Purification of a Growth Factor for Ovarian Cells from Bovine Pituitary Glands (pituitary hormones/ovarian tumor/growth control)

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ABSTRACT The purification of a growth factor from bovine pituitary glands that controls the division of an ovarian cell line maintained in tissue culture is reported. Extraction of bovine pituitary glands at pH 4.5 followed by ammonium sulfate precipitation, chromatography on carboxymethylcellulose, and gel filtration on Sephadex G-50 gives yields of ovarian growth factor of about 7 mg/kg of glands. Contamination by other pituitary hormones was not detected. The preparation obtained has been characterized with respect to biological activity, molecular weight, and amino-acid composition. The implication of this growth factor in the development of ovarian tumors is discussed.

We have reported the isolation of a rat ovarian cell line whose growth *in vitro* is markedly stimulated by partially purified bovine luteinizing hormone (LH, preparation NIH-LH-B7) when the cells are maintained in medium devoid of LH (1, 2). We have shown that NIH-LH-B7 obtained from the Endocrine Study Section, National Institute of Arthritis and Metabolic Diseases, could replace the growth factor(s) removed from serum by immunoadsorption (2). It was not clear that the growth stimulation was due to NIH-LH, since the concentrations required were much higher than those observed *in vivo* and since pure ovine LH prepared as described by Gospodarowicz (3) had no growth stimulating effect. We concluded that the mitogen was a factor in NIH-LH-B7 other than LH.

We are reporting the isolation from bovine pituitary glands of an ovarian cell growth factor (OGF) and presenting evidence which allows us to conclude that it is distinct from known pituitary hormones.

MATERIALS AND METHODS

Materials. Bovine pituitary glands were obtained from Endocrine Research Supply (San Mateo, Calif.). Carboxymethyl-cellulose (CM-cellulose) was from BioRad (Richmond, Calif.), and Sephadex G-50 was obtained from Pharmacia (Piscataway, N.J.).

Assays. Bioassays based on the ability of the different fractions to stimulate the growth of an ovarian cell line will be described elsewhere (4). Bovine NIH-LH-B7 has been taken as reference standard. Follicle stimulating hormone (FSH) activity was determined by the Steelman and Pohley

assay (5), thyroid stimulating hormone (TSH) activity by the McKenzie assay (6), adrenocorticotropin activity by the Saffran and Schally assay (7), melanocyte stimulating activity by the *in vitro* frog skin assay (8), lipolytic activity by the release *in vitro* of free fatty acids from isolated white adipocytes from mice (9), and the LH activity by the ovarian ascorbic-acid depletion test (10).

Dialysis. All dialyses were done against distilled water at 4°. To avoid losing low-molecular-weight peptides, Spectrapor dialysis tubing (Spectrum Med. Ind., Los Angeles, Calif.) with a molecular weight cutoff of 6000-8000 was used.

CM-Cellulose Ion Exchange Chromatography. A column of CM-cellulose (3 × 20 cm) was prepared and equilibrated with 0.01 M sodium phosphate, pH 6.0, at 5°. Proteins adsorbed to the CM-cellulose were eluted by increasing the buffer concentration stepwise from 0.01 M sodium phosphate to 0.1 M sodium phosphate and then from 0.1 M sodium phosphate, 0.1 M NaCl to 0.1 M sodium phosphate, 1 M NaCl.

Sephadex G-50 Chromatography. A 1.5 × 90-cm column of Sephadex G-50 was prepared and equilibrated with 0.05 M sodium phosphate and 0.1 M NaCl at pH 6.0. The column was packed at 40-cm head pressure and operated at 5-cm head pressure with a flow rate of 13.5 ml/hr.

Amino-Acid Analysis. Samples were hydrolyzed in 6 N HCl in evacuated sealed tubes at 110° for 24, 48, or 72 hr. Hydrolysis was also done using 3 N *p*-toluenesulfonic acid containing 0.2% aminoethylindole instead of 6 N HCl to determine the tryptophan content. Analysis was performed with a Spinco 119 amino-acid analyzer by the method of Spackman *et al.* (11).

