A Protein Growth Factor for Mammalian Cells in Culture*

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The amino acid and vitamin requirements of mammalian cells in culture have been carefully defined in the studies of Eagle and coworkers (1) who supplemented their medium with 5 per cent dialyzed serum. Knowledge concerning the growth requirements satisfied by the serum is completely lacking.

In a preliminary report (2), evidence was presented for the protein nature of one of the serum growth factors. Partially purified from bovine serum, the factor has several marked effects upon mammalian cells in culture (appendix A1): (a) it causes adherence of cells to a glass surface; (b) only in its presence cells assume a flattened, epithelial-like appearance; and (c) it stimulates cell multiplication. More recently, Fisher et al. (3), working independently with a different cell culture, HeLa Sa, have reported some of these effects.

The purpose of this report is to describe the purification and properties of the protein growth factor.

MATERIALS AND METHODS

Culture Methods—Cells were grown on glass surfaces in a medium containing the amino acids, inorganic salts, glucose, and antibiotics suggested by Healy et al. (6), plus the vitamin mixture of Eagle (1), and 15 per cent bovine serum.

Materials—Pooled beef serum was prepared from blood procured at a slaughterhouse. The serum was obtained by centrifugation (37°C) 2 to 3 hours after the blood was drawn, and it was stored at -20°C. No variations were noted in the activity of different batches of serum, nor was there a detectable loss in activity over a 6 month period. Human serum and serum from laboratory animals were prepared in a similar manner.

Pancreatic trypsin (1:250) and peptone (Bacto-peptone) were obtained from the Difco Laboratories, Inc., crystalline trypsin and pepsin from the Worthington Biochemical Corporation, and crystalline soy bean trypsin inhibitor from the Nutritional Biochemicals Corporation. Poly-L-lysine (about 30 residues) was a product of the Mann Research Laboratories. A sample of highly purified salmine sulfate was a gift from Dr. W. R. Carroll, pancreatic elastase (80 to 90 per cent pure) was prepared by Warner-Chilcott Laboratories, and crystalline bovine plasma albumin was obtained from the Armour Laboratories. Diethylaminoethyl-cellulose was a product of the Brown Company. z-ethionine and DL-p-fluorphenylalanine were obtained from the Nutritional Biochemicals Corporation, dl-5-methytryptophane from the Sigma Chemical Company.

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1 Appendix A1 is a single clone isolate obtained in Dr. Puck’s laboratory (3) from a culture derived from normal human appendix by Dr. Chang (4).

2 Similar, but less reproducible results were obtained when nontrypsinized cells were used in the assay procedure.

3 Although the test solutions were not treated to free them from microorganisms, no difficulty with microbial contamination was encountered.

Determinations—Cell counts were made with Levy counting chambers. Hexose and hexosamine were estimated according to the procedures described by Winzler (7), and sialic acid was measured directly with Ehrlich’s reagent (8). Protein was estimated by the method of Lowry et al. (9). Radioactivity measurements were made with a gas flow counter after drying the samples in stainless steel dishes. pH determinations were carried out with a Beckman model G pH meter. With cold solutions, the temperature dial was set at 10°C.

Assay of Protein Factor—The protein factor was estimated by determining the lowest concentration of test solution which caused appendix A1 cells to adhere to a glass surface and induced the attached cells to assume a flattened, epithelial-like shape. Unless otherwise indicated, the medium used in the assay procedure was the growth medium minus serum.

The cells were harvested from cultures approaching maximal growth by scraping the culture bottle with a rubber spatula. After two washes by centrifugation, they were incubated for 20 minutes (37°C) in growth medium minus serum containing 0.025 per cent trypsin, and were again twice washed. 1 ml. aliquots of a suspension of the trypsinized cells (1 to 2 X 10⁸ cells per ml.) were then placed in test tubes (16 X 125 mm., fitted with rubberlined, plastic, screw tops) which had previously received various amounts of the test solutions (up to 0.1 ml.). Control tubes containing only the cell suspension, and others with varying amounts of serum, were also prepared. When more purified protein fractions (chromatographic fraction, see “Results”) were tested, trypsin inhibitor (1 µg. per ml.) was added to the cell suspension immediately before dispensation. Whereas no differences were noted with the cruder fractions, with the more purified ones 2 to 4 fold increases in activity resulted from addition of the inhibitor. At the levels tested (1 to 20 µg. per ml.), trypsin inhibitor alone had no effect upon the cells.

