Altered Growth Behavior and Phenotypic Expression of Cells of Mouse and Hamster Cell Lines after Treatment with Polyanions

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GOTO, M., KIMURA, T., SATO, H., SUZUKI, S. and SUZUKI, M. *Altered Growth Behavior and Phenotypic Expression of Cells of Mouse and Hamster Cell Lines after Treatment with Polyanions*. Tohoku J. exp. Med., 1977, 121 (2), 143-148 — Effect of polyanions on saturation density and production of viscous substance of cells of mouse and hamster cell lines was studied. Cell lines used were 3T6, HA-2 and HA-8 cells. Polyanions were dextran sulfate, (3,6-anhydro-4-o- β -galactopyranosyl-2-D-galactopyranose-2,4'-bis (potassium/sodium sulfate) (1 $\rightarrow$ 3) polysaccharide (polygeenan) and dextran phosphate. Dextran sulfate reduced the saturation densities of cell lines. Growth of 3T6 cells was not significantly affected by dextran sulfate. Polygeenan and dextran phosphate could not decrease the saturation densities of all the cell lines tested. The production of viscous substance into medium from the treated cells increased after treatment with all polyanions with the exception of dextran phosphate of molecular weight 40,000. It was suggested that specific structure of polyanion might be necessary for the decrease of saturation density of cell lines. Furthermore, it appears that dextran sulfate decreases saturation density of cell line along with change of phenotypic expression of cells. ——— growth; phenotypic expression; polysaccharide sulfate

Some polyanions are reported to be inhibitory to tumor growth (Lippman 1957; Regelson and Holland 1958; Regelson et al. 1960; Jolles et al. 1963; Costachel et al. 1964; Levy et al. 1969). There are other papers which show growth enhancement of tumor cells by polyanions (Ozzello et al. 1960; Takeuchi 1966). The situation of study seems to be confronted with complicated results.

In the previous papers, it was reported that dextran sulfate restricted the growth of cells of mouse and hamster cell lines but did not affect growth of normal fibroblastic cells derived from embryos (Goto et al. 1972, 1973; Sasaki et al. 1974). Dextran sulfate of high molecular weight decreased the saturation densities of all

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the cell lines tested, while dextran sulfate of low molecular weight did not. It appeared likely that the molecular size of dextran sulfate played an important role in the restriction of growth of cells. The growth rate of treated cells was almost the same as that of untreated cells in the logarithmic phase, and the rate of colony formation by treated cells was similar to that of untreated cells. These findings showed that dextran sulfate was not toxic to the growth of cell lines.

In general, experimental tumor cells have lost the specific function of normal cells. This phenomenon is called deviation or dedifferentiation from the property of normal cells. Transformed cells treated with dextran sulfate ceased growing after reaching confluency at lower cell density than control even with the repeated renewal of medium. It may be suggested that dextran sulfate converts the growth behavior of transformed cells to that characteristic of normal cells. If so, the possibility exists that dextran sulfate might influence phenotypic expression by transformed cells by affecting the cell surface. The ability of dextran sulfate to alter cell surface is shown by the increased electrophoretic mobility of treated cells (Suemasu et al. 1971), inhibitory effect of dextran sulfate on aggregation of embryonic liver cells (Kuroda 1974) and appearance of peaks in the analysis of membrane protein of the treated cells (Gahmberg and Hakomori 1973). Furthermore, evidence that events at the cell surface may be associated with cell differentiation has been presented by some workers (Nevo and Dorfman 1972; Toole et al. 1972; Nameroff et al. 1973).

That the transformed cells treated with dextran sulfate might secrete highly polymerized hyaluronic acid into medium was supported by the following results: 1) the viscosity of the medium in which treated cells had grown disappeared after digestion with bovine testicular hyaluronidase and 2) the precipitation of a "mucin clot", known to be a specific reaction of highly polymerized hyaluronic acid in the presence of protein, was induced by addition of acetic acid to medium in which treated cells had grown (Grossfeld 1957a, b; Koyama and Ono 1970).