Carbohydrate Analysis. The glucosamine and galactosamine contents were determined by chromatographic separation and ninhydrin reaction utilizing the standard resins in the Spinco 119 amino-acid analyzer. Hydrolysis was in 6 N HCl at 110° for 4 hr. Determination of the hexosamine content was also carried out by the method of Gatt and Bermann (12) galactosamine · HCl as a standard.

Disc Gel Electrophoresis. Disc gel electrophoresis was performed at pH 8.5 as described by Davis (13) and at pH 4.5 as described by Reisfeld *et al.* (14). Twelve percent acrylamide gels were run at pH 8.5 and 10% gels at pH 4.5.

Determination of Molecular Weight. Molecular weight was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described by Weber and Osborn

Abbreviations: LH, luteinizing hormone; OGF, ovarian cell growth factor; FSH, follicle stimulating hormone; TSH, thyroid stimulating hormone; CM-cellulose, carboxymethylcellulose; SDS, sodium dodecyl sulfate.

FIG. 1. Scheme of the initial steps of purification of the ovarian growth factor (OGF). The tissue culture plates represent the ovarian cells after 7 days in culture in presence of the different fractions. Bioassay was done as described in (4). At the end of the 7-day period, the cells are fixed with formalin and stained with 0.1% crystal violet. *ADJ* indicates adjusted. (A) Control; (B) 1.0 $\mu\text{g}/\text{ml}$ of crude extract; (C) 1.0 $\mu\text{g}/\text{ml}$ of HPO_3 precipitate (*Ppt*); (D) 1.0 $\mu\text{g}/\text{ml}$ of pH 7.0 supernatant; (E) 1.0 $\mu\text{g}/\text{ml}$ of 2.2 M $(\text{NH}_4)_2\text{SO}_4$ precipitate; (F) 1.0 $\mu\text{g}/\text{ml}$ of 4.9 M $(\text{NH}_4)_2\text{SO}_4$ precipitate (OGF-A); (G) 1.0 $\mu\text{g}/\text{ml}$ OGF-B; (H) 1.0 $\mu\text{g}/\text{ml}$ of the supernatant of the 0.1 M HPO_3 , pH 3.5, precipitate; (I) NIH-LH-B7, 1.0 $\mu\text{g}/\text{ml}$.

(15) and modified by Gospodarowicz (16) using 15% acrylamide gels. Reduction and alkylation of the samples has been described elsewhere (16).

RESULTS

Purification of the OGF. Unless otherwise indicated, all operations were performed at 0–5°. Two kilograms of frozen bovine pituitaries were homogenized in a Waring Blender in the presence of 4 liters of 0.15 M $(\text{NH}_4)_2\text{SO}_4$. The suspension was stirred for 2 hr. Centrifugation at 9000 rpm (Sorvall RC2B centrifuge with GSA rotor) for 20 min removed the residue. The supernatant was then adjusted to pH 3.0 by the addition of freshly prepared 0.5 M HPO_3 , resulting in the formation of an inactive, highly pigmented precipitate. This precipitate was removed by centrifugation and the supernatant was adjusted to a pH of 6.5–7.0 by the addition of 1 N NaOH. Addition of $(\text{NH}_4)_2\text{SO}_4$ (290 g/liter) produced a precipitate which was collected by centrifugation and which contained most of the LH. Little OGF activity was present in this fraction. More $(\text{NH}_4)_2\text{SO}_4$ (250 g/liter) was added to the supernatant, precipitating OGF activity. This precipitate was collected by centrifugation and the supernatant discarded. The precipitate was dissolved in cold distilled water and dialyzed against water for at least 24 hr before

TABLE 1. Recovery of ovarian growth factor from 1 kg of frozen bovine pituitary glands

Fractions	Quantity, mg	Relative activity*	Units
Crude extract	23,700	0.05	1185
pH 3.0, HPO_3 ppt	8,800	ND	—
pH 7.0 sup	15,000	0.07	1050
2.2 M $(\text{NH}_4)_2\text{SO}_4$ ppt	3,200	ND	—
OGF-A	3,700	0.3	1110
OGF-B	2,100	0.4	840
CM-cellulose F_1	770	ND	—
CM-cellulose F_2	553	ND	—
CM-cellulose F_3	75	0.2	15
CM-cellulose F_4	35	1	35
CM-cellulose F_5	25	5–10	75–250
Sephadex G-50 F_4	7	20	140

The procedure used to obtain each fraction is described under *Results*. The ovarian growth factor activity is assayed at two dose levels as described by Jones *et al.* (4) using as a standard NIH-LH-B7. The protein yields and total units recovered are based on 1 kg of frozen bovine pituitary glands as starting material. Supernatant is abbreviated *sup*; precipitate, *ppt*. ND is not detectable.

