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SURFACE SULFATED MUCOPOLYSACCHARIDES OF PRIMARY AND PERMANENT MAMMALIAN CELL LINES

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SUMMARY: The sulfated mucopolysaccharide composition of the mammalian cell lines: HeLa, H.Ep.2, AV3, WI-38, BHK and a cell culture of rabbit lung tissue is reported. It is shown that chondroitin sulfate AC and heparitin sulfate are the main mucopolysaccharides of the permanent cell lines whereas chondroitin sulfate B and heparitin sulfate are the major ones in the primary cultures, with no significant change in their relative concentrations up to seven generations. It is also shown that besides heparitin sulfate , chondroitin sulfate AC and chondroitin sulfate B are located at the surface of the cells. These results are in agreement with the earlier proposals that heparitin sulfate and chondroitin sulfate B might play a role in cell recognition and adhesiveness and that chondroitin sulfate AC might act as a stimulant of cell division.

INTRODUCTION: In recent years several laboratories have shown that heparitin sulfate and chondroitin sulfate AC are the main sulfated mucopolysaccharides present in a wide variety of permanent cell lines in culture (1-4). Chondroitin sulfate B was usually not detected or was present as a minor component in most of these cell lines (1, 2). These results contrasted sharply with the recent evidence that the SMPS[1] obtained from normal mammalian tissues were composed chiefly of chondroitin sulfate B and heparitin sulfate (5-7). In most of these tissues chondroitin sulfate AC was not detected or constituted only a minor fraction of the total tissue mucopolysaccharide.

In view of these findings we have decided to examine the SMPS composition of cell cultures obtained from normal tissues compared with several permanent cell lines. The cell surface SMPS composition of some of the cell lines was also studied.

[1] Abreviations used are: SMPS, sulfated mucopolysaccharides; CHS AC, chondroitin sulfate A and/or C; CHS B, chondroitin sulfate B; HTS, heparitin sulfate; MEM, Minimal essential medium.

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MATERIALS AND METHODS: Substrates and enzymes - Chondroitin sulfates A, B and C, chondroitinases AC and ABC were purchased from Miles Lab. (Elkhart, Ind.), heparitin sulfate, heparitinase, heparitinases I and II, and chondroitinase B were prepared by methods previously described (8-10). Agarose was purchased from L'Industrie Biologique Française (Gennevilliers, France). Diaminopropane was purchased from Aldrich Chemical Co. (Milwaukee, Wis., USA).

Cell lines - The permanent cell lines HeLa (CCL2), H. Ep. (CCL23), AV_3 (CCL21), the limited life span WI-38 (CCL75) were obtained from the cell bank of the Instituto Adolpho Lutz (São Paulo, Brazil) and BHK-21 line from Microbiological Associates (Bethesda, Md.). Lung cultures were prepared from 30-day-old rabbits following the technique described for kidney tissues (11). All the cell cultures were maintained and grown in Eagle's MEM medium (12)with two times the concentration of Eagle's amino acids,non essential amino acids and vitamins, plus 10% of calf serum. The medium also contained 100 U of penicillin, 100 µg of streptomycin and 5 µg of amphotericin per ml. Sterilization of the medium was performed by 0,2 µ Millipore filtration. For the labelling of the SMPS, carrier free $Na_2S^{35}O_4$ (10 µCi/ml) was added to the cultures immediately after inoculation. The cells were then allowed to grow at 37°C up to the completion of the monolayer (4 to 6 days). The cells were scraped from the walls of the flasks with cellophane and collected by centrifugation.

For enzyme treatment the S^{35}-labelled cells were allowed to grow as described above. After completion of the monolayer, the medium was removed and the monolayer washed three times with Dulbecco's phosphate buffer-saline (13). Two ml solutions of trypsin (0.25g %), chondroitinase AC (1 U/ml), chondroitinase ABC (1 U/ml), a mixture of chondroitinases and heparitinases prepared from Flavobacterium heparinum (2 mg/ml) were dissolved in Dulbecco's buffer and added to the monolayer. The cells were then incubated for 10 min at 37°C for trypsin or 60 min at 30° for the other enzymes. After incubation the medium was removed and the cells removed as described above.

Extraction and identification of sulfated mucopolysaccharides - To about 3×10^6 cells, 2 ml of acetone were added and the mixture kept for 2 hours at room temperature. The precipitate formed was collected by centrifugation, dried, resuspended in 100 µl of 0.05 M Tris-HCl buffer, pH 8.0 and incubated for 12 hours at 37°C. After incubation 20 µl of 90% CCl_3COOH were added to the incubation mixture which was then 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 maintained at -20° C for 12 hours. The precipitate formed was collected by centrifugation, washed once with 100 µl of ethanol at 80% and dried. The dried material was dissolved in 20 µl of water and the sulfated mucopolysaccharides analysed by a combination of agarose gel electrophoresis and enzymatic degradation as previously described (5-7). For quantitation of SMPS the radioactive bands corresponding to the migrations of the standards were scraped from the agarose gels (after fixation, drying and staining) and counted in 10 ml of 0.5% PPO toluene solution in a L-S 100 Beckman spectrometer. The SMPS of the cell trypsinates were extracted as follows: to the trypsin incubates CCl_3COOH was added to a final concentration of 15%. After centrifugation, 2 volumes of alcohol were added to the supernatant and the precipitate formed was collected by centrifugation, dried and analysed.

