Vol. 84, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 16, 1978 Pages 794-801

SULFATED MUCOPOLYSACCHARIDES FROM NORMAL SWISS 3T3 CELL LINE AND ITS TUMORIGENIC MUTANT ST1: POSSIBLE ROLE OF CHONDROITIN SULFATES IN NEOPLASTIC TRANSFORMATION

Carl P. Dietrich and Hugo A. Armelin

Departamento de Bioquímica e Farmacologia, Escola Paulista de Medicina, Caixa Postal 20372 and Departamento de Bioquímica, Instituto de Química, Caixa Postal 20780, Universidade de São Paulo, Brazil

Received September 5, 1978

SUMMARY: The sulfated mucopolysaccharide composition of normal Swiss 3T3 cell line and its tumorigenic mutant ST1 is reported. It is shown that chondroitin sulfate B and heparitin sulfate are the sulfated mucopolysaccharides of the normal 3T3 line whereas chondroitin sulfate A and heparitin sulfate are the major ones of the ST1 variant. Degradation of the chondroitin sulfates derived from both cell lines with chondroitinases B and ABC have shown that they contain only 4-sulfated disaccharides differing from each other by the type of uronic acid residue. It is also shown that the chondroitin sulfate A from the tumorigenic variant is mostly located at the cell surface whereas the chondroitin sulfate B from the normal line is less accessible to trypsinization. A relative increase of chondroitin sulfate A was also observed in 3T3 that had lost contact inhibition after successive subcultures,and in the 3T6 cell line. These combined results are in agreement with the earlier proposal that glucuronic acid-containing chondroitin sulfate plays a role in the stimulation of cell division in neoplastic and embryonic tissues.

INTRODUCTION: It is largely accepted that the process of neoplastic transformation involves alterations of the cell surface which might be the primary cause of malignancy (1). However sulfated mucopolysaccharides, ubiquitous components of the cell surface of all cells (2-4), have been negleted in the studies of these alterations. Nonetheless several reports suggest that chondroitin sulfate AC might be involved in neoplasia: a) chondroitin sulfate AC is increased in a wide variety of tumors (5); b) this compound is a stimulant of growth of tumors "in vivo" (6); c) chondroitinases AC and ABC act as inhibitors of growth of Ehrlich ascites tumors (7); d) chondroitin sulfate AC is absent or occurs in small amounts in most of the normal adult tissues (3). Furthermore in cell cultures: a) cell lines derived from tumoral tissues have shown chondroitin sulfate AC as their main surface sulfated mucopolysaccharides (8) whereas primary lung cells have little chondroitin sulfate AC (8); b) absolute and relative increases of this compound were observed in polyoma virus transformed Balb 3T3 cells (Chiarugi and Dietrich, unpublished).

0006-291X/78/0843-0794$01.00/0

Swiss 3T3 cells (9) belong to a line of mouse embryo fibroblasts which exhibit in culture a high degree of contact inhibition and are not able to develop tumors in nude mouse. Recently a glucocorticoid-hormone sensitive mutant (ST1) was isolated from Swiss 3T3 cells (10). Contrary to the parental line, this mutant had no density, anchorage or serum dependence for growth and was able to produce invasive malignant fibrosarcoma in nude mice. However the steroid hormone hydrocortisone induced in ST1 cells a reversible transition from the transformed to the normal phenotype, restoring to the cells the density, anchorage, and serum dependence. The hormone also blocked the growth of ST1 tumors in nude mice (11). Therefore it became of interest: a) to analyse the surface sulfated mucopolysaccharides of the parental Swiss 3T3 cells and its ST1 mutant and b) to verify whether glucocorticoid hormones would lead to changes in the surface sulfated mucopolysaccharides of ST1 cells.

MATERIALS AND METHODS: Substrates and enzymes - Chondroitin sulfates A, B and C, and chondroitinase ABC were purchased from Miles Lab. (Elkhart Ind.). Heparitin sulfate, heparitinases I and II and chondroitinase B were prepared by methods previously described (12,13,15). Agarose was from L'Industrie Biologique Française (Gennevilliers, France). Diaminopropane was from Aldrich Chemical Co. (Milwaukee, Wis., USA).
Cells and growth medium - Swiss 3T3 and 3T6 cells (9) were obtained from the American Type Culture Collection and a stock of frozen samples is maintained in liquid nitrogen. ST1 is a mutant cell line derived from Swiss 3T3 (10). Growth medium consisted of Dulbecco's modified Eagle's medium with 1.2 g/l sodium bicarbonate supplemented with 10% calf or fetal calf serum (Flow Laboratories). Culture procedures have been previously described (10,11).
Extraction and identification of sulfated mucopolysaccharides - For the labeling of mucopolysaccharides, 32-48 hours after plating, carrier free $Na_2{}^{35}SO_4$ (10 μCi/ml) was added to cultures and incubation continued for another 72 hours at which point cells had reached confluency. Cells were then scraped off from the dish surface with a rubber policeman.

