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Isolation and partial molecular characterization of pituitary fibroblast growth factor*

(mesodermal growth factor/microsequencing/vascular endothelial cell proliferation/radioimmunoassay/immunoneutralization)

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ABSTRACT **Fibroblast growth factor (FGF) has been purified to homogeneity from bovine pituitaries by two methods. Starting material for both methods was an FGF preparation partially purified as described by Gospodarowicz [Gospodarowicz, D. (1975) *J. Biol. Chem.* 250, 2515–2520]. Purification procedure I involved cation-exchange and reversed-phase HPLC, while procedure II employed gel filtration and ion-exchange chromatography. Isolation was monitored by testing column fractions for their capacity to stimulate the proliferation of vascular endothelial cells *in vitro*. The growth factor has an approximate molecular weight of 16,000. Its amino-terminal sequence was determined as Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly. Sequence and amino acid composition indicate that the structure of pituitary FGF is different from that of other known growth factors. Pituitary FGF, as isolated under nonacidic conditions (procedure II), has high potency and intrinsic activity to stimulate adult bovine aortic endothelial cells (half-maximal proliferation at 2 pM). Acidic conditions as in procedure I, however, lead to about 90% loss of potency while the intrinsic activity remains intact (identical maximal stimulation values). By all other criteria (molecular weight, amino acid composition, amino-terminal sequence), the two preparations are indistinguishable. Antibodies were raised in rabbits against a synthetic peptide representing the first nine residues of the amino-terminal sequence of the pituitary FGF. The polyclonal antibodies recognize the synthetic peptide and the purified growth factor on an equimolar basis and are capable of inhibiting mitogenic activity *in vitro*. This report describes a partial chemical characterization of a pituitary FGF and demonstrates rigorously that the characterized protein possesses the mitogenic activity commonly referred to as "basic pituitary FGF."**

The pituitary gland has been known to contain mitogenic factors for cultured cells (reviewed in ref. 1). Gospodarowicz (2) substantially purified a mitogen for fibroblasts (thus the term "fibroblast growth factor," FGF), which was subsequently found to stimulate not only the proliferation of fibroblasts but also a variety of normal diploid mesoderm-derived and neural crest-derived cells (3). This pituitary FGF has been shown to be a basic protein (pI 9.6) with an estimated molecular weight of 14,000 (2, 4). Other pituitary-derived growth factors for mesoderm-derived cells have also been described (5–11). It is not known whether any of these growth factors are structurally related because all comparative studies have been restricted to the use of partially purified mitogens. We report here the purification to homogeneity and preliminary molecular characterization of what can be considered the major pituitary FGF in terms of potency and intrinsic activity and also amounts present in the pituitary gland.

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MATERIALS AND METHODS

Preparation of Partially Purified FGF. Frozen bovine pituitaries were obtained from J. R. Scientific (Woodland, CA). Tissue was stored at −80°C and used within a period of 2 weeks. Partially purified FGF was prepared as previously described (2, 12), with minor modifications. Briefly, tissue was homogenized in 0.15 M ammonium sulfate, pH 4.5. Protein was precipitated with ammonium sulfate, the biologically active precipitate was applied to a column of carboxymethyl-Sephadex, and the activity was batch-eluted with 0.5 M NaCl/0.1 M sodium phosphate, pH 6.0. From this material, a partially purified FGF preparation was obtained by gel filtration on Sephadex G-75.

Isolation Procedure I (Cation-Exchange/Reversed-Phase HPLC). The partially purified FGF was subjected to cation-exchange HPLC and two steps of reversed-phase HPLC. Details are contained in the legend to Fig. 1.

Isolation Procedure II (Gel Filtration/Cation-Exchange Chromatography). The partially purified FGF preparation was further fractionated by gel filtration and two steps of cation-exchange chromatography on a Mono S column (Pharmacia). Bioactive material was checked for purity by reversed-phase HPLC. Details are given in the legends to Figs. 2 and 3. Protein was determined by the dye-fixation method (13), using bovine serum albumin as standard, or by amino acid analysis.

