Proc. Nat. Acad. Sci. USA
Vol. 71, No. 9, 3372–3376, September 1974

Biological Activity of a Growth Factor for Ovarian Cells

(pituitary/purification/tumor)

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Communicated by J. Edwin Seegmiller, June 13, 1974

ABSTRACT **The biological activity of a protein growth factor is described. This factor was isolated from bovine pituitary tissue, and its activity was tested on cells of rat ovarian origin. Activity was assayed as growth-promoting potential measured by cell counts and is concentration-dependent. Similar growth stimulation was not produced by several cyclic nucleotides tested, neither was there crossreactivity between this growth factor and a chemically similar one produced by rat liver cells.**

The search for factors that stimulate animal cell growth *in vitro* has been primarily directed at extracting them from serum. Serum factors have been investigated in many cell systems for their effects on cell growth, confluent density, and DNA synthesis (1–4). In 3T3 cells, separate factors have been described for growth and survival (5).

A possible role of pituitary factors in growth *in vitro* was suggested by Holley and Kiernan when they observed that an impure thyrotropin (TSH) preparation stimulated 3T3 cells (6). Corvol *et al.* later demonstrated that partially purified bovine TSH and luteinizing hormone (LH) stimulated the proliferation of primary rabbit chondrocytes in culture (7). This group then documented a growth-stimulating effect of LH (NIH-LH-B7*) on a rat ovarian cell line (8). Armelin later described a similar response in 3T3 cells (9). The observation that more highly purified preparations of LH did not produce the same mitogenic effect (8) led to a search for a growth factor in fresh bovine pituitary tissue. This search has resulted in the isolation of a protein of 13,000 molecular weight, which stimulates rat ovarian cell growth (10).

This paper reports the growth-stimulating activity of fractions from various stages of the purification process. It demonstrates that the factor is more potent than NIH-LH-B7, improves plating efficiency, and does not affect the stimulation of DNA synthesis in chicken embryo fibroblasts produced by a chemically similar factor isolated by Dulak and Temin (11).

MATERIALS AND METHODS

Ovarian cells were from a line originally isolated from a benign ovarian tumor induced in the spleen of a Fisher rat (8). Chick embryo fibroblasts were prepared from 10-day-old chicken embryos by a slight modification of the procedure of Temin (12). A line of rat liver cells originally isolated by Coon (13) from Buffalo rats was provided by Dr. Jerry Schneider. Bovine insulin and bovine serum albumin were obtained from the Sigma Chemical Co. Tryptose phosphate broth was purchased from Difco. Dulbecco's modified Eagle's medium, horse serum, and fetal-calf serum were from Gibco. Calf serum was from Irvine Scientific. Temin's modified minimum essential medium (14) was prepared from component solutions from Gibco. N^6,O^2-dibutyryl adenosine 3′:5′-cyclic monophosphate (dibutyryl cAMP) and guanosine 3′:5′-cyclic monophosphate (cGMP) were from Sigma. 8-Bromo-cGMP was from International Chemical and Nuclear Corp. Cells were subcultured with a solution of Saline A containing 0.5 g/liter of trypsin and 0.2 g/liter of EDTA. Minimal medium for the ovarian cell experiments was made with Dulbecco's modified Eagle's medium plus 10% (v/v) fetal calf serum processed by affinity chromatography as described (15). Eagle–Temin medium was prepared with 80% minimal essential medium and 20% tryptose phosphate broth. Conditioned medium from rat liver cells was obtained by incubating confluent cultures on 100-mm plates with 10 ml of appropriate medium for 48 hr. The medium was removed, immediately filtered, and stored at −20°. The sources and preparation of bovine pituitary and liver fractions have been described (10). All pituitary fractions were weighed, dissolved in phosphate-buffered saline at 30–100 μg/ml, and stored in aliquots at −20°. They were thawed for use and kept at 4° for not longer than 14 days. Activity was stable under these conditions. Insulin was dissolved in 0.85% (w/v) saline at pH 3.0 and stored at 4°. Cell culture was done in Falcon tissue culture dishes from BioQuest and in Linbro Multi-Dishes. Antibiotics were not used. Bovine LH (NIH-LH-B7) was a gift from the Endocrine Study Section of the National Institute of Arthritis and Metabolic Disease. [^{3}H]Thymidine (20 Ci/mmole) was from New England Nuclear Corp.