For the enzyme treatment, ^{35}S-labeled cells were allowed to grow in 15 cm diameter dishes as described above. After ^{35}S incorporation the medium was removed and the cells washed twice with a $Ca^{2+}$and Mg^{2+}free phosphate buffered-saline(16).Trypsinization was carried out by adding 1.5 ml per dish of 0.25% trypsin (ICN Pharmaceuticals,Inc.) in buffered saline.The cells were then incubated at 37°C until they detached from the dish surface which was monitored by phase contrast microscope. ST1 cells took about 3 minutes to come off whereas a monolayer of "normal" 3T3 cells required 40 minutes of incubation to detach. After detachment cells were immediately separated from the supernatant by centrifugation. To the supernatant, trichloroacetic acid was added to a final concentration of 15% and maintained in the cold for 30 min. The precipitate formed was removed by centrifugation and 2 volumes of alcohol were added to the supernatant. The precipitate formed, containing the mucopolysaccharides was collected by centrifugation, washed once with 80% alcohol, dried and analysed as described below. The pellet of viable cells resulting from trypsinization was mixed 10 volumes of acetone, centrifuged and dried. The dried pellet was then resuspended in 1 ml of 0.05 M Tris HCl buffer, pH 8.0 and incubated for 12 hours at 37°C with trypsin (0.25 g %) After incubation, trichloroacetic acid was added to the mixture in a final concentration of 15% and maintained in the cold for 30 minutes. The precipitate formed was removed by centrifugation and two volumes of ethanol were added to the supernatant and main-

tained at -20°C for 12 hours. The precipitate formed was collected by centrifugation, washed once with 100 µl of 80% ethanol, dried and later dissolved in 100 µl of water. The sulfated mucopolysaccharides were analysed by a combination of agarose gel electrophoresis and enzymatic degradation as previously described (12,13).For quantitation the radioactive bands corresponding to the standards were scraped off the agarose gels (after fixation, drying and staining) and counted in 10 ml of 0.5% PPO-toluene solution in a LS 100 Beckman spectrometer. The relative amounts of chondroitin sulfates A, B and C in the mixtures were established by the amounts of unsaturated disaccharides formed from these compounds by the action of chondroitinases as previously described (14).

RESULTS: The S^{35}labelled mucopolysaccharides formed by Swiss 3T3 cells and its tumorigenic mutant ST1 are shown in Fig. 1A. Trypsinates and the cells after trypsinization of normal 3T3 contain two compounds with electrophoretic migrations similar to chondroitin sulfate B and heparitin sulfate whereas both trypsinate and cells after trypsinization of ST1 contain mainly compounds migrating as chondroitin sulfate AC and heparitin sulfate. The nature of these compounds was confirmed by enzymatic degradation (Figs. 1 B and 1 C). The compound from normal 3T3 migrating as chondroitin sulfate B was almost completely degraded by chondroitinase B whereas the compound from the ST1 migrating as chondroitin sulfate AC was resistant to the action of this enzyme but was completely degraded by chondroitinase ABC thus indicating that it is indeed chondroitin sulfate AC. Compounds with the migration of heparitin sulfates were degraded by a mixture of heparitinases I and II (not shown) confirming that they are heparitin sulfates. The relative amounts of the S^{35} sulfated mucopolysaccharides are shown in Table 1. Normal 3T3 cells contain mainly chondroitin sulfate B and heparitin sulfate and small amounts of chondroitin sulfate AC. The ST1 mutant contains mostly chondroitin sulfate AC and heparitin sulfate with small amounts of chondroitin sulfate B. Different relative amounts of the chondroitin sulfates are removed from the two different types of cells. Most of the chondroitin sulfate AC is removed from the ST1 variant whereas most of the chondroitin sulfate B remains in 3T3 cells after trypsinization. The heparitin sulfates from both cells are removed by trypsin to about the same extent.

