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CELL-SURFACE GLYCOSAMINOGLYCANS ARE NOT RELEASED FROM HUMAN DIPLOID FIBROBLASTS BY NON-ENZYMATIC METHODS

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Summary

Attempts were made to release glycosaminoglycans from the surface of intact fibroblasts in culture by changes in pH and ionic strength of the surrounding medium. No additional macromolecular material was released by such 5-min treatments, while 0.01% trypsin released 50 times the amount naturally shed. These results suggest a covalent attachment of glycosaminoglycans to the cell membrane. Cytochalasin B did not effect release of surface components, but a small amount of sulfated glycosaminoglycan was released by inclusion of heparin in the incubation medium.

Glycosaminoglycans are a minor but ubiquitous component of the cell coat of mammalian cells in culture. These glycosaminoglycans include hyaluronic acid, heparan sulfate, and chondroitin sulfates, the proportions of each varying with cell type and culture conditions [1,2]. Glycosaminoglycans can be released from the cell surface by gentle proteolytic treatment of suspension or monolayer cultures, suggesting that they are bound through peptide linkages [3]. Individual glycosaminoglycans can be released from intact cells by specific enzymatic degradation, without the concomitant removal of the others [4]. Their presence at the cell surface does not necessarily indicate that they are intrinsic membrane components, however, for this localization could also result from specific or non-specific adsorption following initial secretion by the cell. In this study the possibility that surface-associated glycosaminoglycans are bound by non-covalent interactions was investigated by using non-degrading methods in attempts to remove them from intact cells.

Human diploid fibroblasts, strain IMR 90 [5] were used in this study. Cells were grown in Eagle's minimal essential medium supplemented with 20%

fetal bovine serum (Grand Island Biological Co.); 10% tryptose phosphate broth (Difco); and low concentrations of antibiotics (Tylan, 50 μg/ml; Neomycin, 75 μg/ml; Mycostatin, 25 μg/ml) at 37°C in an atmosphere of 5% CO_2. All cultures were rapidly growing at a population doubling level below 40 when seeded onto 75-cm^2 Corning polystyrene culture flasks for these experiments.

Monolayer cultures were labeled by addition of [^{3}H]glucosamine (0.5 μCi/ml, 10.13 Ci/mmol) and $Na_2{}^{35}SO_4$ (5 μCi/ml) to medium for 24 h. Radioactive medium was then decanted and the cells were rinsed four times with Earle's balanced salt solution. Flasks were then treated for 5 min at room temperature with 7 ml of balanced salt solution at pH 7.4, 4.0 or 10.0; 1 M NaCl; 4 M guanidinium chloride or 3 M $MgCl_2$ (containing 0.005 M benzamidine, 0.05 M EDTA); or balanced salt solution plus trypsin (0.1 mg/ml, pH 7.4). In other experiments labeled cells were incubated with balanced salt solution containing pig mucosal heparin (500 μg/ml) or cytochalasin B (20 μg/ml) for 30 min at 37°C; this supernatant decanted; and the cells then incubated with trypsin (0.1 mg/ml in balanced salt solution) for an additional 30 min at 37°C.

Supernatants were digested with trypsin plus pronase (0.1 mg/ml, 4 h, 37°C) in the presence of 25 μg each of carrier hyaluronic acid and chondroitin sulfate. Samples were concentrated by lyophilization, applied to a 1 × 36 cm column of Bio Gel P-2 (100—200 mesh), eluted with 0.01 M ammonium acetate, and 0.1 ml of each 2 ml fraction counted. Fractions containing macromolecular material (i.e. eluting before a vitamin B-12 marker, M_W = 1355) were combined for each sample, lyophilized, and then further fractionated by the method of Saarni and Tammi [7]. In this procedure glycosaminoglycans are precipitated with 1% cetylpyridinium chloride (3 h, room temperature, in 0.02 M NaCl) and then passed through an 0.45 μm Millipore filter. That label which is soluble in 1% cetylpyridinium chloride passes through the filter and represents the small carbohydrate moieties of glycopeptides. That which is subsequently eluted from the filter with 0.5 M HCl is hyaluronic acid, and that remaining on the filter represents the sulfated glycosaminoglycans. These results were found to correlate very well with fractionations done by standard methods of DEAE column chromatography.