The assay tubes were incubated at 37°C in an almost horizontal position (the liquid extended about 4 cm. from the bottom of the tube) and were examined after 14 to 16 hours. Adherence to the glass surface was routinely estimated by macroscopic visualization and occasionally by counting the attached cells after detachment with a rubber spatula. Flattening was visualized microscopically (Fig. 1) and, in general, was closely correlated with the ability of the cell population to adhere to the glass surface.

Estimation of protein factor activity was, for the most part, reproducible within the limits of error (about 30 per cent) of the
In occasional assays greater variations were found. Corrections, however, could be readily made using the serum controls since the variations invariably affected all the test fractions equally.

A unit of activity is defined as the smallest amount which induces attachment and flattening of about half the cells in the assay mixture, and specific activity is expressed as units per mg. of protein.

RESULTS

Purification of Protein Factor

Fractionation procedures were carried out at 0–3° unless otherwise indicated.

Precipitation with Ammonium Sulfate—To 960 ml. of beef serum 960 ml. of water followed by 420 gm. of ammonium sulfate were added with stirring. After 10 minutes, the precipitate was discarded by centrifugation and an additional 186 gm. of ammonium sulfate were added to the supernatant fluid. The precipitate, collected after 10 minutes, was dissolved in water to a final volume of 480 ml. and dialyzed against several changes of a solution of NaCl (0.05 M) and glycine buffer (0.005 M, pH 7.6) for 24 hours (ammonium sulfate fraction, Table I).

Precipitation with Ethanol Fraction I—To the dialyzed ammonium sulfate fraction 480 ml. of sodium acetate buffer (0.1 M, pH 5.0) were added with stirring and the small amount of insoluble material which formed was discarded by centrifugation. The clear supernatant fluid was immediately cooled to -1° in an alcohol-ice mixture, and 154 ml. of 95 per cent ethanol (-5°) were added at a rate which allowed the temperature to rise to 2°. The precipitate was obtained by centrifugation (in a room temperature kept at 3°) in precooled (-5°) Servall type SS-1 centrifuges and was dissolved in glycine buffer (0.05 M, pH 8.5) to a final volume of 480 ml. (ethanol fraction I, Table I).

Precipitation with Ethanol Fraction II—Following addition of 24 ml. of sodium acetate buffer (1 M, pH 6.0), the ethanol fraction I was adjusted to pH 6.2 (acetic acid or NaOH, each 1 M), and the solution was cooled to -1° in an alcohol-ice mixture. During an interval of about 20 minutes, 580 ml. of 25 per cent ethanol were added while the temperature was allowed to fall to -5°. After 5 minutes, the precipitate was discarded by centrifugation in Servall type SS-1 centrifuges kept at a room temperature of -5°. To the supernatant fluid was now added a volume (about 500 ml.) of the Reagent 14 of Cohn et al. (10) which was sufficient to lower the pH to 5.5. Collected by centrifugation after 15 minutes, the precipitate was dissolved to a final volume of 80 ml. in glycine buffer (0.05 M, pH 8.5) and adjusted to pH 7 (1 M NaOH) (ethanol fraction II, Table I).

