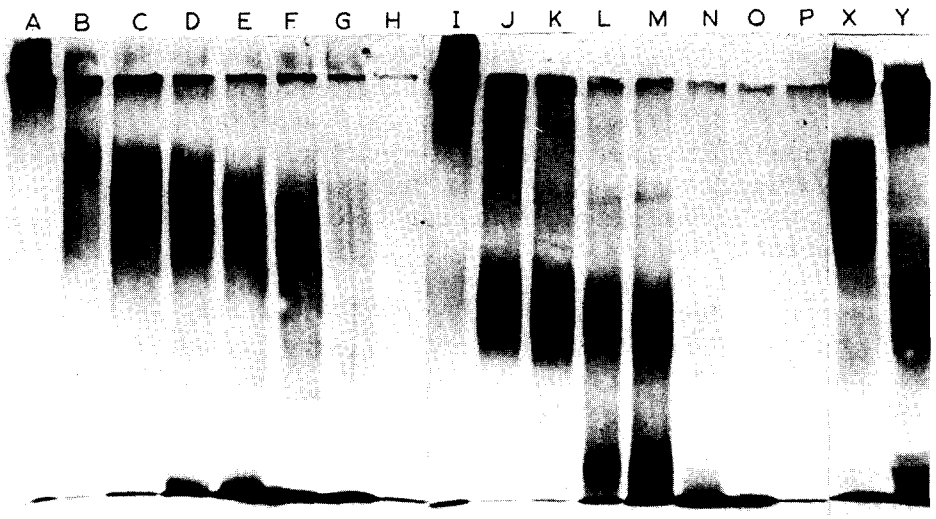

Fig. 3. Selected fractions from the A5m column separation (Fig. 2) were analyzed on 5% slab gels. A—H, NIL8 medium: samples correspond to fractions from A5m column as below. A, Fraction 21; B, Fraction 26; C, Fraction 29, D, Fraction 31; E. Fraction 33; F, Fraction 37; G, Fraction 40; H, Fraction 54. I—P, NIL8-HSV medium: samples correspond to fractions from A5m column as below. I, Fraction 21; J, Fraction 26; K, Fraction 29; L. Fraction 31; M, Fraction 33; N, Fraction 37; O, Fraction 40; P, Fraction 54. In each case, equal volumes were applied to the gel. X and Y are samples of unfractionated medium from NIL8 and NIL8-HSV cells, respectively.

Enzymatic digestions of NIL8 and NIL8-HSV conditioned medium were performed in order to determine quantitative and qualitative differences in the sulfated macromolecules synthesized by the two cell lines (Table II). In both cell types, 20—35% of the labelled material was resistant to chondroitinase abc. This material presumably consists mainly of heparan and heratan sulfates, which are not attacked by this enzyme. NIL8-HSV-conditioned medium con-

TABLE II

ENZYMATIC DIGESTIONS OF NIL8 AND NIL8.HSV $^{35}SO_4$-LABELLED CONDITIONED MEDIUM

Experiment	Enzyme treatment	Trichloroacetic acid-precipitable counts remaining (10-μl aliquots)		Percent degraded	
		NIL8	NIL8.HSV	NIL8	NIL8.HSV
A	Buffer: 20 h, 37°C	2017	3254	*	*
	Hyaluronidase, 100 U/ml, 20 h, 37°C	1297	897	36	72
	Chondroitinase abc, 0.5 U/ml, 20 h, 37°C	724	678	64	79
B	Buffer: 7.5 h, 37°C	2111	2772	*	*
	Chondroitinase ac, 0.5 U/ml, 7.5 h, 37°C	1231	938	42	66
	Chondroitinase abc, 0.5 U/ml, 7.5 h, 37°C	692	925	67	67
C	Buffer: 8.0 h, 37°C	1804	2805	*	*
	Chondroitinase ac, 0.5 U/ml, 8.0 h, 37°C	1080	791	40	72
	Chondroitinase abc, 0.5 U/ml, 8.0 h, 37°C	512	795	72	72