TABLE I

MACROMOLECULAR RADIOACTIVITY RELEASED FROM LABELED CELLS BY 5-MIN TREATMENT AT ROOM TEMPERATURE

Treatment	Total macromolecular radioactivity*		Fractionation**			
			Glycopeptide	Hyaluronic acid	Sulfated glycosaminoglycans	
	^{3}H	^{35}S	^{3}H	^{3}H	^{3}H	^{35}S
Balanced salt solution						
pH 7.4	2 660	550	1.0	1.0	1.0	1.0
pH 4.0	900	330	0.4	0.6	0.7	0.6
pH 10.0	1 220	1 840	0.4	1.9	1.3	1.8
1 M NaCl	2 330	270	1.1	0.8	0.9	0.6
4 M guanidine—HCl	15 050	3 110	7.0	9.7	4.8	3.0
0.01% trypsin	115 400	35 970	53	63	51	48

*Total macromolecular radioactivity (cpm) recovered from Bio Gel P-2 filtrations shown in Fig. 2.
**Cetylpyridinium chloride fractionations of this material with data expressed relative to pH 7.4 control.

Isotopes were purchased from New England Nuclear Corp., guanidine hydrochloride (ultrapure) from Worthington Biochemicals, Chondroitinase ABC from Miles Research Products and other chemicals from Sigma Chem. Co.

Raising or lowering the buffer pH did not affect the cells visibly in 5 min, and did not promote the release of additional macromolecular material (Table I). Cells were beginning to shrivel after 5 min in 1 M NaCl, but labeled material was still not released. The 4 M guanidinium chloride treatment resulted in an approximately 5-fold increase in released macromolecular material and retraction and blebbing of the cells, leaving bits of cellular processes attached to the dish (Fig. 1). Trypsin caused visible retraction but not detach-

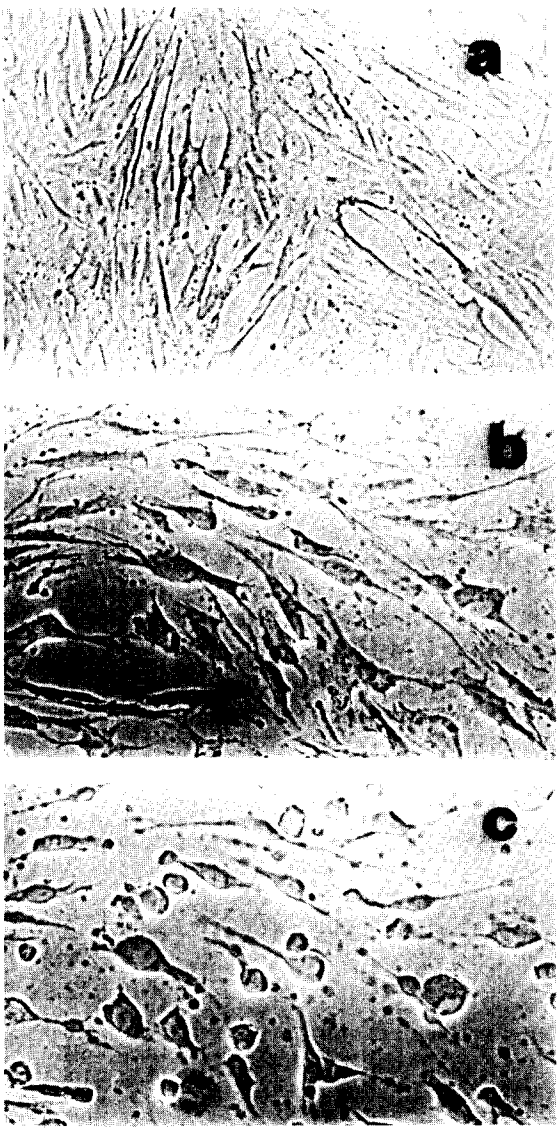

Fig. 1. Phase-contrast microscopy of cells treated with 4 M guanidine hydrochloride. a, control buffer; b, 5 min; c, 10 min. Note pieces of cellular material adherant to the dish. Within 30 min all cell bodies are detached and rupture.