It is important to notice that the time required for trypsinization was quite different for both cell types. ST1 cells required only 3 minutes of incubation to detach from the dish surface while it took 3T3 cells up to 40 minutes to come off. Addition of the hormone hydrocortisone to ST1 cells causes a change in their morphology (11): they become flat, organized without overlapping and more adhesive to the surface of the dish. In spite of this apparently greater adhesion, hormone treated cells did not require more than 3 to 5 minutes to detach from the dish surface under the conditions of trypsinization

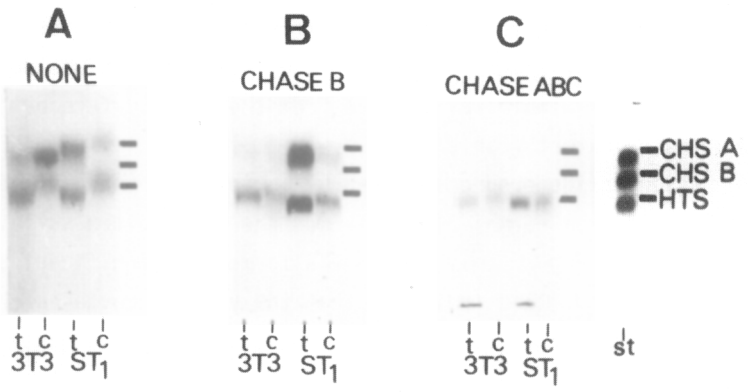

Fig. 1 - Sulfated mucopolysaccharides from normal 3T3 cells and its tumorigenic variant ST1.
About 5.000 cpm of sulfated mucopolysaccharides obtained from the trypsinates (t) and cells after trypsinization (C) were incubated with 0.1 U of chondroitinase B (CHASE B) and 0.1 U of chondroitinase ABC (CHASE ABC) in 0.05 M ethylenediamine acetate buffer, pH 8.0 for 4 hours in a final volume of 20 µl. Aliquots of 5 µl were applied in 5 x 7.5 cm agarose slabs (0.9% agarose in 0.1 M diaminopropane-acetate buffer, pH 9.0, 0.2 cm thick) and subjected to electrophoresis at 100 V for 1 hour. After fixation, drying and staining the gel was exposed to Kodak royal blue X-Ray film for 7 days. St, mixture of standards; 3T3, normal Swiss 3T3 cells; ST1, 3T3 tumorigenic variant; CHS A, chondroitin sulfate A; CHS B, chondroitin sulfate B; HTS, heparitin sulfate.

TABLE I

SULFATED MUCOPOLYSACCHARIDES RELEASED BY TRYPSIN OF NORMAL 3T3 CELLS, ST1 VARIANT AND HYDROCORTISONE TREATED ST_1

CELL LINE	SULFATED MUCOPOLYSACCHARIDES (%)*								
	WHOLE CELLS			TRYPSINATE			CELLS AFTER TRYPSIN		
	CHS A**	CHS B	HTS	CHS A	CHS B	HTS	CHS A	CHS B	HTS
3T3	11	49	40	3	12	23	8	37	17
ST_1	43	13	44	33	9	28	10	4	16
ST_1 + Hydrocortisone	48	10	42	35	4	12	13	6	30

* The results are expressed on % amounts of total S^{35} sulfated mucopolysaccharides present in whole cells.

** CHS A, chondroitin sulfate A; CHS B, chondroitin sulfate B; HTS heparitin sulfate.

described in this paper. Moreover the types of sulfated mucopolysaccharides formed by ST1 cells grown in the presence of hydrocortisone (added 20 hours before labeling) were essentially the same as in untreated controls. It was nevertheless observed that heparitin sulfate was removed from the cell surface by trypsin treatment to a lesser extent (Table I).

The disaccharide products formed by action of chondroitinase ABC upon chondroitin sulfate B from normal 3T3 cells and the chondroitin sulfate from the ST1 variant is shown in Fig.2. Only ΔDi 4S was formed in significant amounts from both chondroitin sulfates. This indicates that the variant contains chondroitin sulfate A rather than chondroitin C and that the chondroitin sulfate B from normal 3T3 cells contains only 4-sulfated disaccharide units. The heparitin sulfate from both cells is not degraded by this enzyme and remain at the origin of the chromatogram (Fig.2). The ST1 variant in the presence of hydrocortisone produced mainly chondroitin sulfate A as did ST1 cells grown in the absence of hydrocortisone (not shown). Therefore even though hydrocortisone restores to ST1 cells density, anchorage and serum dependence it has not changed its composition in sulfated mucopolysaccharides.