Chromatography on Diethylaminoethyl-Cellulose—After dialysis (48 hours) against sodium phosphate buffer (0.01 M, pH 7.1), the ethanol fraction II was applied to a column of diethylaminoethyl-cellulose (Type 20, 57 gm. of adsorbent, height 14 cm., diameter 6.7 cm.) according to the procedure of Sober et al. (11). Inert protein was eluted (15 to 20 ml. per minute)

FIG. 1. Microscopic appearance of cells. The cells were prepared and incubated according to the standard assay procedure. Upper, no protein factor; center, 1 unit; lower, 2 units.
with 760 ml. of a solution of 0.025 M sodium phosphate (pH 7.1)-0.05 M sodium chloride, followed by 450 ml. of a solution containing an increased concentration of NaCl (0.075 M). Gradient elution was then established toward 0.025 M sodium phosphate (pH 7.1)-0.2 M sodium chloride with a 4 liter mixing chamber.

The growth factor, recognized by the standard assay procedure, did not appear as a discrete peak. Rather, it was eluted as a broad plateau between 2150 and 3550 ml. of eluent. Fractions with the highest specific activities were combined, dialyzed against water, and concentrated by lyophilization. The residue, dissolved in water and exhaustively dialyzed against sodium phosphate buffer (0.01 M, pH 7.1), had a final volume of 28 ml. (chromatographic fraction, Table I).

Ultracentrifugal Analysis—Examination of the chromatographic fraction in the analytical ultracentrifuge (Spinco model E) showed three major peaks. The fastest moving fraction (about 35 per cent of the total material as estimated by the area under the peak) sedimented at a rate consistent with a molecular weight of about 1 million, and it was inactive. Of the two remaining major components (about 60 per cent of the total material), the activity appeared to reside in the slower moving one. Thus, complete sedimentation of all but the slowest moving fraction provided a supernatant fluid containing 64 per cent of the recovered activity as estimated in the standard assay procedure (total recovery was 71 per cent), and the specific activities of the supernatant solution and sediment were 2100 and 705, respectively. On the other hand, when the slowest moving component (about 20 per cent of the total material) was, in addition, completely sedimented, 92 per cent of the recovered activity (total recovery was 87 per cent) was present in the sediment. The sedimentation constant of the active component is consistent with a molecular weight of 40,000 to 50,000.

Properties of Protein Factor

Nature of Protein—It is likely that the protein growth factor is a glycoprotein. Purification was accompanied by a marked enrichment of carbohydrate, and the ethanol fraction II and the chromatographic fraction (Table I) both appear to be comprised mainly, or entirely, of glycoprotein. Thus, the percentage content of hexose, hexosamine, and sialic acid of the chromatographic fraction was 5.3, 4.6, and 5.7, respectively. In addition, further purification by ultracentrifugation (see above) led to an increased sialic acid content, i.e. 6.3 per cent.

Effect of Heat and Proteolytic Enzymes—The heat stability of the factor appears to be related to the ionic strength of its solvent. Thus, with the protein (chromatographic fraction, Table I) dissolved in 0.15 M NaCl (pH 7), after heating at 60° (10 minutes), 70° (10 minutes) and 100° (5 minutes), the recoveries of activity were 50, 10, and less than 10 per cent, respectively. On the other hand, when the same fraction was dissolved in water (pH 7), less than 50 per cent of the activity was destroyed by heating at 100° for 10 minutes.

To test the effect of trypsin, 62 µg of protein (chromatographic fraction, Table I) were incubated for 60 minutes (37°, pH 7.2) with 3 µg of the crystalline enzyme. The assay, carried out in the presence of trypsin inhibitor (see "Materials and Methods"), revealed a complete loss of activity. No loss resulted from incubation in the absence of trypsin, nor was the trypsin-treated sample inhibitory. The effect of pepsin was tested in the same manner except that the pH of the incubation mixture was adjusted to 3.0, and after incubation, the proteolytic enzyme was destroyed by heating (80°, 10 minutes) at pH 7. The results were similar to those obtained with trypsin.