Structural Characterization. Molecular weight determination was performed by sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis. Aliquots containing 0.25–0.5 μg of protein were added to the sample buffer composed of 15% (vol/vol) glycerol, 0.1 M dithiothreitol, 2% $NaDodSO_4$, 75 mM Tris·HCl at pH 6.8, 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 1 mM *N*-ethylmaleimide, and 1 mM iodoacetic acid. Samples were boiled for 3 min and then applied to an exponential gradient (10–18%) polyacrylamide slab gel with a 3% stacking gel (14, 15). Gels were fixed and stained using the Bio-Rad silver nitrate staining kit according to the manufacturer's instructions.

Amino acid analyses were performed on a Liquimat III analyzer (Kontron, Zurich, Switzerland), using previously described micromethodology (16).

Amino acid sequence analysis of the unmodified HPLC-purified growth factor was performed with an Applied Biosystems gas/liquid phase microsequenator (17). Phenylthiohydantoin derivatives of amino acids were analyzed by HPLC (18). Protein obtained by procedure I (in HPLC mobile phase) was applied directly to the sequenator. Material from procedure II was dialyzed (1 nmol/ml) against water for 48 hr at 4°C in Spectrapor tubing (molecular weight cut-off at 3500; Spectrum Medical Industries, Los Angeles).

Abbreviation: FGF, fibroblast growth factor.

*Part of this work has been presented at the 37th M. D. Anderson Hospital Symposium on Fundamental Cancer Research, March 6–9, 1984, Houston, TX (32).

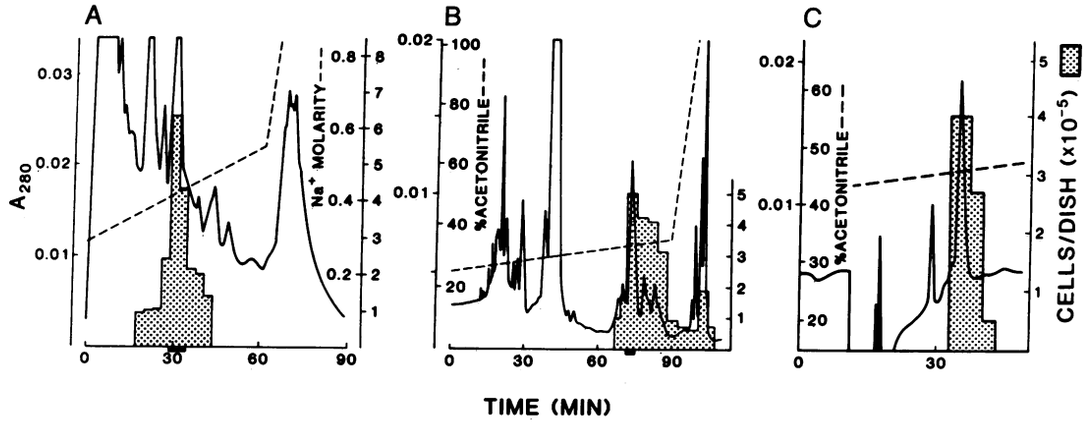

FIG. 1. (*A*) Cation-exchange chromatography on a Mono S column (5 × 0.6 cm; Pharmacia). Partially purified FGF (15 mg, equivalent to 350–750 g of pituitary tissue) was dissolved in 1 ml of 50 mM sodium phosphate, pH 6.7, and injected into the column equilibrated with the same buffer. The column was eluted at room temperature with a 60-min gradient of 0.3–0.55 M NaCl in 50 mM sodium phosphate, pH 6.7. Flow rate was 1 ml/min; 3-ml fractions were collected. Bioactive fractions (horizontal bar) were pooled and subjected to further chromatography. The Mono S column was regenerated by washing with 1 M NaCl/50 mM sodium phosphate, pH 6.7. (*B*) Reversed-phase HPLC on a C_3 column (7.5 × 0.46 cm, 5-μm particle size, 300-Å pore size, Altex, Berkeley, CA). Bioactive material from two Mono S columns was loaded by pumping the sample solution through the column, which had been equilibrated with 0.1% (vol/vol) trifluoroacetic acid. Elution was with a 90-min gradient from 23% to 33% (vol/vol) acetonitrile in 0.1% trifluoroacetic acid at room temperature at a flow rate of 0.6 ml/min. Fractions (1.8 ml) were collected. The fraction marked with a horizontal bar was used for final purification. After a run the column was washed with 99.9% acetonitrile/0.1% trifluoroacetic acid. (*C*) Reversed-phase HPLC on a C_8 column (Aquapore RP-300, 25 × 0.46 cm, 7-μm particle size, 300-Å pore size, Brownlee, Santa Clara, CA). The bioactive fraction from the C_3 column was diluted 1:1 with 0.2% (vol/vol) heptafluorobutyric acid and loaded by pumping the sample solution through the column, which had been equilibrated with 0.2% heptafluorobutyric acid. Chromatography was performed at room temperature with a 90-min gradient from 42% to 52% acetonitrile in 0.2% heptafluorobutyric acid at a flow rate of 1 ml/min. After a run, the column was washed with 99.8% acetonitrile/0.2% heptafluorobutyric acid.