* Expressed in terms of NIH-LH-B7.

being lyophilized. The yield at this stage was 3.7 g of protein per kg of pituitary tissue (OGF-A). A scheme of these preliminary steps as well as the growth observed with the fractions is shown in Fig. 1.

Further purification was achieved by dissolving OGF-A to a final concentration of 10 mg of protein per ml in distilled water and adjusting to a pH of 3.5 with freshly prepared 0.1 M HPO_3 . The precipitate formed (2.1 g/kg) contained the bulk of the OGF activity and was removed by centrifugation (OGF-B). It was redissolved in 0.005 M sodium phosphate (pH 6.0), adjusted to pH 6.0 with 1 N NaOH and dialyzed for 18 hr with frequent changes against 0.005 M sodium phosphate at pH 6.0. When the ionic strength as well as the pH of the dialysate reached that of the dialysis buffer, the resulting clear red solution (approximately 200 ml) was chromatographed on CM-cellulose as described above. Fraction F_1 (Table 1), which contained most of the protein, is inactive. Elution with 0.05 M and 0.1 M sodium phosphate buffer at pH 6.0 removed two other inactive fractions (F_2 , F_3). Elution with 0.1 M sodium phosphate with 0.1 M NaCl removed a fraction with low OGF activity (F_4). Most of the OGF activity was eluted with 0.1 M sodium phosphate with 1 M NaCl (F_5) at pH 6.0. The yield was 25 mg of protein per kg of pituitaries.

Polyacrylamide gel electrophoresis of the different fractions was done at pH 8.5 and at pH 4.5. Fraction 5 looked homogeneous at pH 4.5, but at higher protein concentrations (Fig. 2A) heterogeneity was evident. At pH 8.5, the proteins composing F_5 did not run, and no bands could be detected. This observation confirmed the high isoelectric point of the proteins composing F_5 , a fact already suspected from their elution pattern on CM-cellulose.

Final purification of OGF was achieved by chromatography on Sephadex G-50 as described above. A typical pattern is shown in Fig. 2. Biological activity was present in a zone corresponding to a molecular weight lower than that of the

FIG. 2. Elution pattern of the fractions F_4 and F_5 obtained from carboxymethylcellulose (CMC) chromatography when applied on Sephadex G-50. (A) 80 mg of fraction F_5 is dissolved in 1.2 ml of 0.05 M sodium phosphate, pH 6.9, -0.1 M NaCl and applied on a column (1.5 $\times$ 90 cm) of Sephadex G-50 equilibrated in the same buffer. Fractions (2.5 ml) are collected. In the insert are shown the polyacrylamide patterns at pH 4.5 of the different fractions. From left to right: Input (500 μ g), F_1 (500 μ g), F_2 (500 μ g), F_3 (500 μ g), F_4 (500 μ g), F_5 (500 μ g), input (500 μ g). (B) 50 mg of fraction F_5 is applied under identical conditions to those of F_5 . In the insert are shown the polyacrylamide patterns at pH 4.5 of the different fractions. From left to right: F_1 (500 μ g), F_2 (500 μ g), F_3 (250 μ g). (C) Rerun on Sephadex G-50 of 20 mg of the fraction F_4 obtained when F_5 CMC is chromatographed on Sephadex G-50 (A). In the insert are shown the polyacrylamide patterns at pH 4.5. From left to right: F_4 (250 μ g), F_4 (600 μ g). BD and CYTO. C indicate the elution of blue dextran and cytochrome c, respectively.

cytochrome c marker, 12,400 (Fig. 2A). This zone was not present when F_4 , which contained low OGF activity, was chromatographed under the same conditions (Fig. 2B). When the zone containing OGF activity was rerun under the same conditions a major peak was found. This was dialyzed and lyophilized to obtain the final product. It has been observed that, when the OGF is stored during long periods of time, the electrophoretic pattern of the purified OGF shows minor alterations. A second band which runs more slowly than OGF appears when OGF is submitted to electrophoresis at pH 4.5 (Fig. 2C). This corresponds to a band which has a lower molecular weight than the OGF when submitted to electrophoresis in the presence of SDS (Fig. 3). One may interpret that band as a contaminant, in which case the purity of the OGF based on acrylamide pattern will be on the order of 90%. But since this band appears more pronounced