Tertiary cultures of chick embryo fibroblasts were used for all studies. For DNA incorporation, the cells were plated at about 5×10^4 cells per cm^2 in Linbro 6-well dishes in Eagle–Temin medium with 1% (v/v) calf serum. After 24 hr, the medium was changed to serum-free medium. After another 24 hr, fresh serum-free medium with test substances was added. A 1-hr pulse with [^{3}H]thymidine (1 μCi/ml) was given 16–18 hr later. The cells were washed with phosphate-buffered saline, extracted with 10% trichloroacetic acid, and dissolved in 0.5 M KOH. DNA was precipitated with trichloroacetic acid, collected on glass fiber filters (Schleicher & Scheull), and counted in a liquid scintillation counter in Omnifluor (New England Nuclear Corp.) toluene. Growth experiments with chick fibroblasts were done in 60-mm tissue culture dishes.

Abbreviations: LH, luteinizing hormone; CMC, carboxymethylcellulose; TSH, thyrotropin.

* This designation will be used throughout, although NIH-LH-B7 and NIH-LH-B8 were used. Both were of equal activity.

The cells were plated at 1.8×10^4 cells per cm^2 per plate in Eagle–Temin medium without serum, and test substances were added. Additions were made every 2 days in 0.1 ml of phosphate-buffered saline. After the test period, the plates were washed with phosphate-buffered saline and the cells were removed with trypsin–EDTA and counted in a Coulter model ZBI cell counter.

Stock cultures of rat ovary cells were carried at subconfluence in Dulbecco's modified Eagle's medium with 12.5% horse serum and 2.5% fetal calf serum with 1 μg/ml of NIH-LH. For growth experiments, the cells were subcultured with trypsin–EDTA and plated at concentrations of from 30 to 300 cells per cm^2 in 60-mm dishes in 3 ml of minimal medium. Test substances were added at plating and every 2 days thereafter in 0.1 ml of phosphate-buffered saline. Samples were incubated in a humidified chamber (Wedco) at 37° in 5% CO_2. After test periods of 6–14 days, the cells were removed and counted as above.

Periodically, the ovarian cells underwent morphological changes with decreased growth differential. Fresh cells from earlier passages were then thawed, tested, and used.

Plating efficiency was studied by seeding the cells into 100-mm dishes in minimal medium at 50–100 cells per dish. Additions were made as above. Growth was followed for 7–14 days; the dishes were washed with phosphate-buffered saline, fixed with 10% buffered formalin, and stained with 0.1% crystal violet. Colonies visible to the naked eye were counted.

RESULTS

The cells used in these studies grow as cuboidal cells in closely packed monolayer colonies, as described (8). As mentioned above, with time and under various conditions, the cell line develops a population that appears more fibroblastic, has a less compact and circumscribed colony morphology, and shows less growth difference with growth stimulators.

Variation in cell growth and in responsiveness to growth-promoting fractions is noted with variation in serum concentration and with changes in the initial plating density (J. L. Clark and K. L. Jones, unpublished observations). Different lots of fetal-calf serum have different growth support potential. Lots chosen for experiments are poor supporters of cell growth and produce low plating efficiencies.

Pituitary fractions were prepared as described by Gospodarowicz *et al.* (10), and the same abbreviations were used. Most of the factors were tested at 1 μg/ml of final medium concentration. Concentration dependence can be demonstrated. The more active fractions frequently require testing at lower concentrations because of plateauing at or near the 1 μl/ml of range.

Table 1 lists the activities of several early fractions. Both plating efficiencies and final cell counts are listed. Cell counts were the most sensitive parameter, as plating efficiencies reflect only colony number and not colony size. Plating efficiencies and cell counts do yield reasonably parallel results. The fact that the major effect was not on plating was demonstrated by an experiment in which the ovarian cells were plated in minimal medium with various fractions. After 48 hr, the medium was changed to Dulbecco's modified Eagle's medium with 12.5% horse serum and 2.5% fetal calf serum plus 1 μg/ml of NIH-LH-B7. With this enriched medium there was no difference in the final number of colonies observed.