It may be expected that polyanions of high molecular weight other than dextran sulfate could also affect the growth behavior and/or functional properties of transformed cells, as suggested by the experimental results of cells treated with dextran sulfate. This paper shows that some polyanions other than dextran sulfate stimulate transformed cells to secrete viscous substance into the medium but do not restrict the growth behavior of transformed cells.

MATERIALS AND METHODS

Polyanions used in this study were dextran sulfate, (3,6-anhydro-4- α -D-galactopyranosyl-2-D-galactopyranose-2,4'-bis (potassium/sodium sulfate) (1 $\rightarrow$ 3) polysaccharide (polygeenan) and dextran phosphate. The mean molecular weight of dextran sulfate was 20,000 and that of polygeenan was 20,000–40,000. The sulfur content of dextran sulfate was 17–18% and that of polygeenan was 12.3%. The mean molecular weights of dextran phosphate were 40,000 and 110,000. Phosphorus contents of these were 24.4% and 36.4%, respectively. Dextran sulfate was kindly supplied by the Kowa Co., Ltd., Nagoya, Japan. Cell lines used were 3T6 (Todaro and Green 1963), HA-2 and HA-8 cells. HA-2 and HA-8

cells were hamster embryonic fibroblasts transformed by a chemical carcinogen (Kuroki and Sato 1968). Cells were cultured in modified Eagle's minimum essential medium supplemented with 10% calf serum in 35 mm Falcon plastic dishes as previously described (Goto et al. 1972, 1973). Media were changed every 2nd day. The decrease of saturation density induced by polyanion was estimated by comparing growth curves of treated and untreated cells. Each point on the growth curve was determined by the average number of cells in 3 dishes counted with a hemocytometer after 0.025% pronase digestion. Values in the table indicate ratios of saturation density of untreated cells to that of treated cells. The mucin clot test was a modification of Grossfeld's method (1957a, b). Culture medium was centrifuged to remove cell debris and 10 ml of supernatant was chilled in an ice-cold water. To it was added 0.1 ml of acetic acid followed by agitation of the mixture with a glass rod. When a thread-like precipitate around the rod was detected, the test was considered to be positive. When positive, the medium might contain more than 30 $\mu\text{g/ml}$ of hyaluronic acid (Koyama and Ono 1970).

RESULTS

Dextran sulfate of molecular weight 20,000 decreased the saturation densities of HA-2 and HA-8 cells. The growth of 3T6 cells was not significantly affected by dextran sulfate of molecular weight 20,000 (Table 1). Polygeenan and dextran phosphate could hardly reduce the saturation densities of all the cell lines tested. Dextran sulfate, a sulfated polymer of glucose, reduced the saturation density of cells, while polygeenan, a sulfated polymer of galactose and its derivative, could not decrease the saturation densities of both HA-2 and HA-8 cells, suggesting the existence of a structure-activity relationship in the restrictive growth of cells by polyanions.

During the course of the preceding experiments we observed an interesting phenomenon that the culture medium of 3T6 cells became viscous after treatment with dextran sulfate. The viscosity of the medium disappeared after incubation of 5 ml of medium with 500 U of bovine testicular hyaluronidase for 5 min at 37°C. Furthermore, the mucin clot test, known to be a specific reaction of highly polymerized hyaluronic acid, was positive, indicating that the viscous substance

TABLE 1. Saturation density of cell line and mucin clot test of culture medium after treatment with polyanions

Polyanion	Concentration ($\mu\text{g/ml}$)	3T6		HA-2		HA-8	
		Saturation density(%)	Mucin clot	Saturation density(%)	Mucin clot	Saturation density(%)	Mucin clot
Dextran sulfate	20	68	+	20	+	42	-
	40	65	+	NT	NT	36	-
Polygeenan	20	100	+	92, 98	+	82	+
	40	NT	NT	90	+	74	+
	50	97	+	74	+	NT	NT
Dextran phosphate (mol wt 40,000)	20	NT	NT	95	-	90	-
	40	NT	NT	100	-	84	-
Dextran phosphate (mol wt 110,000)	10	NT	NT	87	+	NT	NT
	20	NT	NT	84	+	NT	NT