tained very little labelled material (7%) sensitive to chondroitinase abc but not to hyaluronidase, and virtually no labelled material (0—1%) sensitive to chondroitinase abc but not to chondroitinase ac. NIL8 conditioned medium, however, contained 28% of the former labelled material, and from 25—32% of the latter. Since chondroitinase abc degrades chondroitin sulfate and dermatan sulfate, while hyaluronidase and chondroitinase ac degrade only chondroitin sulfates A and C, the labelled material found in NIL8 conditioned medium which is absent from (or at low concentrations in) NIL8-HSV medium is probably dermatan sulfate (chondroitin sulfate B).

Autoradiograms from the enzymatic digestion experiments are shown in Fig. 4. The broad band observed in NIL8-conditioned medium but not in NIL8-HSV-conditioned medium was completely degraded by chondroitinase abc but was resistant to chondroitinase ac and hyaluronidase (not shown) and therefore is probably dermatan sulfate. In addition, the broad band from NIL8-HSV-conditioned medium (which migrates farther into the running gel than the band from NIL8 conditioned medium) is degraded by all three enzymes and therefore consists of chondroitin sulfates A and/or C.

Enzymatic digestion of NIL8 conditioned medium with chondroitinase abc reveals the distinct, more clearly resolved band in the autoradiograms from SDS polyacrylamide gels (Fig. 4). This band can be seen (after treatment with chondrotinase abc) to co-migrate with ^{125}I-labelled LETS glycoprotein under both reducing (monomer, molecular weight = 230 000) and nonreducing (dimer, molecular weight = 460 000) conditions (Fig. 5). This band is also present in the cell lysates from NIL8 cultures, but absent from NIL8-HSV cells (Fig. 1). Digestion of NIL8-HSV conditioned medium reveals a similar band, but with a

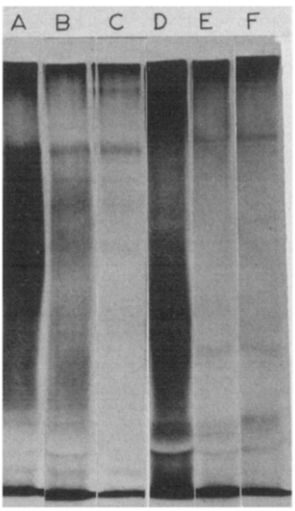

Fig. 4. Acrylamide gel analysis of enzymatic digestions of NIL8 and NIL8-HSV conditioned medium. Equal samples were treated as below and applied to the gel. A, NIL8 conditioned medium: buffer, 8 h, 37°C. B, NIL8 conditioned medium: chondroitinase ac, 0.5 U/ml, 8 h, 37°C. C, NIL8 conditioned medium: chondroitinase abc, 0.5 U/ml, 8 h, 37°C.D, NIL8-HSV conditioned medium: buffer, 8 h, 37°C. E, NIL8-HSV conditioned medium: chondroitinase ac, 0.5 U/ml, 8 h, 37°C. F, NIL8-HSV conditioned medium: chondroitinase abc, 0.5 U/ml, 8 h, 37°C.

