PROPERTIES OF A GROWTH STIMULANT (GS), PRODUCED BY SERUM-FREE FIBROBLAST CULTURES, FOR HUMAN CELLS IN VITRO

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INTRODUCTION - A report on the biological synthesis of a growth factor substance for cultures of mammalian cells was recently published (Alfred and Pumper, 1960). It was shown that this growth-stimulating substance (GS) was found not intracellularly, but in the growth fluid of actively proliferating fibroblast cultures, and had the ability to replace the serum requirement for growth of cells of human liver origin. Other reports (Ozzello, 1960 ; and Lieberman et al, 1959) have shown the importance of mucopolysaccharides and fetuin in the growth of mammalian cell cultures, but a quantitative assay of biological activity of these macromolecules has not yet been described. The present investigation was concerned with the purification, characterization and the in vitro assay of a GS produced in cell cultures.

METHODS - 1. Monolayer cell cultures : The serum-free mouse lung fibroblast cultures were from a cell line which had been adapted to a peptone medium (Pumper, 1958). Certain growth phases of these serum-free cells have been described (Pumper and Alfred, 1961). Human liver cells (Chang, 1954) were cultivated in a medium consisting of 90 % medium 199 plus 10 % fetal calf serum.

2. Separation and purification of the GS : The entire procedure was carried out at 4°C. The pooled growth fluid from 6-10 day old serum-free fibroblast cultures was dialyzed against running distilled H_2O for 4 days and lyophilized (Crude GS). The attached cell sheets were overlaid with 10 % Hanks balanced salt solution (BSS), dislodged by scraping, the free cells washed 3-4 times with 10 % BSS, resuspended, counted by use of a hemocytometer, and aliquots were saved for protein and hexuronic acid determinations. A sample of washed cells was lysed by grinding in alumina powder. The lyophilized crude GS fraction was partially purified by

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precipitating the active material from solution with solid $(NH_4)_2SO_4$ to obtain the 30-50 % fraction (AS_{30-50}). The precipitate was redissolved in 10 % BSS, dialyzed as above and lyophilized. An aqueous solution of the AS_{30-50} fraction was adsorbed onto calcium phosphate gel (prepared according to Main et al, 1959) to give a gel : protein ratio of 2 : 1. The gel-protein complex was washed with 0.005 M sodium phosphate buffer pH 6.7, and the active GS substance was eluted from the gel with 0.175 M phosphate buffer at pH 6.7, dialyzed as above and lyophilized (Gel Fraction II). 3. Growth stimulation assay technique : A washed cell inoculum was prepared from 4-5 day old human liver cell cultures by decanting the serum-containing growth medium, rinsing the cell sheet twice with 10 % BSS, scraping off the cells with a rubber policeman, and washing the dislodged cells 3 times with 10 % BSS. The assay was carried out by adding varying dry weight amounts of GS or other known materials (chondroitin sulfate, hyaluronate) to a standard number of liver cells suspended in medium 199. Controls for base line growth consisted of the same number of liver cells plus the standard calf serum-medium 199 or medium 199 alone. The cell-mixtures were incubated for 48 hours at 37°C. and observed for the presence of cytoplasmic outgrowth. The response of the stimulated liver cells was quantitated by a measurement of the cell protein (method of Oyama and Eagle, 1956) after 48 hours and the values compared to those of the controls. The latter measurement consisted of a determination of the protein content of the stimulated cells after the growth medium had been discarded and the cells washed with 10 % BSS. The response was expressed as the F/S ratio according to the following relationship : $\frac{F - C}{S - C} = F/S$, where F = protein of cells stimulated by the GS, S = protein of cells stimulated by serum, and C = protein of cells in deficient medium 199. One unit of stimulatory activity was defined as the dry weight of GS that gave an F/S of 0.5 (50 % stimulation) in 48 hours at 37°C. Specific activity was defined as the number of units of activity per mg. of GS protein 4. Chemical determinations : The protein content of crude and partially purified fractions of the GS material was determined by the method of Lowry et al, (1951), using bovine serum albumin as a standard. Hexuronic acid was determined according to Dische (1947). Hexosamine was determined according to the method of Elson and Morgan (1933).

RESULTS - The growth-stimulating activity first appeared in the growth fluid of serum-free fibroblast cultures on the 3rd day during the lag phase at a time when the cell number was relatively constant, but the production of protein per cell was maximum (Table I).