Sulfated mucopolysaccharides from 3T3 cells after "spontaneous transformation". The ST1 line is a cloned transformed mutant of Swiss 3T3 with unique properties (11). However it is known that "normal" Swiss 3T3 cells undergo spontaneous transformation with time in culture. The cause for this transformation is unclear and the time required to obtain a transformed population depends on the cell strain and on culture conditions. A sample of frozen stock of "normal" 3T3 cells was maintained until the first signs of transformation appeared. After three months in culture these 3T3 cells presented the following indications of an early stage of transformation: a) abundant criss-crossing and a tendency to pile up; b) rapid trypsinization of confluent plates (less than 3 minutes at 37°C), c) higher saturation density (9 x 10^4 cells per cm^2), twice as much as the "normal" 3T3. Correlating with these early signs of transformation a relative increase of chondroitin sulfate A and a concomitant decrease of chondroitin sulfate B in these cells was observed (Table II). The sulfated mucopolysaccharides of 3T6 cells which also derived from Swiss mouse embryo (9) and which exhibit in culture a transformed phenotype were analysed. The strain of 3T6 cells used presented the following characteristics: a) great tendency to pile up; b) saturation density of 16 x 10^4 cells per cm^2; c) rapid trypsinization of confluent cultures (3 minutes at 37°C). This strain of 3T6 cells also contains much higher relative amounts of chondroitin sulfate A (table II). It is noteworthy that both transformed cultures of 3T3 cells of the experiment described in Table II and the 3T6 cells were unable to grow macroscopic colonies in suspension cultures of methocel (M.C.S. Armelin, un-

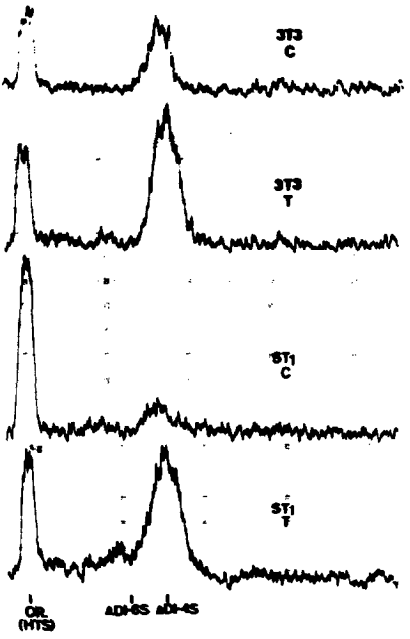

Fig. 2 - Disaccharide products formed from the sulfated mucopolysaccharides of normal 3T3 cells and variant ST1 by the action of chondroitinase ABC.

The incubations were performed as described in Fig.1. 15 µl aliquots of the mixtures were applied in Whatman nº 1 filter paper and subjected to descending chromatography in isobutyric acid-1 M NH_3, 5:3, v/v for 48 hours. The radioactive compounds were detected in strips of the chromatograms by a Packard radioactivity scanner. HTS, heparitin sulfate; ΔDi-4S, unsaturated 4-sulfated disaccharide derived from chondroitin sulfates A and B; ΔDi-6S, unsaturated 6-sulfated disaccharide derived from chondroitin sulfate C; C, cells after trypsinization; T, trypsinate.

T A B L E II

SULFATED MUCOPOLYSACCHARIDES FROM 3T3 CELL LINES SPONTANEOUSLY TRANSFORMED

CELL LINE	SULFATED MUCOPOLYSACCHARIDES (%)		
	Chondroitin sulfate A	Chondroitin sulfate B	Heparitin sulfate
3T3 (Normal)	11	49	40
3T3 (spontaneously transformed)	23	34	43
3T6	45	24	31

published results). These results indicate that these cultures of 3T3 and 3T6 cells still retain anchorage dependency.

DISCUSSION: The results reported in this paper have shown that normal Swiss 3T3 cells contains mainly chondroitin sulfate B whereas its tumorigenic mutant ST1 contains chondroitin sulfate A. Besides these, both cells contain heparitin sulfate in equivalent amounts. Furthermore, "normal" 3T3 which became partially transformed and 3T6 cells that have no contact inhibition also presented a relative increase in chondroitin sulfate A. It can thus be concluded that transformation of 3T3 cells is acompanied by changes in the type of chondroitin sulfate at the cell surface.