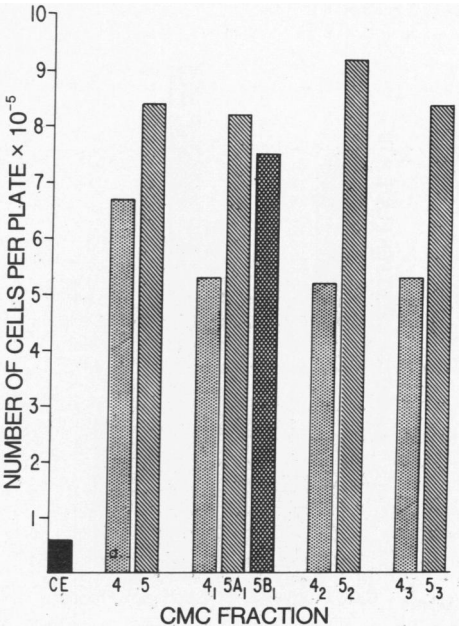

FIG. 1. The relative activities of CMC F_4 and CMC F_5 from four separate chromatography separations. All samples were added at final medium concentrations of 1 μg/ml. A comparison of crude extract at 10 μg/ml is included. $5A_1$ and $5B_1$ represent gradient elution rather than stepwise elution of the column (see text). CMC, carboxymethyl-cellulose.

Precipitation with 80% ammonium sulfate produced a significant increase in activity. In several preparations this precipitate showed a 3- to 6-fold increase over crude pituitary extract. Further purification was achieved with metaphosphate precipitation and carboxymethyl-cellulose chromatography (see Table 1). Subsequent preparations separated CMC F_4 into two fractions—F_4 and F_5—with cleaner gel electrophoresis patterns. F_5 was the more active fraction (Fig. 1). The third column shows an F_{5A} and F_{5B}. This pattern represents a gradient elution of F_5 to separate the more rapidly and more slowly eluting companions to the major protein band. Activity in both fractions negates a significant contribution to activity by these minor contaminants.

Final purification was achieved by chromatography on Sephadex G-50 (10). Fig. 2 shows the relative activities of the

TABLE 1. *Effect of pituitary fractions on rat ovarian cell growth*

Fraction	Cell count	Plating efficiency (%)
Crude extract	3.2×10^4	3.5
2.2 M $(NH_4)_2SO_4$ ppt	2.3×10^4	3.5
4.0 M $(NH_4)_2SO_4$ ppt	1.2×10^5	6.5
CMC F_1	6.3×10^4	6.0
CMC F_2	7.0×10^4	5.0
CMC F_3	9.6×10^4	6.0
CMC F_4	3.1×10^6	43.0

The effect of various pituitary fractions on the final cell count and on the plating efficiency of rat ovarian cells. The purification steps have been detailed (10). Cells were plated at 250 cells per cm^2 or as described for plating efficiency. Growth period was 10 days. CMC F_4 represents pooling of CMC F_4 and CMC F_5 of later preparations. CMC, carboxymethyl-cellulose.

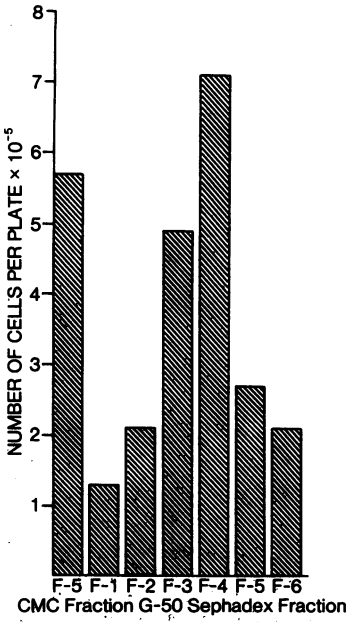

FIG. 2. The relative growth response to CMC F_5 and the Sephadex G-50 fractions. The cells were plated at 35 cells per cm^2 in minimum medium and allowed to grow for 7 days.

column input from F_5 of the carboxymethyl-cellulose column and the Sephadex G-50 fractions. The activity is clustered around the G-50 F_4 fraction, with maximum activity in that fraction.

