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Surface Glycosaminoglycans and Calcium Distribution in 3T3 Cells

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Resting 3T3 cells have relatively more sulphated glycosaminoglycans and Ca^{2+} in their cell coat than do growing or SV40-virus-transformed cells. It is suggested that the different Ca^{2+}-binding capacity controls cellular activities by affecting the distribution of Ca^{2+} between the intra- and ecto-cellular compartments.

The relative amounts of sulphated and non-sulphated glycosaminoglycans of the cell coat vary widely with some fundamental aspects of cell behaviour, including growth (Vannucchi & Chiarugi, 1977), transformation by oncogenic viruses (Chiarugi *et al.*, 1974) and differentiation (Augusti-Tocco & Chiarugi, 1976). Such evidence led to a model based on a cell-cycle-dependent shedding of heparan sulphate, where *N*-sulphated glycosaminoglycans inhibit growth and favour homoeostasis and differentiation (Chiarugi & Vannucchi, 1976).

We extended out interest from sulphated glycosaminoglycans to Ca^{2+} ions for a variety of reasons: (1) *N*-sulphated glycosaminoglycans are good ligands of Ca^{2+} (Casu, 1975); (2) much of the cellular Ca^{2+} and glycosaminoglycans are found at the outer cellular coat (Borle, 1975*a*; Kraemer, 1971); (3) sulphated glycosaminoglycans are bound to the cell coat either with a proteinase-sensitive bond or with a Ca^{2+} bridge sensitive to chelators of bivalent cations (Chiarugi *et al.*, 1974); (4) the shedding of the cell coat that takes place during sea-urchin egg fertilization is characterized by Ca^{2+} release from the coat itself and by Ca^{2+} influx into the cytoplasm (Kinoshita, 1969). There are thus grounds for the hypothesis that Ca^{2+} affects cell control in opposite ways, according to its location at the surface (cell coat) or in the cytoplasm.

With this in mind, experiments have been carried out with the 3T3 cell line to check whether the changes in the composition of the cell-coat glycosaminoglycans taking place during growth and transformation are paralleled by changes in the distribution of cellular Ca^{2+}.

Experimental

Materials

Plastic bottles were obtained from Falcon Division of Becton, Dickinson and Co., Cockeysville, MA, U.S.A.; modified Eagle's minimum essential medium (Dulbecco & Freeman, 1959) and 0.25% trypsin (1:250) were from Grand Island Biological Co., Grand Island, NY, U.S.A.; calf serum was prepared from pooled calf blood obtained at the local abattoir. [^{3}H]Glucosamine (12Ci/mmol), inorganic [^{35}S]sulphate (268Ci/mol) and $^{45}CaCl_2$ (1Ci/mmol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K.; the AG 1-X2 resin was from Bio-Rad Laboratories, Richmond, CA, U.S.A.; dialysis tubing (cut-off mol.wt. 3500) was from Arthur H. Thomas Co., Philadelphia, PA, U.S.A.; GF/A filters were from Whatman Biochemicals, Maidstone, Kent, U.K.; hyaluronic acid (from human umbilical cord) was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; heparin (from calf lung) was from Serva Feinbiochemica, Heidelberg, Germany; heparan sulphate (from calf lung) was a gift from the Upjohn Co., Kalamazoo, MI, U.S.A.; chondroitin sulphates A, B and C, all from shark cartilage, were purchased from Seigakaku Kogyo Co., Nihombashi-Honcho, Chuo-Ku, Tokyo, Japan. Other reagents were from E. Merck A.G., Darmstadt, Germany. The reagents were of analytical grade and of the highest purity available.

Cell cultures

The mouse cell line 3T3 (Balb/C) and the same transformed by SV40 virus were obtained from Dr. P. Amati, Laboratorio Internazionale di Genetica e Biofisica, Naples, Italy. All cells were grown at 37°C under CO_2/air (1:19) in modified Eagle's minimum essential medium, with 10% (v/v) calf serum. Resting cells were prepared as previously described (Vannucchi & Chiarugi, 1977).

For labelling, pregrown cultures received 1μCi of [^{3}H]glucosamine/ml and 20μCi of inorganic [^{35}S]-sulphate/ml, and were further incubated for 36–48h. Alternatively 10μCi of $^{45}CaCl_2$/ml was added, and incubation continued for 24h.

Harvesting of cultures and trypsin treatment of cells

^{3}H- and ^{35}S-labelled media were decanted, and the attached cells were washed five times with phosphate-buffered saline (Dulbecco & Vogt, 1954); they were then incubated with 2ml of trypsin for 15min at 37°C. The resulting cell suspension was centrifuged at 1000*g* for 10min; cells in the pellet were counted with a haemocytometer. The supernatants were analysed by ion-exchange chromatography.