Bioassay. Growth factor purification was monitored by testing chromatography fractions for their ability to stimulate the proliferation of adult bovine aortic arch endothelial (BAAE) cells (19, 20). Briefly, stock cultures, maintained in the presence of Dulbecco's modified Eagle's medium H-16 (GIBCO) supplemented with 10% calf serum (HyClone, Logan, UT) and antibiotics [gentamycin (Schering) at 50 μg/ml and Fungizone (Squibb) at 0.25 μg/ml] and a partially purified

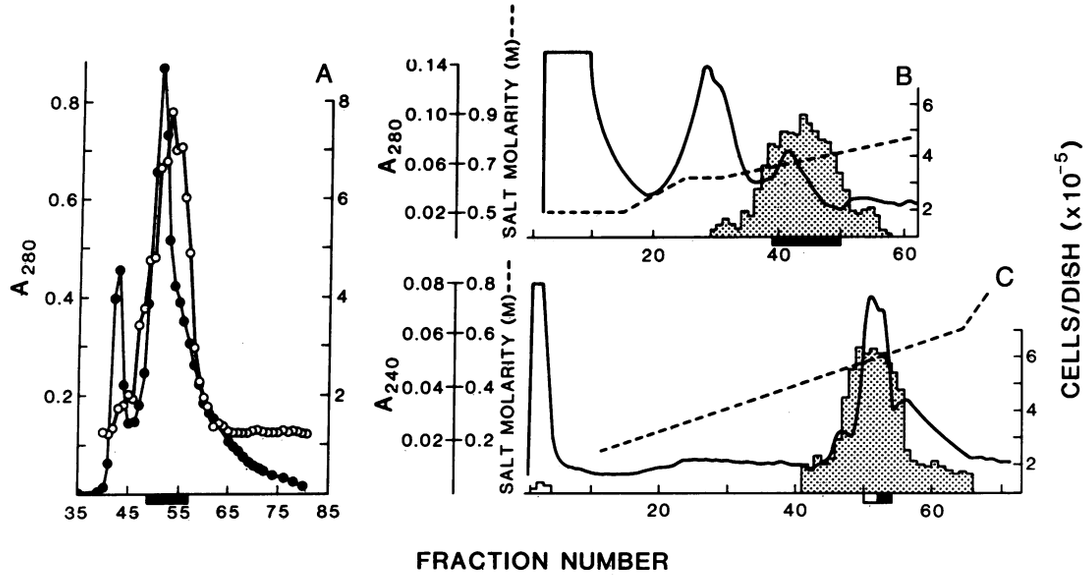

FIG. 2. (*A*) Gel filtration on Bio-Gel P-10 (95 × 1 cm; Bio-Rad). Partially purified FGF (37 mg) was dissolved in 3.5 ml of 0.1 M ammonium bicarbonate, pH 9.2, loaded onto the column, and eluted with the same buffer at 4°C at 5 ml/hr. Fractions (1 ml) were collected and assayed for protein content (UV absorbance, ●) and bioactivity (○). Bioactive fractions (horizontal bar) were pooled and lyophilized. (*B*) Cation-exchange chromatography on Mono S. Material from the Bio-Gel column (30 mg) was dissolved in 2 ml of 0.5 M ammonium formate, pH 6.0, and injected into the column, which was equilibrated with the same buffer. Protein was eluted at room temperature with a multilinear gradient of ammonium formate at pH 6.0 (0.5 M for 15 min, 0.5–0.65 M in 10 min, 0.65 M for 5 min, and 0.65–0.9 M in 50 min). The flow rate was 1 ml/min. Fractions (1 ml) were collected and assayed for bioactivity (shaded area). Bioactive fractions (horizontal bar) were pooled and lyophilized. (*C*) Rechromatography on Mono S. Bioactive material from the first Mono S chromatography (1 mg) was dissolved in 2 ml of 20 mM Hepes, pH 8.3/0.1 M NaCl, injected into the column (equilibrated with the same buffer), and eluted at room temperature with a 60-min gradient of 0.1–0.6 M NaCl in 20 mM Hepes, pH 8.3. The flow rate was 1 ml/min; 1-ml fractions were collected and bioassayed (shaded area). Material from pooled fractions 53–54 (solid horizontal bar) was used for structural characterization.