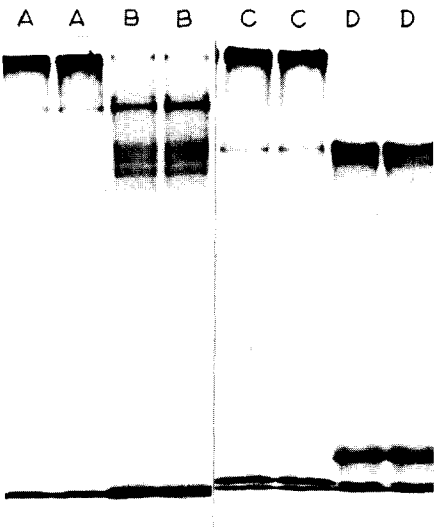

Fig. 5. Autoradiogram of slab gel analysis of reduced and non-reduced NIL8-conditioned medium after treatment with chondroitinase abc. A. Non-reduced, $^{35}SO_4$-labelled cells. B. Non-reduced, ^{125}I-labelled cells. C. Reduced, $^{35}SO_4$-labelled cells. D. Reduced ^{125}I-labelled cells. The predominant ^{125}I-labelled band is LETS protein released from the cells into the medium. It comigrates with the chondroitinase-resistant $^{35}SO_4$-labelled band whether reduced or not.

slightly higher apparent molecular weight (Fig. 4). The band from NIL8-HSV medium, however, does not change mobility upon reduction.

The results of parallel enzymatic digestions of NIL8 $^{35}SO_4$ and ^{125}I-labelled conditioned medium are shown in Table III and Fig. 6. The comigrating $^{35}SO_4$- and ^{125}I-labelled bands behaved similarly with each treatment. These results suggest that the discrete SO_4-labelled species is the same as that detected by iodination, namely, LETS glycoprotein.

To confirm this identity, NIL8-conditioned medium was incubated with

TABLE III

ENZYMATIC DIGESTIONS OF NIL8 $^{35}SO_4$- AND ^{125}I-LABELLED CONDITIONED MEDIUM

PBS, phosphate-buffered saline.

Enzyme treatment	Trichloroacetic acid-precipitable counts remaining		Percent degraded	
	^{125}I	^{35}S	^{125}I	^{35}S
PBS, 16 h, 37°C	1200	1203	*	*
Trypsin, 10 μg/ml, 10 min, room temp.	812	969	32	19
Hyaluronidase, 100 μg/ml, 16 h, 37°C	1186	408	1	66
Hyaluronidase, 100 μg/ml, 16 h, 37°C then Trypsin: 10 μg/ml, 10 min, room temp.	762	245	36	80
Chondroitinase abc, 0.5 U/ml, 16 h, 37°C	1163	211	3	82
Chondroitinase abc, 0.5 U/ml, 16 h, 37°C, then Trypsin, 10 μg/ml, 10 min, room temp.	694	138	42	89