Changes in concentration produce comparable increases in cell growth in most fractions. Fraction CMC F_5 shows little difference between the activity at 0.25 μg/ml and 1 μg/ml in many experiments, suggesting that plateauing with this material was occurring at about 0.25 μg/ml. Fig. 3 illustrates the relative concentration effects of CMC F_5 and NIH LH-B7. The former material shows a 10 to 15-fold greater activity over a range of concentrations. The pituitary fraction begins to show activity at just greater than 0.01 μg/ml and peaks at between 0.1 and 1.0 μg/ml. NIH-LH-B7 begins and ends at

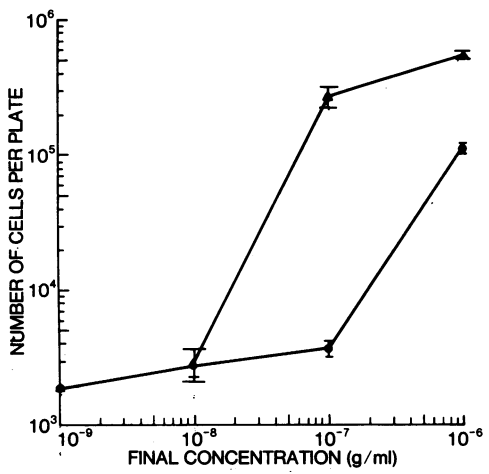

FIG. 3. Relative concentration effects of NIH-LH-B7 (●) and CMC F_5 (▲). The cells were plated in minimal medium at 300 cells per cm^2 and allowed to grow 13 days. Additions were made every 2 days, and cells were harvested and counted as described. Standard deviations are shown.

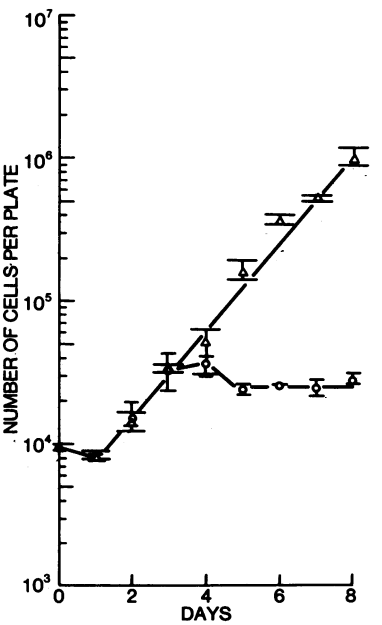

FIG. 4. Growth rate of rat ovarian cells in minimal medium (○) and in minimal medium plus 1.0 μg/ml of CMC F_5 (△). Cells were plated, harvested, and counted as described. There was no medium change, and additions were made every 2 days. Standard deviations are shown.

about one logarithm unit higher concentration. The maximum differential occurs at 0.1 μg/ml, with CMC F_5 producing a 70-fold greater effect than NIH-LH-B7.

The cells grow with a doubling time of about 24 hr in minimal medium. Fig. 4 traces a growth curve in minimal medium with and without 0.25 μg/ml of CMC F_5. The slopes are parallel through the first 3 days until the counts stabilize in the unsupplemented plates, while logarithmic growth continues with CMC F_5. Addition of growth factor to arrested plates produces a new growth spurt.

The relative contribution of the growth factor to growth and to survival is not clear at this time. In unsupplemented minimal medium, cells form colonies and then die. There are some cells that continue to grow and form healthy colonies. This growth and death is probably responsible for the apparently static phase in Fig. 4. It is unlikely that there is a

TABLE 2. *Effect of factors on DNA synthesis in chick embryo fibroblasts*

Additive	cpm	% Increase
Control	175 ± 21	—
Insulin, 1 μg/ml	1534 ± 218	780
CMC F_5, 1 μg/ml	388 ± 80	120
G-50 F_4, 1 μg/ml	208 ± 49	20
BRL, conditioned	2068 ± 174	1080
1% Calf serum	3163 ± 149	1710
Bovine serum albumin	346 ± 163	100

Effect of various factors on the incorporation of [^{3}H]thymidine into DNA. Cells were plated at 5 × 10^5 as described in *Materials and Methods*. Exposure to test medium was for 16 hr and incorporation for 1 hr with isotope concentration of 1 μCi/ml. BRL, Buffalo rat liver cells.

growth arrest similar to that shown by some fibroblasts after serum deprivation or with density-dependent inhibition.