Saturation densities of 3T6, HA-2 and HA-8 cells were about 6×10^5 cells/cm², about 4×10^5 cells/cm² and about 1×10^6 cells/cm². NT, not tested; +, positive; -, negative.

secreted into the treated medium might be hyaluronic acid. Viscosity and mucin clot test were negative in the medium of untreated cells. Results of the mucin clot test on medium from cell cultures treated with polyanions are summarized in Table 1. When positive, the media of the treated cells gave a positive mucin clot test 2-4 days after beginning treatment with polyanions. The results of mucin clot test of cells treated with dextran sulfate were positive with the exception of HA-8 cells. The mucin clot tests of the media from cells treated with polygeenan and dextran phosphate were positive with the exception of the medium from cells treated with dextran phosphate of molecular weight 40,000.

DISCUSSION

It has been reported that transformed fibroblastic cells growing in the presence of some substances such as dibutyryl adenosine-3',5'-cyclic monophosphate (Johnson et al. 1971), trypsinized concanavalin A (Burger and Noonan 1970), and phenetyl alcohol (Wright et al. 1973) show morphological characteristics of normal cells. Dextran sulfate also causes the reduction of saturation density of transformed cells without affecting growth of normal fibroblastic cells. The temporal reversion of transformed fibroblastic cells has been studied chiefly from the morphological point of view. It is likely that morphological reversion of transformed cells may accompany some functional change of cells. Hsie et al. (1971) showed that the addition of dibutyryl cAMP caused an increase in collagen synthesis of transformed cells. Johnson and Pastan (1972) showed that dibutyryl cAMP increased the production of melanin of cells. It was suggested in this study that dextran sulfate affected the functional property of transformed cells along with the decrease of saturation density of cells, because the addition of dextran sulfate to cultures increased the production of viscous substance of cells into medium.

Among three polyanions tested in this study, only dextran sulfate reduced the saturation density of transformed cells, while other polyanions, polygeenan and dextran phosphate, failed. This result indicates that not only specific configuration of substance is necessary for the decrease of saturation density of cells but also site(s) of cells combining with dextran sulfate may be specific.

There are some papers showing that polysaccharide sulfates inhibit peptic activity as a result of reacting not only with enzyme but also with substance (Levey and Sheinfeld 1954; Anderson 1961; Ravin et al. 1962). Anderson (1961) showed that different kinds of protein had its own affinity of forming complex with carageenan. It was reported that polysaccharide sulfate inhibited the activity of polynucleotide kinase (Wu 1971). Moreover, polysaccharide sulfates form complex with lipoprotein of serum (Bernfeld et al. 1960; Nishida and Cogan 1970).

Suzuki et al. (1971) reported that polysaccharide phosphate induced the interferon activity in rabbits. Scholnick et al. (1973) reported that there were three groups of sulfated polysaccharide which affected glycolysis and P_i uptake by ascites tumor cells. Dextran sulfate and amylopectin sulfate inhibited glycolysis

and P_i uptake. Amylopectin sulfate decreased the saturation density of a cell line and this polymer of large molecular weight was obviously toxic to the growth of other cell lines (unpublished data). Compounds such as heparin, cellulose sulfate and hyaluronic acid sulfate inhibited P_i uptake with little effect on glycolysis. Chondroitin sulfate and hyaluronic acid inhibited neither glycolysis nor P_i uptake. This result also suggests that pharmacological activity of polyanion depends on structure. Ogata and Kondo (1972) reported that dextran sulfate inhibited the respiratory activity of rat liver mitochondria incubated in media of low ionic strength as a result of preventing mitochondrial utilization of externally added ADP. It may be interesting that increasing K^+ concentration obliterates inhibition of glycolysis of cells and respiration of mitochondria by dextran sulfate.

It seems likely that dextran sulfate leads growth of transformed cells to that of normal cells by combining with certain loci on cell surface along with change of functional property of cells.

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