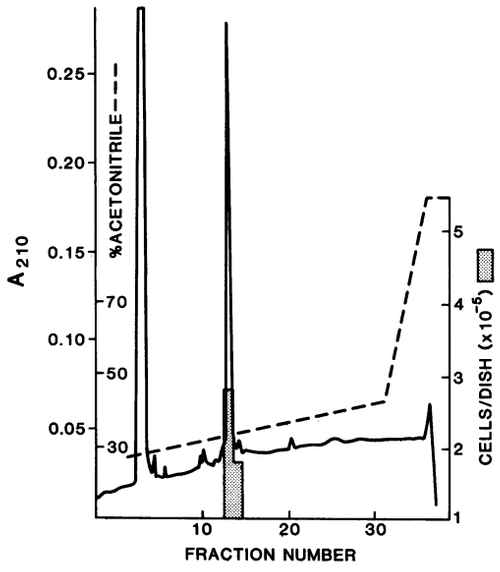

FIG. 3. Reversed-phase HPLC of FGF isolated by procedure II (purity test). An aliquot of pooled Mono S fractions 53–54 (Fig. 2*C*) corresponding to 9 μg of protein was dissolved in 1 ml of 0.2 M acetic acid and chromatographed on a Vydac C_4 column (25 × 0.46 cm, 5-μm particle size, 300-Å pore size, The Separations Group, Hesperia, CA) using 0.1% trifluoroacetic acid/acetonitrile (90-min gradient from 28% to 42% acetonitrile) as mobile phase at 0.6 ml/min. Fractions (1.8 ml) were collected. Column equilibration and regeneration was performed as described for Fig. 1*B*.

FGF fraction as defined above (100 ng/ml, added every 48 hr), were passaged weekly at a split ratio of 1:64. For mitogenic assay, cell monolayers from stock plates (at passage 3–10) were dissociated by exposure (2–3 min, 24°C) to STV solution (GIBCO; 0.9% NaCl/0.01 M sodium phosphate, pH 7.4/0.05% trypsin/0.02% EDTA). Cells were then seeded at an initial density of 2 × 10^4 cells per 35-mm plastic culture dish (Falcon) containing 2 ml of Dulbecco's modified Eagle's medium H-16/10% calf serum and antibiotics as described above. Diluted mitogen samples (10 μl) were added to triplicate dishes every 48 hr. After 4 days of culture, plates were treated with trypsin and cells were counted in a Coulter particle counter.

Antibody Production and Radioimmunoassay (RIA). Amino-terminally directed antibodies against FGF were obtained by immunizing 3-month-old male and female New Zealand White rabbits against a bis-diazotized (21) derivative of the synthetic decapeptide Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Tyr—i.e., FGF-(1–9)-Tyr^{10} conjugated to bovine serum albumin. The peptide was synthesized by solid-phase methodology (22). Rabbits were injected with 1 mg of peptide conjugate as described (23) and boosted by injection of 200 μg of peptide conjugate at intervals of 2–3 weeks. After the third boost antisera were examined for their capacity to bind radioiodinated hapten decapeptide prepared by the chloramine-T method (24) and purified by DEAE-cellulose anion-exchange chromatography. An RIA (25) was established, using this radioiodinated peptide as a tracer and antiserum (716B3) at a final dilution of 1:5000.

RESULTS

FGF Isolated by Procedure I. The purification to apparent homogeneity of FGF from a partially purified FGF preparation (as defined in *Materials and Methods*) is shown in Fig. 1. The bioactivity profiles of the gel filtration and the C_3 reversed-phase HPLC fractions suggest that pituitary extracts contain multiple molecular species with proliferative activity for vascular endothelial cells. Although some of these entities can be readily separated by reversed-phase HPLC (Fig. 1*B*), only the protein corresponding to the major activity was purified to homogeneity and further studied. FGF was judged to be >90% pure on the basis of peak appearance in reversed-phase HPLC (Fig. 1*C*), polyacrylamide gel electrophoresis (not shown), and sequence analysis data (see below, Table 2). The yield of pure mitogen averaged to about 3 nmol per kg of pituitary tissue.