Other Cells—Several other cell cultures, heart (12), HeLa (13), lung E2 (14), and "altered" kidney (15), were tested to determine whether they would respond to the protein factor. Under the conditions of the assay, the epithelial-like cells, heart, HeLa, and lung behaved similarly to appendix Al except that few of the glass-attached lung cells became flattened (about 20 per cent), even with relatively large concentrations of the protein factor (8 units per ml). Contrariwise, the fibroblast-like "altered" kidney cells showed no protein requirement for attachment to a glass surface.

Cellulose Nitrate Surface—To determine whether the protein factor can induce attachment and flattening of cells on a surface other than glass, cellulose nitrate tubes were used. In an assay with 137,000 cells per tube, with no protein addition, and with 1 and 2 units of protein factor (chromatographic fraction, Table I), 8,000, 67,000, and 116,000 cells were attached to the plastic surface, respectively. As with a glass surface, the cells became flattened only in the presence of the factor.

Activity of Various Sera

Other sera tested in the standard assay, i.e. human, guinea pig, rabbit, rat, and fetal calf, also possessed protein factor activity. The levels of activity in several individuals of two of the serum sources, adult humans and fetal calves, were carefully measured and were found to be indistinguishable from the level in beef serum.

Furthermore, the solubility characteristics of the human serum activity were essentially the same as the bovine one. Thus, the specific activities of the human serum fractions compared to the bovine serum fractions, respectively, were as fol-

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume of solution</th>
<th>Total units</th>
<th>Total protein</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef serum</td>
<td>960</td>
<td>1,728,000</td>
<td>62,400</td>
<td>27.7</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>480</td>
<td>960,000</td>
<td>8,640</td>
<td>111</td>
</tr>
<tr>
<td>Ethanol I</td>
<td>480</td>
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<td>52</td>
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<td>1,440</td>
<td>901</td>
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<tr>
<td>Chromatographic</td>
<td>28</td>
<td>100,000</td>
<td>86.8</td>
<td>1132</td>
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</table>

4 To free it from contaminants from the cellulose column which might interfere with the estimation of hexose, an aliquot of the chromatographic fraction was dialyzed against sodium acetate buffer (0.01 M, pH 5), and the precipitate (containing all the protein factor) was washed with the same buffer solution. Diethylaminoethylcellulose (up to 10 mg.) did not react in the color tests for hexosamine and sialic acid.

4 The heart (normal monkey) and HeLa (human cervical carcinoma) cultures were generously provided by Dr. J. E. Salk; the lung (human fetal) culture was kindly supplied by Dr. T. T. Puck and Dr. S. J. Cieciura; and the kidney ("altered" monkey) culture was obtained through the kindness of Dr. L. Siminovitch and Dr. R. C. Parker.
TABLE II

Growth with the protein factor

The appendix A1 cells had been kept for 2 days in a medium containing protein factor (ethanol fraction II, 120 µg. per ml.) and peptone (0.1 ml. of a 5 per cent solution per ml.) in place of serum.* They were harvested by scraping with a rubber spatula and were washed once by centrifugation. 0.5 ml. aliquots of the cell suspension (50,000 cells) were placed in T-flasks containing 2.0 ml. of the synthetic growth medium supplemented with protein factor (chromatographic fraction, Table I), peptone (as above), crystalline bovine plasma albumin (2.5 mg.), and bovine serum as indicated. Of the supplements, only the peptone solution had been sterilized (autoclaved 15 minutes, 15 pounds pressure). Growth was estimated by counting with a Levy counting chamber after incubation for 110 hours at 37°.

