Stimulation of HeLa Cell Growth by a Serum Fraction with Sulfation Factor Activity (35370)

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(Introduced by Roger M. Des Prez)

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Serial propagation of most mammalian cells requires serum proteins. The functions of the latter remain unclear but utilization for synthesis of cell proteins is relatively insignificant (1). Attempts have been made to replace the serum with fractions thereof or with purified macromolecules. Lieberman and Ove have reported the use of a serumless medium containing a protein growth factor, catalase, insulin, and bovine serum albumin for culture of HeLa and human appendix A1 cells (2). The protein growth factor appeared to function at the cell surface to permit adherence to glass, cause flattening, and increase cell multiplication. The catalase served to prevent accumulation of peroxide. The growth-stimulating effect of insulin is pertinent to the present studies for reasons discussed below.

It has been shown that a fraction of serum from normal rats stimulates *in vitro* incorporation of leucine into protein, sulfate into mucopolysaccharide, uridine into RNA, and thymidine into DNA by cartilage from hypophysectomized rats (3). Those effects are due to a factor dependent upon, but distinct from, pituitary growth hormone. Since the factor was first identified in whole serum on the basis of an action to increase sulfate incorporation into cartilage mucopolysaccharide, it was called the serum sulfation factor (4). Recently sulfation factor has been found to stimulate incorporation of leucine into protein of muscle as well as cartilage from hypophysectomized rats (5). The actions of sulfation factor have been reproduced by insulin (5, 6), but quantitative and immunological differences distinguish the two. Furthermore, sulfation factor activity has been demonstrated in whole serum from alloxan-diabetic hypophysectomized rats treated with growth hormone (7). The similarities in effects of sulfation factor and insulin on rat cartilage and muscle and the growth promoting effect of insulin in cell cultures stimulated the present investigation of the effect of sulfation factor on growth of HeLa cells.

Methods. HeLa cells were grown in stock culture in a medium composed of 90% Eagle's minimum essential medium supplemented with "nonessential" amino acids (MEMN; Hyland) and 10% fetal calf serum (Hyland). Cell suspensions were prepared for subculture and counting by washing cell layers twice with calcium- and magnesium-free Dulbecco's phosphate-buffered saline (8) containing 0.1% glucose (PSG) and incubating them for 30 min with 0.02% collagenase (Worthington Biochemical Corporation) in PSG. Cell counts were made in a hemocytometer. Approximately 10^5 cells in 1 ml of stock medium were transferred to each of the required number of 16 × 125-mm culture tubes. Incubation was carried out in an atmosphere of 95% air–5% carbon dioxide at 35°. On the following day the attached cells were washed once with Hanks' solution (Hyland), and 1 ml of test medium was applied to individual cultures in quadruplicate for each treatment. Incubation was continued as before with daily changes of media. Protein determinations on cells were made by the method of Oyama and Eagle (9) after washing the layers twice with PSG.

Calf serum, regular (Hyland), which had been shown previously to contain sulfation factor activity, was used for all experiments. A fraction with sulfation factor activity was

prepared by the following procedure. Sixty ml of serum was diluted with 120 ml of distilled water and 180 ml of 0.05 *M* sodium acetate buffer, pH 5.5. The pH was adjusted to 5.5 roughly with 2 *N* and finally with 0.5–1 *N* acetic acid. The acidified serum was heated with mixing in a sealed flask immersed in boiling water for 15 min, cooled, and centrifuged to remove coagulated protein. The supernate was adjusted to pH 5.5, measured, and applied to a 2 × 20-cm column of DEAE-Sephadex equilibrated with 0.05 *M* sodium acetate buffer, pH 5.5. The column was washed with 125 ml of buffer. All effluent was collected and applied to a 2 × 20 column of CM-Sephadex equilibrated with 0.05 *M* sodium acetate buffer, pH 5.5. This column was washed with 125 ml of 0.05 *M* ammonium acetate buffer, pH 5.5 and the washing was discarded. Then, 175 ml of 0.2 *M* ammonium acetate (adjusted to pH 7.5 with ammonium hydroxide) was applied to the column, and the eluate was collected and lyophilized. The dried residue was dissolved in magnesium sulfate-free Krebs' phosphate-buffered saline (KPS; 10), placed together with several glass beads into cellophane tubing which had been washed repeatedly in boiling 0.15 *M* sodium bicarbonate solution, and dialyzed against KPS for 2.5 hr at 4°. Whole serum was dialyzed in the same manner as the serum fraction or, in Expt. 2 of Table II, used in unaltered form. The serum fraction was added as indicated in a concentration sufficient to provide the amount which was obtained from 0.2 ml of whole serum/ml of final culture medium. This represented 23 μg of protein as determined by the method of Lowry *et al.* (11). The protein concentration of the whole serum as determined by the same method was 79 mg/ml. The test media for the experiment of Table I and Expt. 1, Table II, were composed of 8.9 vol of MEMN; 0.1 vol of Hanks' solution containing crystalline bovine serum albumin (Nutritional Biochemicals Corporation), 0.3 g/ml; and 1 vol of either dialyzed serum, serum fraction, or KPS. In Expt. 2, Table II, the media were composed of 8.5 vol of MEMN; 0.1 vol of Hanks' solution containing bovine serum albumin; 0.4 vol of either serum fraction or KPS; and 1 vol of either whole serum or a 1:100 dilution of the same in Hanks' solution.