RESULTS: Sulfated mucopolysaccharides of permanent cell lines

Figure 1 A shows the agarose gel electrophoresis of the S^{35}-labelled mucopolysaccharides formed by HeLa, H.Ep.2, AV_3, WI-38 and BHK-21 cell lines. Two main bands are present in all extracts with the migrations of chondroitin sulfate AC and heparitin

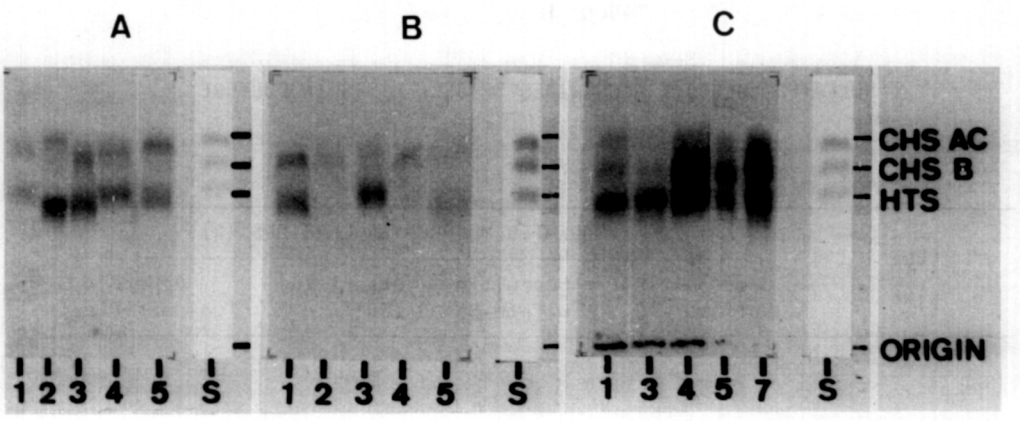

Fig. 1 - Agarose gel electrophoresis of sulfated mucopolysaccharides from primary and permanent cell lines.

5 µl aliquots of SMPS extracts (about 1,000 cpm) were applied on 5 x 7.5 cm (0.2 cm-thick) agarose gel slabs (0.9% agarose in 0.05M propanediamine-acetate, pH 9.0) at 1 cm from the negative electrode. The agarose gel was then subjected to electrophoresis for 1 hour at 120 V. The SMPS in the gel were fixed with CETAVLON and stained with toluidine blue. Radioautograms of the slides were prepared with Kodak Royal blue X ray film exposed for 10 days. S. Mixture of standards. A - SMPS extracts from: 1, AV_3; 2, H.Ep.2; 3, HeLa; 4, BHK-21; 5, WI-38. B - SMPS extracts from: 1, HeLa; 2, HeLa after chondroitinase AC + heparitinases; 3, HeLa after chondroitinase AC; 4, HeLa after trypsin; 5, HeLa tripsinate (For further details see methods). C - 1,3,4,5 and 7 - sulfated mucopolysaccharides of rabbit lung cells from the 1st, 3rd, 4th, 5th and 7th generation respectively.

sulfate standards. The band with the migration of chondroitin sulfate AC from all the cell lines was completely degraded by chondroitinase AC. The band with the migration of heparitin sulfate was degraded by a mixture of heparitinase I and II, but not by heparinase or chondroitinase ABC. These combined results indicate that all the cell lines analysed contain mainly chondroitin sulfate AC and heparitin sulfate. WI-38 and H.Ep.2 cells contain also a minor component with the migration of chondroitin sulfate B standard. This band was degraded only by chondroitinase B but not by chondroitinase AC. This indicates that chondroitin sulfate B is also present in these cells. The relative proportions of the mucopolysaccharides formed by the cell lines as well as L cells, mouse embryo, and rat embryo, which were described previously (1) are shown in Table I.

The cell surface mucopolysaccharides obtained by trypsinization of HeLa cells are shown in Fig. 1 B. Heparitin

TABLE I

SULFATED MUCOPOLYSACCHARIDES OF CELL LINES IN CULTURE

CELL LINE	SULFATED MUCOPOLYSACCHARIDES (%)			
	Chondroitin Sulfate AC	Chondroitin Sulfate B	Heparitin Sulfate	Other (unidentified)
HeLa	59	<2	41	-
AV_3	51	<2	49	-
H.Ep.2	24	7	69	-
WI-38	26	6	68	-
BHK-21	40	<2	60	-
L *	48	<2	40	12
Mouse Embryo*	56	<2	37	7
Rat Embryo*	44	<2	41	15

* Data obtained from ref. 1

sulfate and chondroitin sulfate AC are partially removed by trypsin (Table II). This indicates that both SMPS are at least in part located at the cell surface. This is corroborated by the finding that chondroitinase AC also degrades surface chondroitin sulfate AC of intact cells; a mixture of chondroitinase AC and heparitinases degrade most of the mucopolysaccharides (Fig. 1 B). The cell membranes of the enzyme-treated cells were apparently intact since no uptake of trypan blue could be observed. Besides, these cells still exhibited normal growth after the treatment with the enzymes. In another experiment H.Ep.2 cells were treated with trypsin; all the three SMPS, namely chondroitin sulfate AC, chondroitin sulfate B and heparitin sulfate were partially removed by the enzyme (Table II).