Hydrocortisone when added to ST1 cultures is able to restore to these cells some of the properties of the "normal" 3T3 line, namely growth dependency for serum, density and anchorage (11). However the hormone failed to restore the original sulfated mucopolysaccharide composition of the "normal" 3T3 line. On the other hand the trypsinization indicated that the heparitin sulfate from the surface of hydrocortisone treated ST1 is less susceptible to the protease action (Table I). This last fact suggests that the hormone leads to changes in components of the cell surface other than sulfated mucopolysaccharides. Actually it has been observed that treatment of ST1 cells with hydrocortisone increases the amount of iodinatable proteins of the cell surface detected by the ^{131}I-lactoperoxidase method (M.C.S. Armelin, unpublished results).

Apparently the only difference between the two types of chondroitin sulfates is the amount of iduronic acid residues of the polymers, chondroitin sulfate B containing about 3/4 of iduronic acid and 1/4 of glucuronic acid residues (17). The hexosamine moiety of the two compounds is identical, i.e., 4-sulfated N-acetyl galactosamine. According to the currently available experimental data, chondroitin sulfate B is formed in the tissues by a C-5 epimerization reaction of the glucuronic acid residues of chondroitin sulfate A at the polymeric level (18). If the proposition is correct we could postulate that the transformation of the 3T3 cells to the ST1 variant is at least partially due to the loss of the C-5 epimerase responsible for the formation of chondroitin sulfate B.

The correlation of the exposure of glucuronic acid-containing chondroitin sulfate at the cell surface with the tumorigenic activity and loss of contact inhibition supports the previous suggestion of a possible role for this chondroitin sulfate in the stimulation of cell division in embryonary and cancer tissues (5,19). This stimulation, as previously pointed out, could be brought about by the disruption of the recognition sites (probably mediated by heparitin sulfate) through the intercalation of the newly formed chondroitin sulfate A.

ACKNOWLEDGEMENTS : This work was supported by grants from FINEP (Financiadora de Estudos e Projetos), FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico). The authors express their gratitude to Dr. S.M.C. Dietrich for help in the preparation of the manuscript and Dr. M.C.S. Armelin for making available her unpublished results, and for critical reading of the manuscript.

REFERENCES

1 - Hynes, R.O. (1976). Biochim.Biophys.Acta 458, 73-107.
2 - Dietrich, C.P., Sampaio, L.O. and Toledo, O.M.S. (1976). Biochim.Biophys. Res.Commun. 71, 1-10.
3 - Toledo, O.M.S. and Dietrich, C.P. (1977). Biochim.Biophys.Acta 498, 114-122.
4 - Cassaro, C.M.F. and Dietrich, C.P. (1977). J.Biol.Chem. 252, 2254-2261.
5 - Sampaio, L.O., Dietrich, C.P. and Giannotti FQ, O. (1977). Biochim.Biophys. Acta 498, 123-131.
6 - Takeuchi, J. (1968). Canc.Res. 28, 1520-1523.
7 - Takeuchi, J. (1972). Br.J.Cancer 26, 115-119.
8 - Dietrich, C.P. and Montes de Oca, H. (1978). Biochim.Biophys.Res.Commun. 30, 805-812.
9 - Todaro, G. and Green, H. (1963). J.Cell Biol. 17, 299-313.
10 - Armelin, M.C.S. and Armelin, H.A. (1977). Nature, 265, 148-151.
11 - Armelin, M.C.S. and Armelin, H.A. (1978). Proc.Natl.Acad.Sci. 75, 2805-2809.
12 - Dietrich, C.P. and Dietrich, S.M.C. (1972). Anal.Biochem 46, 209-215.
13 - Dietrich, C.P. and Dietrich, S.M.C. (1976). Anal.Biochem 70, 645-647.
14 - Michelacci, Y.M. and Dietrich, C.P. (1976). J.Biol.Chem. 251, 1154-1158.
15 - Michelacci, Y.M. and Dietrich, C.P. (1975). Biochem.J. 151, 121-129.
16 - Dulbeccp, R. and Vogt, M. (1954). J.Exptl.Med. 99, 167-182.
17 - Michelacci, Y.M. and Dietrich, C.P. (1976). Biochim.Biophys.Acta 451, 436-443.
18 - Malmström, A. and Fransson, L. (1975). J.Biol.Chem. 250, 3419-3425.
19 - Dietrich, C.P., Sampaio, L.O., Toledo, O.M.S. and Cassaro, C.M.F. (1977). Biochem.Biophys.Res.Commun. 75, 329-336.