Table I

Mean Cellular Content of Protein and HA*, and Content of Extracellular HA during a 12 Day Growth Period of Serum-Free Fibroblast Cultures

Days of Incubation	Cell Count x 10^5/ml.	Protein, mg. x 10^{-9}/cell	HA, mg. x 10^{-9}/cell	HA, mg./ml. Growth Fluid
0	2.0	400.0	7.5	0
1	2.0	960.0	8.75	0.0100
2	2.5	992.0	8.9	0.0225
3	2.3	1350.0	12.5	0.0175
4	3.5	938.0	15.0	0.0120
6	6.8	564.0	8.9	0.0100
8	15.0	306.0	2.5	0.0100
10	31.0	150.0	2.3	0.0110
12	20.0	230.0	2.5	0.0080

*HA : Hexuronic Acid

These fibroblasts produced both intra and extracellular hexuronic acid (HA) during the period of production of the GS substance (Table I). The latter results were consistent with the presence of carbohydrate staining particulates located in the cytoplasm of these fibroblasts (Pumper, unpublished).

The response of washed liver cells to varying amounts of GS or to other macromolecular substances was determined qualitatively by the degree of outgrowth during a 48 hour incubation period. The response was then quantitated by a measurement of the protein of stimulated cells, and the results of a typical experiment are shown in Table II.

The lowest amounts (in terms of protein) of crude and partially purified fraction of GS material necessary to produce a 4 + liver cell response in 48 hours were compa to show the differences in specific activity. The results of the latter comparison are shown in Table III.

The fractions of GS substances were compared with respect to the content of protein, hexuronic acid and hexosamine. These determinations were performed on the material in an attempt to relate the composition of the active substance to its biologica activity, and the results are shown in Table IV.

Table II

Response of Human Liver Cells to the Crude and Partially Purified Fractions of GS Material and Other Substances

Culture Tube Number	Test Material	Conc. test Material µg per ml.	Response of Liver Cells: Outgrowth in Hours 4	24	48	µg Cell Protein per culture in 48 hours	F/S
1	Crude	4000.0	4 +	4 +	4 +	247.0	1.53
2	"	2000.0	0	4 +	4 +	169.0	0.7
3	"	500.0	0	1 +	1 +	119.0	0.15
4	AS_{30-50}	300.0	4 +	4 +	4 +	215.0	1.20
5	"	150.0	4 +	4 +	4 +	220.0	1.26
6	"	50.0	0	0	0	121.0	0.16
7	Gel II	200.0	4 +	4 +	4 +	166.0	0.67
8	"	50.0	4 +	4 +	4 +	190.0	0.93
9	"	10.0	4 +	4 +	4 +	140.0	0.38
10	Cell lysate*	1000.0	0	0	0	100.0	ND**
11	Hyaluronate	1000.0	0	0	0	100.0	ND
12	"	500.0	0	0	0	94.0	ND
13	Chon. SO_4C	100.0	4 +	4 +	4 +	160.0	0.6
14	"	50.0	1 +	4 +	4 +	151.0	0.5
15***	Medium 199	-	0	0	0	105.0	-
16****	Calf Serum	-	0	4 +	4 +	196.0	-

* Lysate from serum-free fibroblasts

** Not determined

*** Synthetic medium 199 only, which served as a deficient medium control = 0 (absence of outgrowth)

**** Serum-containing (10 % v/v) control = 4 + (maximum outgrowth)

Assay mixture : This mixture contained the test material dissolved in synthetic mediun plus a washed liver cell inoculum. The inoculum contained 2.0 x 10^5 cells (equivalent to 100.0 ug of protein) per ml. of medium. The total volume per culture was 1.0 ml. (Leighton tube).

Table III

Comparison of the Stimulatory Activity of the Fractions of GS Material

Purification Step	Fraction	Protein Conc. mg. per ml.	F/S Ratio	Units per ml.	Specific Activity
I	Crude	1.280	1.53	6.12	2.3
II	AS30-50	0.050	1.26	50.4	46.7
III	Gel II	0.010	0.38	14.0	107.6

Table IV

The Content of Protein and Certain Carbohydrates of the Nondialyzable Portions of GS Material and of Uninoculated Medium

Fraction	% Protein	% Hexosamine	% Hexuronic acid
Uninoc. Medium*	93.0	0	0
Crude	82.5	2.0	1.5
AS_{30-50}	61.0	1.0	1.6
Gel II	88.0	0	0

*Uninoculated Medium : The nondialyzable portion of complete serum-free medium prior to growth of fibroblasts (time zero).

DISCUSSION - A proteinaceous growth-stimulant for human liver cell in vitro was recovered from the growth fluid of serum-free mouse lung fibroblast cultures. The GS substance first appeared during the lag phase of growth during which time protein synthesis per cell was maximum, and hexuronic acid was found to be present both intra and extracellularly. The results show an increase in protein of liver cells stimulated by GS material as compared to no net increase of protein in cells maintained in synthetic medium 199. The specific activity of the GS was significantly increased by fractionation with $(NH_4)_2SO_4$ and calcium phosphate gel. The mechanism of action of this substance ha not yet been defined, but immunochemical studies are in progress which may explain thi phenomenon. These results may also aid in determining whether or not the stimulation o growth of one cell type by the metabolic product (s) of another type represents a transfer of cell specialization.

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