FIG. 3. Tubes 1 to 4: SDS-polyacrylamide gel electrophoresis of ovarian growth factors and different marker proteins of known molecular weight. (1) LH (20 μ g), LH_α , LH_β , cytochrome c (Cyto C) (10 μ g). The second band from the top is a contaminant present in the cytochrome c. The LH_α and LH_β are the results of the dissociation of LH into its subunits in the presence of SDS (16). The apparent molecular weights of the different proteins on SDS-polyacrylamide gel electrophoresis are: LH (34350), LH_α (18750), LH_β (16000), cytochrome c (12700) (16). (2) OGF (10 μ g); (3) OGF (20 μ g); (4) LH (20 μ g), LH_α , LH_β , cytochrome c (5 μ g). Electrophoresis was for 6 hr at 6 mA per tube. Staining was with Coomassie brilliant blue. Tubes 5 and 6: polyacrylamide gel electrophoresis at pH 4.5 of the ovarian growth factor. (5) OGF (250 μ g); (6) OGF (600 μ g). Tubes 7-9: comparison of the electrophoretic pattern at pH 4.5 of OGF with that of NIH-LH-B7. (7) NIH-LH-B7 (1 mg); (8) OGF (300 μ g); (9) NIH-LH-B7 (1 mg). LH_α and LH_β in NIH-LH-B7 have been identified on the basis of previous electrophoresis (16). Other bands are contaminants. For tubes 5-9 electrophoresis was 75 min at 9 mA per tube. The gels were stained with 1% Amido Schwartz in 7% acetic acid solution.

after storage of the OGF in the dry state, it may also be a degradation product of OGF, as suggested by its lower molecular weight. The final yield of ovarian growth factor averaged 7 mg/kg of glands. Table 1 shows the biological activity of several intermediate fractions in the purification.

Properties of the OGF. The purified ovarian growth factor migrates as a single major component on polyacrylamide gels both at pH 4.5 (Fig. 3) and when electrophoresed in the presence of SDS (Fig. 3). The mobility of the OGF in SDS-polyacrylamide gels relative to other standard proteins suggests a molecular weight of 13,400 and is in direct contrast with the apparent molecular weight observed by gel filtration on Sephadex G-50, which suggests a molecular weight of less than 12,400 (Fig. 2). Since the OGF is a basic peptide and since Sephadex contains some carboxyl groups, it is likely that OGF chromatographs by ion exchange as well as molecular sieving on Sephadex G-50 at pH 6.9. The amino-acid composition of OGF (Table 2) shows a marked difference when compared to that of other growth factors, such as epithelial growth factor (17) or nerve growth factor (18). The basicity of the OGF is reflected in its high content of lysine and arginine. No glucosamine or galactosamine could be detected. Bioassay of different hormone activities (Table 3) shows that it is free of contamination by other known pituitary hormones. Since OGF activity was first observed with NIH-TSH and NIH-LH preparations (1, 4), the absence of these activities in the final OGF preparation is of particular interest. This is further confirmed by comparing the electrophoretic pattern of bovine NIH-LH-B7 and of OGF on polyacrylamide gel at pH 4.5 (Fig. 3). OGF migrates faster than LH

TABLE 2. Amino-acid composition of ovarian growth factor

Amino acid (or sugar)	nmoles of amino acid*
Lysine	8
Histidine	1
Arginine	10
Aspartic acid	7.9
Threonine	4
Serine	2.6
Glutamic acid	5.7
Proline	10.4
Glycine	9.5
Alanine	8.4
Valine	2.6
Methionine	1.5
Isoleucine	1.5
Leucine	3.5
Tyrosine	5.3
Phenylalanine	5.6
Cystine	4.8
Tryptophan†	ND
Glucosamine	ND
Galactosamine	ND

Amino-acid determinations were performed as described under *Methods*. Corrections are made for destruction or incomplete hydrolysis. ND is not detectable.