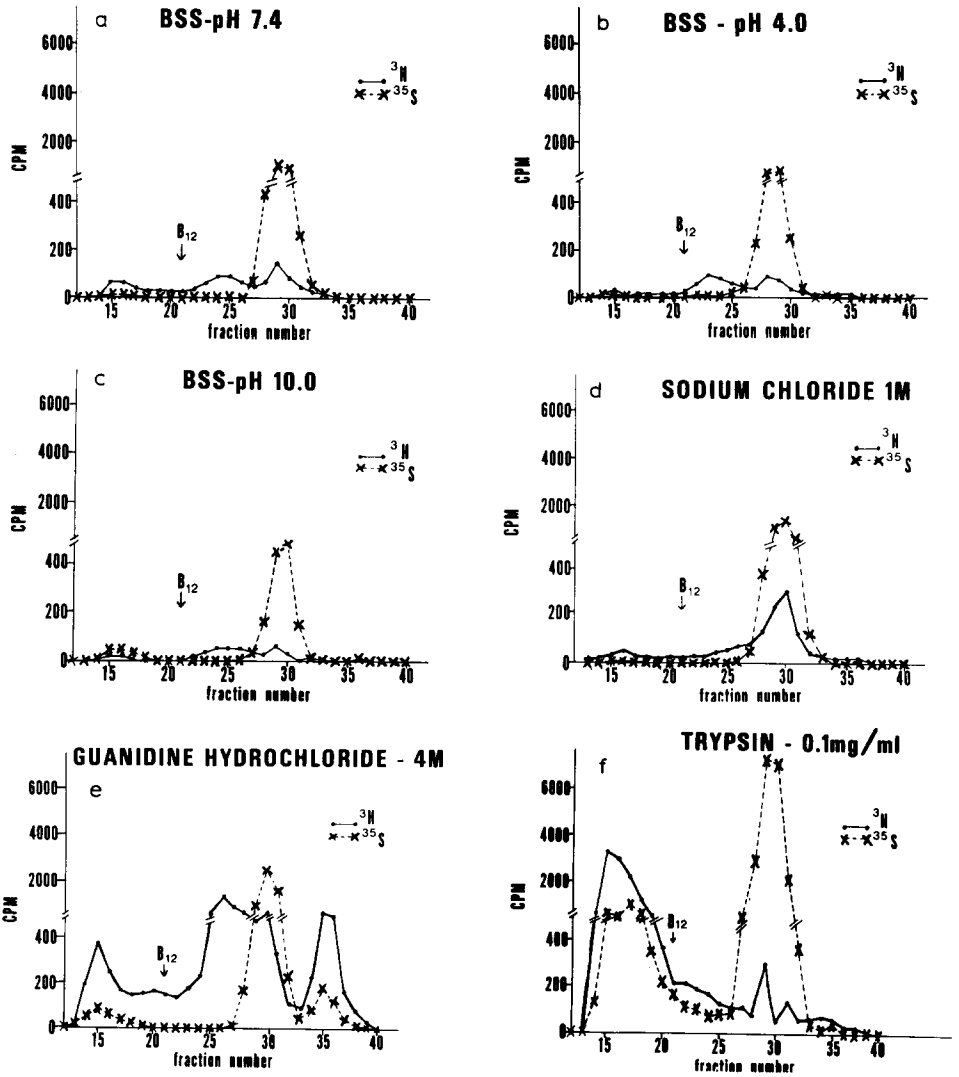

Fig. 2. Bio gel P-2 filtration profiles of material released from prelabeled cells during 5 min treatment at room temperature. Macromolecular material elutes before the vitamin B-12 marker; 0.1-ml fractions of each 2-ml sample were counted. BSS, balanced salt solution.

ment of cells, and released about 50 times the amount of labeled macromolecular material as did the buffer alone.

In the filtration profile of each supernatant (Fig. 2) macromolecular material elutes before the vitamin B-12 marker. A glucosamine-labeled peak follows this marker when whole-cell homogenates are filtered, but is lacking in the material released from intact (trypan-blue impermeable) cells. This cytoplasmic component has been identified by Kraemer as UDP-*N*-acetylhexosamine [8]. Its appearance in significant quantities in the medium indicates membrane leakiness. These peaks are followed by the radioactivity of unincorporated sulfate and glucosamine. The control pH 7.4 sample shows that a small amount of tritium- and sulfate-labeled macromolecular material is

continually released from these cells, along with a small amount of slightly retarded material and a variable amount of unincorporated sulfate. This profile is basically unchanged when the pH is altered, or following treatment with 1 M NaCl. The putative nucleotide sugar peak is high only in the guanidinium chloride sample. Additional low molecular weight peaks also appear in this sample, further indicating that membrane damage has occurred. The trypsin profile shows that a large amount of macromolecular material has been released without causing membrane damage. Fractionation of these samples did not suggest the specific release of any component by any of the methods used. In both control and trypsin samples of these 5-min incubations 88% of the radioactive macromolecular material released represented glycopeptides while only 6% was hyaluronic acid and 6% sulfated glycosaminoglycans.

Extended extraction with guanidinium chloride caused total disintegration of the cells. A greater proportion of the label was solubilized, but after 24 h at 4°C approx. 25% of the macromolecular label still remained with insoluble cellular debris (data not shown). Cells extracted extensively with 3 M $MgCl_2$ (20 h, 4°C) remained surprisingly intact though clearly dead. After such an extraction 48% of the total radioactivity remained with the cells. Filtration of the extract showed that it was 99% low molecular weight material.