Table 1. Amino acid composition of pituitary FGF

Residue	Prepared by procedure I*	Prepared by procedure II†	Integers assumed
Asx	11.3	9.4	10
Thr	3.6	3.5	4
Ser	8.6	8.5	9
Glx	12.9	11.5	12
Pro	8.6	9.7	9
Gly	15.2	16.7	16
Ala	8.7	8.6	9
Cys‡	5.4	5.6	6
Val	5.4	5.0	5
Met	1.5	1.5	2
Ile	3.1	2.3	3
Leu	12.2	10.5	11
Tyr	6.3	7.1	7
Phe	6.8	7.3	7
His	2.2	3.5	3
Trp	0.4	1.0	1
Lys	12.7	13.9	13
Arg	10.6	11.9	11
			138

Values represent residues per molecule determined from 24-hr hydrolysates of 5–15 pmol of protein and are not corrected for hydrolysis losses. Compositions are calculated for a 138-amino acid protein, which is in agreement with the observed molecular weight.
*Results are means obtained from duplicate determinations of four different batches (see Fig. 1*C*).
†Results are means from duplicate determinations from a single batch (pool of fractions 53 and 54, Fig. 2*C*).
‡Determined as cysteic acid.

Polyacrylamide gel electrophoresis of FGF showed a single band corresponding to a protein with an apparent molecular weight of 16,000 (data not shown). The amino acid composition of the protein is given in Table 1. Microsequencing with a gas/liquid phase sequenator established the amino-terminal sequence of the first nine residues of FGF as Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser (Table 2).

FGF is highly active in stimulating the proliferation of adult bovine aortic endothelial cells (Fig. 4); maximal stimulation of cell proliferation is achieved at a concentration of 2 ng/ml; half-maximal response occurs at 0.25 ng/ml, as quantitated by amino acid analysis. The activity of the mitogen is stable under the acidic conditions (pH 2) of the HPLC mobile phase used. However, the biological activity of the isolated mitogen represents only 1–2% of the total activity present in the partially purified FGF preparation used as starting material. Most of the activity (90–95%) but not the protein mass is lost when the material is subjected to the initial step of reversed-phase chromatography, while recovery of biological activity in the other HPLC steps is good. Loss of this activity correlates with exposure of the growth factor preparation to acidic conditions. This was further established by showing that incubation of partially purified FGF in the reversed-phase HPLC mobile phase (0.1% trifluoroacetic acid, pH 2.0/50% acetonitrile in water) causes comparable losses in biological activity. These results are in agreement with earlier reports (12, 20) that pituitary FGF preparations are >90% inactivated below pH 4.

Table 2. Amino-terminal sequence analysis of FGF

Cycle	FGF from procedure I* PTH-AA	Quantity, pmol	FGF from procedure II† PTH-AA	Quantity, pmol
1	Pro	105	Pro	150
2	Ala	47	Ala	62
3	Leu	46	Leu	70
4	Pro	35	Pro	73
5	Glu	24	Glu	48
6	Asp	17	Asp	23
7	Gly	8	Gly	47
8	Gly	27	Gly	9
9	Ser	21	Ser	21
10	—	—	Gly	34
11			Ala	50
12			Phe	42
13			Pro	38
14			Pro	9
15			Gly	28
16			—	—

PTH-AA, phenylthiohydantoin amino acid; analysis of phenylthiohydantoin derivatives showed no evidence for >10% protein contamination of samples.
*Applied to sequenator: 500 pmol.
†Applied to sequenator: 250 pmol.