* nmoles of amino acid per mole of OGF (molecular weight assumed to be 13,400).

† *p*-toluenesulfonic acid hydrolysis.

and its α or β subunits. A weak band representing one of the contaminants of NIH-LH can be seen to correspond to OGF. It is probably that component which is responsible for the stimulation of ovarian cell division by NIH-LH-B7.

DISCUSSION

Intrasplenic transplantation of ovarian fragments results in the development of ovarian tumors (19, 20). It was originally suggested (19, 20) that the development of such tumors is induced by elevated levels of LH and FSH.

We have noted that neither highly purified LH (1) nor FSH (K. Jones and D. Gospodarowicz, unpublished observation) triggers the growth of an ovarian cell line obtained from these intrasplenic ovarian tumors. This observation stands in contrast with the stimulation observed with impure preparations of LH or TSH. The present data demonstrate that a mitogenic factor different from LH or FSH can be purified from pituitary glands and allows us to conclude that the ovarian tumors obtained by the method of Biskind and Biskind (19, 20) probably are not provoked by a hypersecretion of gonadotropins but by a hypersecretion of OGF.

It seems unlikely that LH and FSH can be held responsible for the stimulation of division of ovarian cells, since they are known to increase the intracellular concentration of 3':5' cyclic AMP (21, 22) and since increased cyclic AMP has been associated with inhibition of cellular division (23-28). When bovine luteal cells are maintained in tissue culture (29) in the presence of LH or high concentrations of cyclic AMP, a decrease or arrest in the rate of cell division is observed (30).

It would seem, from our data, that pituitary glands may secrete growth factors which are responsible for the control

TABLE 3. Maximal contamination of the purified OGF by other pituitary hormones

	Activity*, %
Follicle stimulating hormone	<0.05
Luteinizing hormone	<0.05
Adrenocorticotropin	<0.1
Melanocyte stimulating hormone	<0.1
Thyrotropin	<0.1
Lipolytic activity†	<0.1

* Results are expressed to show the maximum concentration of different biological activity found in the OGF. In no case did the degree of contamination by other pituitary hormones exceed 0.1%. Biological assays were conducted as described under *Methods*.

† Lipolytic activity is expressed in terms of that of purified TSH.

of target cell division. Other evidence is available. Holley and Kiernan (31) have detected growth activity for 3T3 cells in a commercial thyroid stimulating hormone preparation. Armelin found similar activity in bovine NIH-LH and TSH preparations (32). Corvol *et al.* (33) have reported that NIH-LH and TSH preparations exert a strong mitogenic action on articular chondrocytes, and Van Wyk (personal communication) has observed that among crude extracts of different organs, only that of pituitary glands has higher sulfation and mitogenic activities than serum.

To establish that OGF present in the pituitary does not merely reflect factors present in the blood supply, we have extracted bovine liver in parallel using the same method. The liver was chosen for two reasons: (1) It has been described as the site of synthesis of somatomedin, a potent mitogenic agent, and (2) its blood supply is richer than that of the pituitary. The different liver fractions thus obtained have less activity in stimulating ovarian cell division. OGF also lacks sulfation and mitogenic activity when tested on rat cartilage (Van Wyk, personal communication).

When the ovarian growth factor is compared to somatomedin (34, 35) or a growth factor acting on fibroblasts (36), a number of similarities are found to exist. All three proteins are characterized by a high isoelectric point as seen from their behavior on ion exchange chromatography and on acrylamide gel electrophoresis. When chromatographed on Sephadex G-50 or G-75 they elute in a position which suggests a molecular weight of 8000. In the case of OGF this may reflect retardation on the column due to secondary, ion exchange effects. The same may also apply to the fibroblast growth factor of Dulak and Temin (36).

It should also be pointed out that while we have purified a growth factor with OGF activity, it is by no means the only one present in the pituitary gland, as evidence from our data as well as that of others (28-30). We have been able to detect growth activity for glial cells in the pituitary (Gospodarowicz, unpublished observation) and have identified a fibroblast growth factor (37). Also, it should be kept in mind that since pituitary hormones can present strong homologies, the same could also be true for growth factors present in the pituitary. Only when those different activities are purified will it be possible to compare their structure to that of other known pituitary hormones to see what relationship, if any, does exist.

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