^{45}Ca-labelled cultures were decanted, cell layers were washed with 3×100ml of 1.2% (w/v) NaCl, and the cells were scraped off with a 'rubber policeman' and collected in 2ml of the same solution. A sample was processed for the determination of the total cellular radioactivity and of cell number, and the bulk of the material was incubated with 0.05% trypsin in 1.2% (w/v) NaCl for 4min, then filtered through a Whatman GF/A filter and finally washed with 100ml of 1.2% NaCl. Samples of the filtrate and filtered cells were dried and counted for radioactivity by liquid scintillation.

Determination of Ca^{2+} influx during trypsin treatment

Unlabelled cells, suspended by scraping them off the bottle wall, were treated with trypsin (as described above) in the presence of 1 μCi of carrier-free $^{45}CaCl_2$/ml, then processed as indicated above.

Ion-exchange chromatography

The trypsin digests were loaded on columns (1cm×3cm) of AG 1-X2 resin and eluted stepwise with increasing concentrations of NaCl, as previously described (Vannucchi & Chiarugi, 1977). Fractions (1.5ml) were collected, dried and counted for radioactivity by liquid scintillation.

Equilibrium dialysis

Samples (0.1mg) of each commercial glycosaminoglycan were dissolved in 10ml of water. The solutions were introduced into separate dialysis bags, which were immersed together and stirred for 24h in 2500ml of water to which 250 μCi of $^{45}CaCl_2$ had been added. Samples of the solutions remaining in the bags were then counted for radioactivity.

Results and Discussion

Table 1 shows that resting 3T3 cells release, on digestion by trypsin, relatively more sulphated glycosaminoglycans and less hyaluronic acid than do either the growing or the SV40-virus-transformed cells. These findings agree with our experience in several other systems, and are considered to reflect a different distribution of sulphated and unsulphated glycosaminoglycans in the cell coats.

Under comparable culture conditions an attempt has been made to measure the total cell-associated Ca^{2+} and to study its distribution. Table 2 shows that resting 3T3 cells have more Ca^{2+} than do growing or transformed cells; on trypsin treatment of the suspended cells, most of the cell-associated Ca^{2+} is released into the supernatant, and only a minor fraction, especially in the resting cells, remains cell-associated. We believe that the latter fraction grossly overstimulates the true initial intracellular Ca^{2+} concentration, whose direct measurement is fraught with technical difficulties. In fact we have found that during the trypsin treatment there is a net influx of Ca^{2+}, as shown in Table 3; in these experiments, washed unlabelled cells were treated with trypsin in the presence of tracer amounts of $^{45}CaCl_2$, so that the molar concentration of Ca^{2+} is only slightly increased over that of the Ca^{2+} initially associated with the cell. It appears that resting cells take up more Ca^{2+}

Table 1. *Composition of the cell-coat glycosaminoglycans of the 3T3 cells in the resting, growing and virus-transformed cells*

The cell-coat glycosaminoglycans are revealed by the stepwise elution of [^{3}H]glucosamine- and inorganic [^{35}S]sulphate-labelled materials removed by trypsin from the cell surface. Non-sulphated glycosaminoglycans are eluted at 0.5M-NaCl and sulphated glycosaminoglycans at 1.0, 1.3, 1.5 and 2.0M-NaCl. The values are means ± S.E.M. for five experiments.

	Peak area (% of total)	
Cell line	Non-sulphated glycosaminoglycans	Sulphated glycosaminoglycans
3T3, resting	24.4±2.3	74.3±3.1
3T3, growing	44.5±4.1	54.0±4.2
SV40-virus-transformed 3T3	47.7±7.0	51.0±3.8

Table 2. *Ca^{2+} content of the whole cells, of the trypsin-treated cells, and of the cell coat in resting, growing and transformed 3T3 cells*

The incubation medium contains 1.8mM-$CaCl_2$ (non-radioactive) and 10 μCi of $^{45}CaCl_2$/ml. The values given are means ± S.E.M. for three experiments.

	[Ca^{2+}] (nmol/10^6 cells)		
Cell line	Total	Trypsin-treated cells	Cell coat
3T3, resting	3.72±0.32	0.70±0.15	3.39±0.45
3T3, growing	1.62±0.07	0.34±0.08	1.58±0.20
SV40-virus-transformed 3T3	1.34±0.05	0.30±0.12	1.26±0.31

Table 3. *Uptake of $^{45}Ca^{2+}$ during trypsin treatment in resting, growing and transformed 3T3 cells*

The uptake of ^{45}Ca is carried out in trypsin solution with 1 μCi of $^{45}CaCl_2$/ml. The values are means ± S.E.M. for the numbers of determinations in parentheses.