Heparin did not affect the shape or attachment of the cells. There was consistently some indication of enhanced release of sulfate-labeled glycosaminoglycans (Table II), amounting to less than 10% of the amount subsequently released by trypsin. The absence of this amount from the trypsin-removable material was never detected. Chondroitinase ABC digestion of the sulfate-labeled macromolecular material released into buffer rendered 70% of the label non-precipitable by cetylpyridinium chloride. This digestion solubilized only 25% of the material released into buffer plus heparin, while approx. 40% of the macromolecular material released by trypsin from both samples was made soluble. Treatment of cells with cytochalasin B for 30 min caused a visible contraction of the cells without detachment or membrane damage. There was no evidence for specific release of any of the surface components.

TABLE II

COMPOSITION OF MACROMOLECULAR RADIOACTIVITY (cpm) RELEASED FROM CELLS BY INCLUSION OF HEPARIN IN INCUBATION BUFFER

	^3H*			^{35}S**
	Glycopeptide	Hyaluronic acid	Glycosaminoglycan	Sulfated glycosaminoglycan
Buffer				
—	2 436	476	171	3 205
+ Heparin	3 376	160	214	6 687
Trypsin				
—	23 808	2 606	1 291	39 584
+ Heparin	24 896	1 900	1 250	38 848

*Each flask ($\simeq 3 \cdot 10^6$ cells, 0.55 mg protein) was labeled for 24 h, rinsed four times with buffer, then incubated in buffer or buffer plus heparin (500 μg/ml, 30 min, 37°C) followed by incubation in buffer plus trypsin (0.1 mg/ml, 30 min, 37°C) to assess the trypsin-removable fraction. Macromolecular material was obtained by Bio-Gel P-2 filtration and fractionated.

**Cultures were labeled with ^{35}S only (15 μCi/ml).

Fibroblasts grown in culture have glycosaminoglycans as well as glycoproteins at their cell surface. Both of these components can be removed by gentle treatment of the cells with trypsin. They are also continually shed into the extracellular medium, apparently without being degraded [9]. The intact release of these molecules could suggest a membrane shedding process [10] or it could be explained as indicating that the molecules are not covalently bound to the membrane in the first place. Recent reports of saturable, reversible binding of exogenous heparin and heparan sulfate to rat liver cells [11] and heparin-induced release of heparan sulfate from the surface of cultured Chinese hamster cells [12] suggest specific exchangeable receptor sites for this glycosaminoglycan at the surface.

We have been unable to release surface-associated glycosaminoglycans from cultured human embryo fibroblasts by changes in pH or ionic strength. This very straightforward result suggests that these molecules are not held in association with the surface by ionic or electrostatic bonds. We recognize, of course, that other explanations are possible. For example, if glycosaminoglycans are synthesized as proteoglycans with a hydrophobic protein portion, the salt and pH treatments would not solubilize it. Alternatively, binding of the high molecular weight linear polysaccharides to multiple receptor sites would greatly decrease the probability of releasing an entire molecule simultaneously.

Guanidine hydrochloride was used because it is very effective for extracting intact proteoglycan molecules from cartilage [13]. It was hoped that such an extraction would lead to a rapid method for extraction of cell-surface glycosaminoglycans or intact proteoglycans. In fact, brief 4 M guanidinium chloride treatment extracts only a fraction of the glycosaminoglycans which are removed by trypsin, and the evidence for cell leakage suggests that this may be from cytoplasmic pools. Extended extraction with guanidinium chloride may, however, solubilize a larger portion of the total cellular glycosaminoglycan pool. Extraction with 3 M $MgCl_2$ was quite unsuccessful.

Our inability to detect a greatly enhanced release of sulfated glycosaminoglycans by incubating cells in the presence of heparin may contradict the report of Kraemer [12]. We have often noted, however, that human embryo fibroblasts do not seem to have as great a percentage of heparan sulfate in their cell-associated radioactivity as do Chinese hamster ovary cells [4]. In these fibroblasts trypsin-released sulfated glycosaminoglycan is no more than 60% heparan sulfate and label representing less than 20% of that was released by incubation with heparin. Our kinetic data suggests that the sulfated glycosaminoglycans are synthesized and appear at the cell surface prior to their release into the extracellular medium [13]. More information is needed before it will be possible to decide whether all the glycosaminoglycans at the surface of a given cell are synthesized as integral parts of the membrane, or whether some are non-covalently bound components. Since less than 50% of the trypsin-removable heparan sulfate was displaced during incubation of Chinese hamster ovary cells with heparin [12], there may be two pools of cell-surface heparan sulfate with differing modes of attachment.

We were not surprised to find that surface material was not released by treatment with cytochalasin B, for we have previously shown that EDTA

caused extensive contraction of the cells without releasing significant surface material [6]. It now seems clear that both EDTA and cytochalasin B have their primary effect on the cytoskeleton of the cell and do not effect morphological changes by removal of cell-surface components. The quantity of fibronectin reported to be released from skin fibroblasts by treatment with cytochalasin B [15] would not be detected in this assay.

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