FGF Isolated by Procedure II. Fig. 2 illustrates the chromatographic results obtained with this procedure. In the final Mono S chromatography the biological activity eluted with a well-defined peak of UV-light absorbing material (fractions 50–54, Fig. 2*C*). The yield of FGF was 5 nmol/kg of pituitary tissue. Overall recovery of bioactivity was 45% (gel filtration, 100%; Mono S ion-exchange chromatography, 65–70%). Although the peak corresponding to the major bioactivity does not appear to be homogeneous, the protein in the pooled fractions migrated in $NaDodSO_4$ gel electrophoresis as a single band with an apparent molecular weight of 16,000 (data not shown). No other significant band was found even when the gel was overloaded. However, the apparent heterogeneity of the protein peak could be due to the presence of multiple polypeptides with similar molecular weights. This possibility was tested by analyzing aliquots of pools of fractions from the ascending (fractions 50–52) and descending (fractions 52–54) parts of the peak by highly resolutive reversed-phase HPLC on a C_4 column. Fig. 3 demonstrates that the protein in fractions 53–54 is essentially homogeneous (the breakthrough peak did not contain protein as judged by amino acid analysis). Protein in fractions 50–52 elutes from the C_4 column with the same retention time as that of fractions 53–54 but contains a well-separated impurity (10% by peak height) eluting as a sharp peak immediately after the main component (data not shown).

The amino acid composition of the Mono S-purified mitogen (fractions 53–54, Fig. 2*C*) is shown in Table 1. It is strikingly similar to that of reversed-phase HPLC-purified FGF. Furthermore, the amino acid composition of this material is very similar to that of the protein in fractions 50–52 and also agrees with that obtained with procedure II after reversed-phase HPLC (Fig. 3). The sequence analysis of protein in fractions 53–54 established the first 15 residues of the amino-terminal sequence as Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly (Table 2), which is in agreement with the sequence of procedure I-derived FGF.

The intrinsic activity of fractions 53–54 to stimulate the proliferation of vascular endothelial cells is identical to that of FGF purified by reversed-phase HPLC (Fig. 4). However, the two preparations are clearly different with respect to their potencies: FGF purified by procedure II is 8-fold more potent (half-maximual stimulation of cell proliferation at 30 pg/ml—i.e., <2 pM). When this preparation is exposed to acidic conditions (0.2 M acetic acid) or subjected to reversed-phase HPLC (Fig. 3), it loses 90–97% of its potency, depending on conditions, while no loss of peptide occurs.

Immunological Characterization. An RIA was established for the quantitation of FGF by using the synthetic peptide FGF-(1–9)-Tyr^{10} as immunogen and FGF-(1–9)-$Tyr^{10}(^{125}I)$ as tracer. Synthetic peptide and native FGF displace the tracer in a parallel fashion and are recognized by the antibodies on an equimolar basis (unpublished data). The sensitivity of the

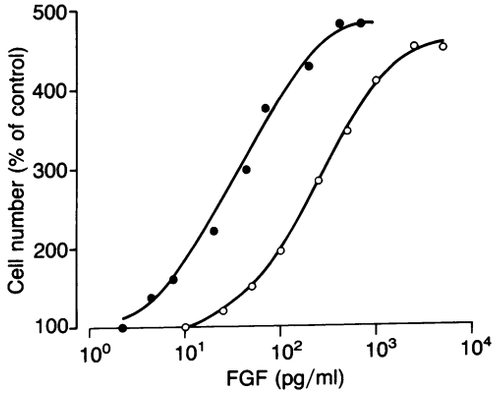

FIG. 4. Mitogenic activity of FGF on adult bovine aortic arch endothelial cells: dose–response assays. ○, FGF purified by ion-exchange/reversed-phase HPLC (procedure I); ●, FGF purified by gel filtration/ion-exchange chromatography (procedure II). Individual points are averages from duplicate determinations. Replicates were within 6% of mean values. The two preparations were tested in different assays; maximal cell densities were reached at 7.2×10^5 and 5×10^5 cells per dish for FGFs prepared by procedures I and II, respectively.