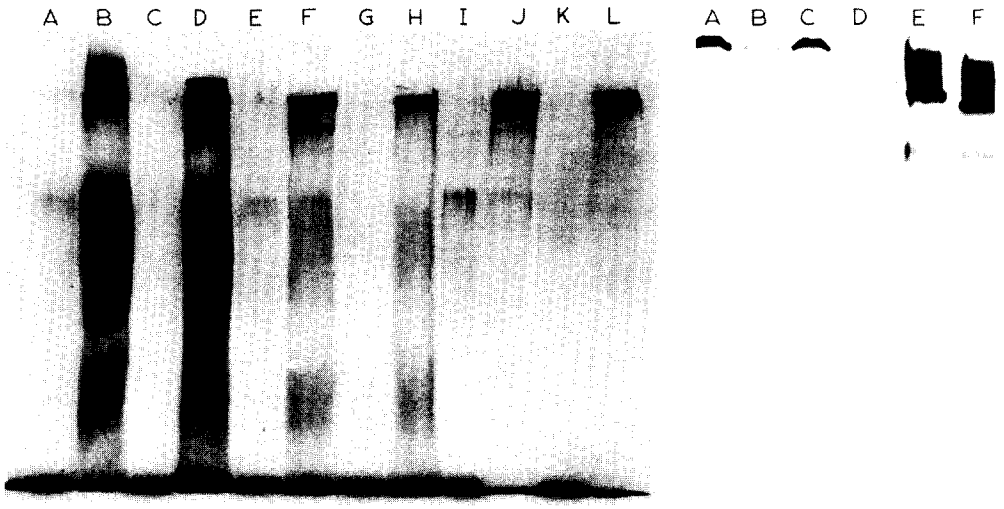

Fig. 6. Autoradiogram of slab gel of enzymatic digestions of $^{35}SO_4$- and ^{125}I-labelled conditioned medium. A. ^{125}I-labelled medium: phosphate-buffered saline control (16 h, 37°C). B. $^{35}SO_4$-labelled medium: phosphate-buffered saline control (16 h, 37°C). C. ^{125}I-labelled medium: trypsin (10 μg/ml, 10 min, room temp. D. ^{35}S-labelled medium: trypsin (10 μg/ml, 10 min, room temp.). E. ^{125}I-labelled medium: hyaluronidase (100 μg/ml, 16 h, 37°C). F. ^{35}S-labelled medium: hyaluronidase (100 μg/ml, 16 h, 37°C). G. ^{125}I-labelled medium: hyaluronidase (100 μg/ml, 16 h, 37°C) then trypsin (10 μg/ml, 10 min, room temp.). H. ^{35}S-labelled medium: hyaluronidase (100 μg/ml, 16 h, 37°C) then trypsin (10 μg/ml, 10 min, room temp.). I. ^{125}I-labelled medium: chondroitinase abc (0.5 U/ml, 16 h, 37°C). J. ^{35}S-labelled medium: chondroitinase abc (0.5 U/ml, 16 h, 37°C). K. ^{125}I-labelled medium: chondroitinase abc (0.5 U/ml, 16 h, 37°C) then trypsin (10 μg/ml, 10 min, room temp.). L. ^{35}S-labelled medium: chondroitinase abc (0.5 U/ml, 16 h, 37°C) then trypsin (10 μg/ml, 10 min, room temp.).

Fig. 7. Autoradiograms of slab gel analyses of immune and ammonium sulfate precipitation of $^{35}SO_4$-labelled NIL8-conditioned medium. A. Immune precipitate from NIL8-conditioned medium. B. Pre-immune precipitate from MIL8-conditioned medium. C. Immune precipitate from NIL8-HSV-conditioned medium. D. Pre-immune precipitate from NIL8-HSV-conditioned medium. E. Ammonium sulfate precipitate from NIL8-conditioned medium treated with chondrotinase abc. F. Ammonium sulfate precipitate from NIL8-conditioned medium.

rabbit antiserum to LETS glycoprotein followed by incubation with goat anti-rabbit antiserum. 4.7% of the total trichloroacetic acid-precipitable radioactivity was precipitated. A control precipitation using preimmune serum precipitated only 2.0%. Incubations using NIL8-HSV conditioned medium precipitated 1.8 and 0.5% respectively of the total trichloroacetic acid-precipitable counts in the medium. Subsequent analysis on SDS-polyacrylamide gels revealed specific immunoprecipitation of a $^{35}SO_4$-labelled species which co-migrated with LETS glycoprotein (Fig. 7). Again the band in NIL8-HSV medium migrated slightly slower than that released by NIL8 cells.

Precipitation of LETS glycoprotein from $^{35}SO_4$-labelled NIL8-conditioned medium was also performed by the addition of 30% ammonium sulfate (see Materials and Methods). Addition of ammonium sulfate was found to precipitate 6.4% of the total trichloroacetic acid-precipitable counts in untreated NIL8-conditioned medium, and 22.0% of the total counts in NIL8-conditioned medium previously treated with chondroitinase abc. Chondroitinase treatment of medium was performed before precipitation in order to remove sulfate-

labelled proteoglycans which may have been precipitated with the LETS glycoprotein. However, the pre-treatment caused no change in the material precipitated. Ammonium sulfate pellets of both treated and untreated NIL8-conditioned medium can be seen to contain a $^{35}SO_4$-labelled species which comigrated with LETS glycoprotein (Fig. 7).

Taken together, these results indicate that the large, external, transformation-sensitive (LETS) glycoprotein is sulfated in cultures of NIL8 hamster fibroblasts.