Cell line	$^{45}CaCl_2$ (c.p.m./10^6 cells)
3T3, resting	17548 ± 484 (3)
3T3, growing	13266 ± 467 (7)
SV40-virus-transformed 3T3	9674 ± 129 (3)

Table 4. *Equilibrium dialysis of standard glycosaminoglycan solutions against $^{45}Ca^{2+}$*

The values express the Ca^{2+}-binding capacity of the sulphated glycosaminoglycans relative to that of hyaluronic acid. Binding capacity was estimated by the difference, at equilibrium, between the radioactivities inside and outside dialysis bags.

Commercial glycosaminoglycan	Relative Ca^{2+}-binding capacity
Hyaluronic acid	1.00
Heparin	2.76
Heparan sulphate	2.00
Chondroitin sulphate A	1.60
Chondroitin sulphate B	1.67
Chondroitin sulphate C	1.80

than do growing or transformed ones, and probably their true initial intracellular concentration is correspondingly lower. However indirect, this argument accords well with the idea that trypsin brings about its mitogenic effect by causing a Ca^{2+} influx into the cytoplasm (Parker, 1974); if internal Ca^{2+} must reach a threshold concentration to be effective, a higher influx should be needed into the cytoplasm of resting cells because of their lower initial concentration.

Table 4 shows the results of the equilibrium dialysis: clearly, *N*-sulphated glycosaminoglycans in general, and heparin in particular, trap more Ca^{2+} than does hyaluronic acid.

Taken together, our results indicate that: (1) resting 3T3 cells are covered with glycosaminoglycans among which the sulphated species predominate, and they also have increased amounts of ectocellular (i.e. removable by trypsin) Ca^{2+}, whereas their intracellular concentration might be lower, as suggested by the high Ca^{2+} influx on treatment with trypsin; (2) the variations in the glycosaminoglycan composition of the cell coat could well affect the content and the distribution of Ca^{2+} in and around any given cell, by virtue of the different avidities of the cell-coat components for this ion.

Our evidence supports the idea that the role of Ca^{2+} in the control of cellular activities is regulated by its cellular distribution, and that its intra- and ectocellular concentrations may be inversely related. On a first approximation, Ca^{2+} in the cell coat leads to cell adhesion and growth inhibition, whereas internal Ca^{2+} is mitogenic. We are aware that even direct measurement of the total intracellular Ca^{2+} may be too coarse for the study of its role in cell control. It is emerging that Ca^{2+} inside membrane compartments and free Ca^{2+} in the cytosol might play different roles and be interrelated in complex ways. It is only the free Ca^{2+} in the cytosol that probably acts as a 'second messenger' in the regulation of the cellular activity as do cyclic nucleotides (Borle, 1975*b*).

N-Sulphated glycosaminoglycans of the cell coat could exert their negative effect on growth via their strong sorption of Ca^{2+} ions, thus influencing the trapping ability of the cell surface towards bivalent cations and modulating the intracellular influx of Ca^{2+}.

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References

Augusti-Tocco, G. & Chiarugi, V. P. (1976) *Cell Differ.* **5**, 161–170

Borle, A. B. (1975*a*) *Methods Enzymol.* **39**, 513–573

Borle, A. B. (1975*b*) in *Calcium Transport in Contraction and Secretion* (Carafoli, E., Clementi, F., Draibikowsky, W. & Margreth, A., eds.), pp. 77–86, American Elsevier, New York

Casu, B. (1975) *Congr. Naz. Soc. Ital. Biochim. 1st, Napoli*, 29–31 (abstr. Td4)

Chiarugi, V. P. & Vannucchi, S. (1976) *J. Theor. Biol.* **61**, 459–475

Chiarugi, V. P., Vannucchi, S. & Urbano, P. (1974) *Biochim. Biophys. Acta* **345**, 283–293

Dulbecco, R. & Freedman, G. (1959) *Virology* **8**, 396–397

Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167–173

Kinoshita, S. (1969) *Exp. Cell Res.* **56**, 359–366

Kraemer, P. M. (1971) *Biochemistry* **10**, 1445–1451

Parker, C. W. (1974) *Biochem. Biophys. Res. Commun.* **61**, 1180–1186

Vannucchi, S. & Chiarugi, V. P. (1977) *J. Cell. Physiol.* **90**, 503–510