<table>
<thead>
<tr>
<th>Protein factor</th>
<th>Serum</th>
<th>Peptone</th>
<th>Albumin</th>
<th>Number of cells†</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg. protein/ml.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12.4</td>
<td>+</td>
<td>+</td>
<td></td>
<td>55,000</td>
</tr>
<tr>
<td>2.5</td>
<td>-</td>
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<td>49,000</td>
</tr>
<tr>
<td>5.0</td>
<td>+</td>
<td>+</td>
<td></td>
<td>106,000</td>
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<td>7.4</td>
<td>+</td>
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<td>187,000</td>
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<tr>
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<tr>
<td>130</td>
<td>+</td>
<td>-</td>
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<td>55,000</td>
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<tr>
<td>260</td>
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<td>-</td>
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<td>520</td>
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<td>-</td>
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<td>780</td>
<td>+</td>
<td>-</td>
<td></td>
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</tr>
<tr>
<td>1300</td>
<td>-</td>
<td>-</td>
<td></td>
<td>80,000</td>
</tr>
<tr>
<td>2600</td>
<td>-</td>
<td>-</td>
<td></td>
<td>154,000</td>
</tr>
<tr>
<td>5200</td>
<td>-</td>
<td>-</td>
<td></td>
<td>256,000</td>
</tr>
</tbody>
</table>

* This procedure did not exercise any selection pressure. However, more consistent results, with serum as well as with other supplements, were obtained with cells treated in this manner.

† Cell numbers represent the total cells present, attached or floating in the medium. Nuclear counts made by a modification of the method of Sanford et al. (16) were in excellent agreement with the cell counts.

Additions

Mechanism of Action

Requirements for Activity—For attachment to a glass surface and flattening no requirement for added amino acids or vitamins was evidenced by the trypsinized appendix A1 cells. To study the effect of other components of the growth medium, a simple medium was prepared from which various components could be readily omitted. The simple medium contained only the inorganic salts, glucose, cysteine, and antibiotics of the growth medium.

Omission of inorganic phosphate from the simple medium, SO₄²⁻, or HCO₃⁻ (glycylglycine buffer was used), had no effect upon the response of the cells to the protein factor, but Mg²⁺ or Ca²⁺ was essential. In the absence of added glucose, the cells initially adhered to the glass surface but became detached after several hours.

Metabolic Activities—To determine whether the role played by the protein factor involves phosphate metabolism, cells were incubated (14 hours) with and without the factor in simple medium containing inorganic P⁳² and no added nonradioactive phosphate. Essentially all the cells attached to the glass surface, and they assumed an epithelioid form only in the presence of the factor. However, the washed cells of each group contained similar amounts of radioactivity, 45 per cent (without factor) and 34 per cent (with factor) of the added counts. Similar results were found with C⁴-labeled glucose.

No evidence could be obtained for the involvement of protein synthesis. Thus, after incubation (22 hours) in simple medium containing C⁴-L-aspartate, rounded, nonattached cells contained the same number of counts as the flattened, glass-attached ones, approximately 5 per cent of the added radioactivity.

The effect of three amino acid analogues, L-ethionine, DL-5-methyltryptophane, and DL-p-fluorphenylalanine on the attachment of cells in simple medium was studied as a function of time (Table III). As shown in the table, the initial attachment (1.5 hours) of the trypsinized cells was not prevented by the analogues. With two of them, p-fluorophenylalanine and 5-methyltryptophane, some of the cells later became detached.

Examination of the cells for flattening at 3.5 hours revealed only slight inhibition by two of the analogues and a greater, but not complete, inhibition by the third analogue, i.e. fluorophenylalanine. Comparison with the control cells at 18 hours yielded the following picture: essentially no effect with ethionine, a marked but far from complete inhibition by methyltryptophane, and an almost complete absence of flattening with the tyrosine analogue.

The authors are indebted to Dr. J. D. Myers and Dr. H. D. Davis for blood from hospital patients and for their diagnoses, and to Dr. W. J. Kuhns for human blood from normal subjects.
exposed to trypsin for a longer time (40 minutes) yielded identical
results except that the initiation of flattening was delayed, and at
3.5 hours p-fluorophenylalanine had not yet caused detachment
of any cells.

Preincubation—Following incubation (up to 90 minutes)
of trypsinized or nontrypsinized cells in growth medium con-
taining the protein factor, no loss of activity could be detected
in the supernatant fluid. The cells, removed from the preincubation
medium by centrifugation, were able to attach to a
glass surface only upon the addition of protein factor or the
supernatant preincubation fluid.