Because of the reported importance of HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) to pituitary growth factors in 3T3 cells (9), several experiments were done with 15 mM HEPES buffer in minimal medium. This buffer did increase the final cell concentration in both test and control plates. There was no augmentation of the response to growth promoters. On several occasions the increased control growth in HEPES-containing medium decreased growth differentials.

As most known pituitary hormones are mediated by cAMP (16), various concentrations of dibutyryl cAMP were tested on the ovarian cells. Concentrations of 10^{-4}–10^{-3} M produced significant inhibition of growth. Lower concentrations had no positive or negative effects. cGMP and 8-bromo-cGMP were also tested at 10^{-3} M and lower concentrations with no growth stimulation.

In view of the chemical similarity of the pituitary factor to the multiplication stimulating activity isolated by Dulak and Temin (17), several fractions were tested on chick fibroblasts. Table 2 illustrates the stimulation of thymidine incorporation by various factors. Although there is some increase over control by CMC F_5, it is much less than known stimulators and about equal to bovine serum albumin. The additional purification on Sephadex produced a decrease per mg of protein rather than an increase in activity. The substances were also tested in a growth experiment with chick cells. The differences were not as marked, but still showed no stimulation of growth by the fractions which were active in rat ovarian cell experiments. In the reverse experiment, minimal medium was conditioned by a 48-hr exposure to both Buffalo rat liver cells and to ovarian cells. Neither conditioned medium produced a growth stimulation when compared to fresh medium.

DISCUSSIONS

This paper reports the growth response of a cell line of rat ovarian origin to a substance isolated from bovine pituitary glands. It was suspected from earlier work (8) and is now established (10) that the growth-stimulating substance present in bovine NIH-LH and NIH-TSH is not a known anterior pituitary hormone and does not cross-react in specific assays. Nor is it similar in its amino-acid composition (10) to bovine TSH, LH, or their subunits (17) or to active peptides of bovine growth hormone (18).

The growth factor, or substances with similar activity, is present in serum and must be removed from the serum supplement in order to demonstrate the growth response. Its mechanism of action is not yet clear. It has been suggested that the impure factor (NIH-LH) can drive 3T3 cells in G1 into S phase (9). Growth curve patterns and step-up plating efficiency experiments suggest that there is no major contribution by a role in attachment. Relative contributions to growth and/or survival are also not clear at this time.

Because of similarities to the factor described by Dulak and Temin (19), we looked for interchangeability in the rat ovary cell and chick fibroblast systems. It is apparent that ovarian growth factor has no effect in the chick system, and likewise, conditioning of medium with rat liver cells does not produce any growth stimulation of ovarian cells. We did not purify the multiplication stimulating activity, so that a definite conclusion cannot be drawn from that particular experiment. We do not know the effect of serum on the synthesis or release of the Temin factor.

It is interesting that dibutyryl cAMP inhibits growth in this system as it has in most other systems tested (20–22). This argues that ovarian growth factor does not act through adenyl cyclase activation as do most anterior pituitary tropic hormones. The identification of ovarian growth factor and the previous work demonstrating adrenal growth inhibition by adrenocorticotropic hormone (23) calls into question the role of tropic hormones in target cell growth. Direct extrapolation of work *in vitro* to the whole animal is impossible, and ovarian growth factor will have to be tested in animals before definite information about a physiologic role can be gained.

Physiologic significance is further clouded by the suggested lack of cell specificity. The work by Armelin (9) and Corvol *et al.* (7) suggests that this factor may produce cell division in 3T3 cells and rabbit chondrocytes. It may also be that the pituitary contains a family of growth-promoting peptides, similar to those described by Dulak and Temin (19) from rat liver cells. These may have different cell specificities.

This description of ovarian growth factor further increases the list of peptides with growth-promoting activity. These include insulin (14, 24), epidermal growth factor (25, 26), nerve growth factor (27), chick fibroblast growth factor, and somatomedin A and B described by Uthne (28).

We acknowledge the excellent technical assistance of Ms. J. Addison and Mr. P. Curtis. The support of grants from the National Institutes of Health (HD 07651 and GM 17702), from the American Cancer Society (IN 93CO), and from the Rockefeller Foundation is most gratefully acknowledged.

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