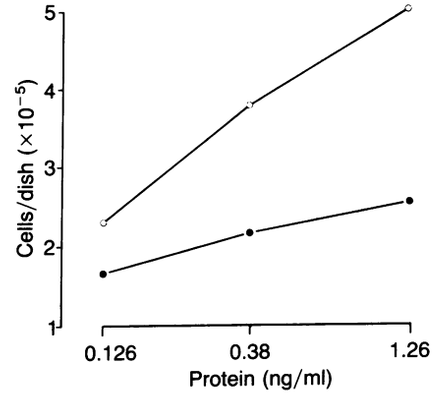

FIG. 5. Immunoneutralization of FGF proliferative activity on adult bovine aortic endothelial cells in culture. Cells were incubated in the presence (●) or absence (○) of 20 μl of antibody-containing globulin fraction [obtained by adding saturated ammonium sulfate solution (26) to 60 ml of serum and dissolving the precipitate in 30 ml of 0.1 M boric acid/0.025 M sodium tetraborate/0.075 M NaCl, pH 8.4] with various amounts of FGF (prepared by procedure II). Ammonium sulfate-precipitated antibodies were used instead of antiserum to exclude the possibility of artefactual stimulation of cells by growth factors present in the rabbit serum. Antibodies and growth factor (in 10 μl) were added together at days 1 and 3. Cells were counted on day 4. Individual points are averages from duplicate determinations. Replicates were within 6% of mean values. Cell numbers of control cultures (in presence or absence of antibodies) were approximately 1×10^5 per dish. In a control experiment, ammonium sulfate-precipitated globulins from normal rabbit serum had no effect on cell proliferation.

RIA enables the detection of approximately 30 fmol of FGF. The antibodies are capable of inhibiting the mitogenic activity of FGF *in vitro*, as shown in Fig. 5. These data demonstrate that the isolated and partially characterized molecule is indeed endowed with the mitogenic activity observed.

DISCUSSION

The presence of a substance in pituitary extracts capable of stimulating the proliferation of fibroblasts was described in 1968 by Holley and Kiernan (27). Subsequently, Gospodarowicz (2, 4) partially purified from bovine pituitary a fibroblast growth factor (FGF). Pituitary FGF has been shown to stimulate a wide variety of mesoderm-derived cells (3), including vascular endothelial cells. Preparations of FGF from the pituitary (and also the brain), none totally homogeneous, are being widely used in cell biology to elucidate mechanisms associated with cell growth and division. Because of the inherent heterogeneity of these FGF preparations, it has not been clear whether the activities attributed to FGF correspond to one and the same molecular entity. We conclude that FGF, as isolated to homogeneity and characterized in the work described here, is the molecule responsible for most, if not all, biological activities usually ascribed to the "basic pituitary FGF." The mitogen has very high intrinsic activity to stimulate proliferation of vascular endothelial cells (half-maximal stimulation at a concentration of 2 pM). Like partially purified FGF, it stimulates other mesodermal cells—e.g., human umbilical vein endothelial cells, bovine vascular smooth muscle, adrenal cortex, granulosa cells, and chondrocytes as well as neural crest-derived corneal endothelial cells—but not endodermal and ectodermal cells—e.g., basal epithelial cells (unpublished data). Like crude FGF preparations, homogeneous pituitary FGF as isolated here suffers loss of potency when exposed to acid but does not lose its intrinsic mitogenic properties and spectrum of activity on various mesoderm-derived cells. Finally, the FGF isolated here possesses an isoelectric point of 9.6 (data not shown), which is in agreement with that found for the principal mitogenic activity contained in FGF preparations prepared according to ref. 2. The FGFs isolated by either of the two procedures reported here are identical by all criteria tested (polyacrylamide gel electrophoresis, immunoreactivity, intrinsic biological activity, amino acid composition, and amino-terminal sequence) except for their differences in potency. A likely explanation for this discrepancy is an acid-induced change of the three-dimensional protein structure, possibly leading to reduced receptor binding; the observation is not surprising for a protein of such molecular weight and has many precedents.

Amino acid composition and amino-terminal sequence indicate that the FGF reported here is structurally different from all known growth factors. It also appears to be different from a basic pituitary fibroblast growth factor reported by Lemmon and Bradshaw (28) and from other bovine pituitary polypeptides with mitogenic activity for fibroblasts (5) for which an amino acid composition was reported. Finally, the structural properties of this FGF distinguish it from such extrapituitary FGF-like molecules as acidic brain FGF (29), basic brain FGF (30), or cartilage-derived growth factor (31).

This report describes the preliminary molecular characterizations (including amino-terminal sequence) of a pituitary FGF. Furthermore, it demonstrates by a rigorous criterion (immunoneutralization of mitogenic activity) that the biological activity observed is indeed associated with the characterized protein rather than with an unrecognized impurity. Until now the term "FGF" has been used somewhat ambiguously for several biologically related, but chemically distinct, FGFs of both pituitary and brain origin—e.g., basic, neutral, and acidic FGFs. It is not known to what extent those biologically similar FGFs are related chemically to the FGF described here. The establishment of a partial sequence for "basic pituitary FGF" is therefore an important step towards a better understanding of the growth factors represented in the FGF family.

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