Other Compounds—A variety of serum proteins including
albumin, γ-globulin, and three glycoproteins* (18) from the
supernatant fluid of Fraction V of Cohn et al. (19) had no protein
factor activity. Low concentrations of two basic peptides,
salmine sulfate and poly-L-lysine (about 2 µg. per ml.) and
higher concentrations of elastase (20 µg. per ml., isoelectric
point = 9.5 (20)), however, induced glass attachment. In
addition, although the extent was less than with protein factor,
flattening of the attached cells occurred, particularly with
the synthetic compound. With regard to cell multiplication,
none of the compounds was effective.

Other compounds with basic groups were tested, L-lysine
(0.001 M), L-arginine (0.001 M), lyszyme (33 µg. per ml., isoelec-
tric point = 10.5 (21)), aminoquinidine (0.002 M), ethyl-
enediamine (0.001 M), and the various isomers of phenylenedi-
amine (0.001 M). None was active or inhibitory.

Time—Attachment to a glass surface as a function of time
was studied with the procedure of the standard assay except
that 0.5 ml. aliquots of cell suspension were used and the tubes
were incubated horizontally to provide a thin liquid layer.
After incubation (37°), the tubes were gently shaken, and un-
attached cells were discarded. In an experiment with 300,000
cells and 4 units of protein factor, the number of attached cells
after 5, 10 and 20 minutes was 4,000, 30,000 and 89,000, respec-
tively. The values represent the per cent of attached cells related to con-
trols containing protein factor and no amino acid analogue.

† Percentage of attached cells. In the absence of protein
factor, 2 to 3 per cent of the cells attached to the glass surface.

The appendix Al cell, like many or all animal cells in culture,
can be readily detached from a glass surface by exposure for
several minutes to low concentrations of either crystalline trypsin
or Versene (ethylenediaminetetraacetate). The activity of these
unrelated agents suggests that a surface protein and a poly-
valent cation are intimately related to the process of attach-
ment. From the phenomena induced by the protein factor,
adherence of cells to a glass surface and their assumption of
an epithelioid form, it would seem likely that the factor plays
an important role in the synthesis or maintenance of the surface
protein or is itself the surface protein.

Induction of the same phenomena by several basic peptides
* Kindly supplied by Dr. K. Schmid.

Table III

<table>
<thead>
<tr>
<th>Analogue</th>
<th>Hours of incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.5</td>
</tr>
<tr>
<td>L-Ethionine</td>
<td>100</td>
</tr>
<tr>
<td>DL-5-Methyltryptophane</td>
<td>100</td>
</tr>
<tr>
<td>DL-p-Fluorophenylalanine</td>
<td>100</td>
</tr>
</tbody>
</table>

* The simple medium contained only the inorganic salts, glu-
cose, cysteine, and antibiotics of the growth medium.

strongly suggests that attachment and flattening involve the
conversion of a net negatively charged cell surface to a posi-
tively charged one. With at least one of the basic peptides,
salmine, the rapidity of its action makes it appear likely that
the altered charge results simply from adsorption of the peptide
onto the cell surface, rather than by a more indirect mechanism.

DISCUSSION

The appendix Al cell, like many or all animal cells in culture,
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Induction of the same phenomena by several basic peptides
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centration in fetal calf serum (about 45 per cent of the serum protein (22, 23)), has protein factor activity. However, results that will be reported later by the present authors indicate that fetuin can be separated from the protein factor by chromatography on a diethylaminoethyl-cellulose column.

SUMMARY

1. A protein growth factor for a human cell in culture (appendix A1) has been purified about 40-fold from bovine serum. The assay used to follow the purification depends upon two marked effects of the growth factor: it causes adherence of the cells to a glass surface, and only in its presence do cells assume a flattened, epithelial-like shape.

REFERENCES

A Protein Growth Factor for Mammalian Cells in Culture
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