Sulfated mucopolysaccharides of lung cell cultures - Figure 1 C shows the sulfated mucopolysaccharides obtained from cell

TABLE II

SULFATED MUCOPOLYSACCHARIDES RELEASED BY TRYPSINIZATION OF CELLS IN CULTURE

SULFATED MUCOPOLYSACCHARIDES	SULFATED MUCOPOLYSACCHARIDES (%)*					
	HeLa		H.Ep.2		Rabbit lung	
	Whole cells	Trypsinate	Whole cells	Trypsinate	Whole cells	Trypsinate
Chondroitin sulfate AC	59	25	23	13	20	18
Chondroitin sulfate B	<2	<2	7	4	28	20
Heparitin sulfate	41	35	70	55	52	35

* The results are expressed in percent amounts of total radioactive SMPS present in whole cells.

cultures of 30-day-old rabbit lung tissue. It is clear from the figure that the three main SMPS are present in about the same relative amounts during at least seven generations. Except for small differences, the percent amounts of the three mucopolysaccharides of the cultures are similar to the relative amounts of these compounds found in normal lung tissue of rabbits (Fig. 2).

The results of trypsinization of rabbit lung cells (seventh generation) are shown in Table II. The three SMPS were removed from the cells in amounts varying from 60 to 90%. Chondroitinase ABC treatment of intact cells degraded 75% of chondroitin sulfate B and 85% of chondroitin sulfate AC (not shown). Like the HeLa, the lung cells did not stain with trypan blue after enzyme treatment.

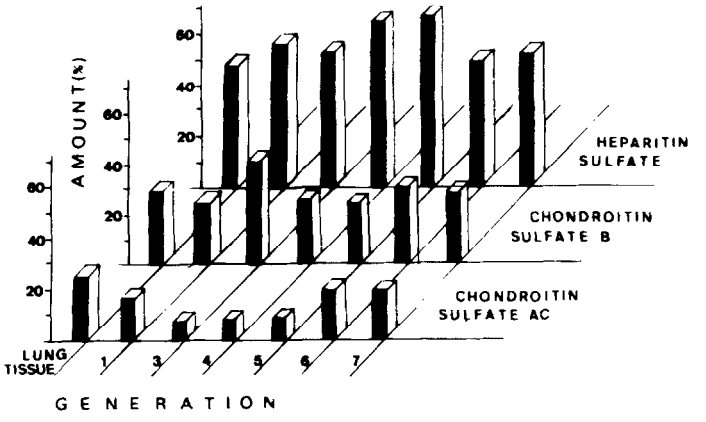

Fig. 2 - Relative proportions of sulfated mucopolysaccharides obtained from rabbit lung cells of different generations.

The radioactive bands shown in Fig. 1 C were counted as described in methods and the results expressed as percent amounts of total SMPS. The SMPS from the lung tissue were measured by densitometry as described in ref. 5.

DISCUSSION: The results reported in this paper have shown that the permanent cell lines contain mainly chondroitin sulfate AC and heparitin sulfate whereas the rabbit lung cell culture, similarly to their tissue of origin, contains chondroitin sulfate B, heparitin sulfate, and chondroitin sulfate AC. The SMPS composition of the permanent cell lines resemble somewhat the SMPS composition of the tumoral and embryonic tissues recently reported (6, 14). It is noteworthy that the permanent cell lines here reported were either derived from tumor (HeLa, H.Ep.2) or embryonic(WI-38, AV_3) tissues. No significant changes were observed in the SMPS composition of the cultures of lung tissue at least during seven generations. These combined results suggest that the SMPS composition of a given cell line is stable and does not change during successive generations. In agreement with this hypothesis are the results previously reported for cultures of mouse and rat embryos which, like the original embryonic tissues, contain mainly chondroitin sulfate AC and heparitin sulfate (1).

It has been reported that heparitin sulfate is the SMPS present in the cell coat (3, 4). This paper provides evidence that besides this mucopolysaccharide, chondroitin sulfate B and

chondroitin sulfate AC are also present at the cell surface. The presence of chondroitin sulfate B at the cell surface reinforces the previous suggestion that this compound together with heparitin sulfate might be involved in the process of cell recognition and adhesiveness (6).

The finding that chondroitin sulfate AC is also present at the cell surface is in agreement with the suggestion that this compound might act as stimulant of cell division in tumoral and embryonic tissues (14).

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