TABLE I. Effect of Sulfation Factor on Cell Count in HeLa Cell Culture.

Test medium	Cell count[a] 3 days[b]	Cell count[a] 6 days[b]
Control	103,000	118,000
Sulfation factor	161,000	373,000
Dialyzed calf serum 10%	200,000	972,000

[a] Mean of 4 cultures.
[b] Time of incubation in test medium.

TABLE II. Effect of Sulfation Factor on Total Protein in HeLa Cell Culture.

Expt.	Test medium	Protein[a] (μg) 0 days[b]	Protein[a] (μg) 6 days[b]
1	—	29.1	
	Control		73.4
	Sulfation factor		174
	Dialyzed calf serum, 10%		350
2	—	32.0	
	Calf serum, 0.1%		48.0
	Sulfation factor + calf serum, 0.1%		106
	Calf serum, 10%		240

[a] Mean of 4 cultures.
[b] Time of incubation in test medium.

Results. Under the conditions of the present experiments, cells in the medium containing 10% whole or dialyzed serum formed a dense monolayer in approximately 6 days. When serum was omitted and the only protein contained in the medium was 0.3% bovine serum albumin (control medium), a profound reduction in growth of cells occurred, as assessed by either cell count (Table I) or total protein (Table II). Addition of sulfation factor increased cell count within 3 days (Table I). The final cell count of cultures after 6 days' incubation with sulfation factor was more than twice that of cultures incubated in the control medium but less than half that of cultures incubated in the medium containing 10% dialyzed serum. In a subsequent experiment, roughly parallel results were obtained when cell growth was assessed by total protein. Sulfation factor increased

total protein of cultures during 6 days to more than twice the level of cultures incubated in the control medium, but the former was only about half the total protein of cultures incubated in the medium containing 10% dialyzed serum (Table II, Expt. 1). These observations are not explained by a nonspecific effect of serum proteins. As indicated above, all media contained 0.3% bovine serum albumin. In another experiment (Table II, Expt. 2), 0.1% whole serum was added to the control medium, which represented an addition of 79 μg of whole serum proteins/ml of incubation medium. Still, sulfation factor (23 μg of serum fraction protein/ml of incubation medium) more than doubled total protein of cultures during 6 days by comparison with cultures incubated in the medium containing 0.1% serum but no additional sulfation factor.

Discussion. Jacquez and Barry have investigated the growth-promoting activity of serum proteins in cultures of rat fibroblasts (12). Of particular interest was their observation that serum from hypophysectomized rats was as active as serum from normal rats in stimulating growth of their cells. The finding is somewhat obscured by the statement that only one of four rats used as serum donors was completely hypophysectomized. However, it is clear from the present experiments that sulfation factor does not replace all the functions of serum proteins in promoting growth of HeLa cells, even after attachment of cells and in the presence of serum albumin. Consequently, loss of serum sulfation factor activity following hypophysectomy of rats cannot be equated with elimination of serum growth-promoting activity.

The present results establish another similarity in actions of sulfation factor and insulin, in that both are capable of stimulating growth of HeLa cells. Insulin was reported by Gey and Thalheimer in 1924 to stimulate growth of chick fibroblasts (13). Since that time numerous investigators have documented effects of insulin on various cells and tissues in culture and included it as a component of cell culture media (14). Quantitative considerations made it unlikely that the insulin content of serum is important for the effects of serum proteins, a point emphasized by Temin in reporting experiments with chick embryo fibroblasts (15). This investigator has indicated, however, that serum insulin-like activity might be responsible for an effect of serum proteins. The possible relationship between sulfation factor and insulin-like activity in serum has been discussed elsewhere (5).

The influence of pituitary growth hormone on sulfation factor activity in serum has been established in the rat and man (4, 16). Although this factor is distinct from growth hormone, the latter also has been found to stimulate growth of a line of human liver cells (17) and strain L mouse fibroblasts but not HeLa cells (18). Also, myogenesis in cultures of chick embryo breast muscle was increased by addition of growth hormone to a medium containing insulin (19). Since the concentrations of growth hormone used in the various studies (5–300 μg/ml) greatly exceed the concentration in serum, the findings are of doubtful relevance to the growth-promoting effect of serum proteins.

Summary. A calf serum fraction with sulfation factor activity was shown to stimulate growth of HeLa cells in Eagle's minimum essential medium supplemented with "nonessential" amino acids and bovine serum albumin. The results are discussed in light of the influence of pituitary growth hormone on sulfation factor activity in serum and of similarities in effects of the latter and insulin.

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