Discussion

The results described indicate the following differences between normal (NIL8) cells and transformed (NIL8-HSV) cells.

(1) The transformed cells retain a smaller proportion of the total sulfated macromolecules synthesized: 6% instead of 18—20%. In both cell types most of the cell-associated label is in very high molecular weight species.

(2) NIL8 cells have a discrete sulfate-labelled band which comigrates with iodinated LETS glycoprotein: NIL8-HSV cells do not.

(3) The majority ($\leq$80%) of the sulfated macromolecules are released into the culture medium as proteoglycans in both cell types.

(4) The size classes of these proteoglycans and the proportions of different glycosaminoglycans differ between NIL8 and NIL8-HSV. In both cases, 20—35% of the sulfate is in the form of heparan sulfate or keratan sulfate, most of the rest is chondroitin sulfates A, B and C. Previous reports on other cell lines have indicated extensive synthesis of heparan sulfate by the cells [26,27] and release into the medium [28]. NIL8 cultures contain 30% of the total extracellular sulfated macromolecules in the form of dermatan sulfate (chondroitin sulfate B), while NIL8-HSV cells synthesize very little of this glycosaminoglycan.

(5) The dermatan sulfate is found predominantly in a heterogeneous class of proteoglycans (mol. wt. 150 000—250 000) released by normal NIL8 cells. This size class is absent from NIL8-HSV cells and is replaced by a smaller molecular weight class of protoglycans (mol. wt. 100 000—180 000) consisting largely of chondroitin sulfates A and/or C. This size difference could reflect either incomplete synthesis or degradation of dermatan sulfate. Preliminary experiments involving mixing labelled NIL8 medium with transformed cell medium or cells failed to show any evidence of degradation (unpublished results). Therefore, the difference probably results from a reduced synthesis of dermatan sulfate by the transformed cells.

(6) Normal NIL8 cells also release a discrete sulfate-labelled species into the medium. This appears to be identical with the LETS glycoprotein detected by iodination by several criteria: comigration, sensitivity to a variety of degradative enzymes, and to reduction, precipitation by ammonium sulfate and specific antisera to LETS glycoprotein. Thus, one can conclude that LETS glycoprotein released by these cells contains sulfate. The results confirm and extend earlier data [3] showing that LETS glycoprotein does not contain glycosaminiglycans sensitive to the enzymes tested. The possibility remains that it contains heparan or keratan sulfates. Alternatively, the sulfate may be attached to a gly-

coprotein core, as in the case of certain viral glycoproteins [29] or to tyrosine as in the case of fibronogen [30] and certain gastric hormones [31]. A similar, discrete, sulfate-labelled band is observed on the normal cells and is presumably also LETS glycoprotein. Its absence from the NIL8-HSV cells is consistent with this idea. The presence of a discrete sulfate-labelled band in NIL8-HSV medium is of some interest. This band is of similar size, although it appears to be of slightly lower mobility and is also precipitable by antibody to LETS glycoprotein. This band, however, migrates similarly under both reducing and non-reducing conditions, and therefore is not the same as LETS protein found in NIL8 conditioned medium. It may represent a modified form of LETS protein or an unrelated macromolecule which is also recognised by the antiserum.

LETS glycoprotein can be isolated in a dense non-membranous surface coat fraction [32] which also contains proteoglycans as judged by its high affinity for Ruthenium Red [33]. Also under certain conditions, cells can be released by chelating agents, leaving behind material on the substratum [11]. This substrate-attached material contains proteoglycans [11,12] as well as LETS glycoprotein [34,35]. There are therefore, several reasons for suspecting a relationship between LETS glycoprotein and proteoglycans. The observation that transformed cells which lack cell-bound LETS glycoprotein also retain a lower proportion of their newly synthesized proteoglycans suggests the possibility that the two phenomena might be related. Although there is, at present, no evidence to support such a relationship, the techniques are available to test this hypothesis.

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