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Acidic fibroblast growth factor (FGF) from bovine brain: amino-terminal sequence and comparison with basic FGF

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Acidic fibroblast growth factor (FGF) from bovine brain has been isolated by a combination of salt precipitation, ion-exchange chromatography, heparin-Sepharose affinity chromatography and reverse phase h.p.l.c. The amino acid composition of the mitogen is indistinguishable from that of acidic FGF previously purified. The amino-terminal sequence of acidic FGF was established as Phe-Asn-Leu-Pro-Leu-Gly-Asn-Tyr-Lys-Lys-Pro-Lys-Leu-X-Tyr-X-Ser-Asn-Gly-X-Tyr-Phe-Leu-Arg-Ile-Leu-Pro-Asp-Gly. Acidic FGF is structurally different from basic FGF as judged by mol. wt., amino acid composition and sequence. *In vitro* biological comparison of the two growth factors indicates that acidic and basic FGFs possess the same intrinsic activities to stimulate the proliferation of aorta, vein or capillary endothelial cells and adrenal cortex cells, but acidic FGF is 30–100 times less potent, depending on the cell type.

Key words: isolation/heparin-Sepharose affinity chromatography/mitogenic activity/endothelial cell growth factor

Introduction

Since the purification of fibroblast growth factor (FGF) from bovine pituitary and brain (Gospodarowicz, 1975; Gospodarowicz *et al.*, 1978a) and the demonstration that this basic mitogen (pI 9.6) stimulates the proliferation of a wide variety of mesoderm-derived cells *in vitro* (Gospodarowicz *et al.*, 1978b), other growth factors with similar biological activities have been found in brain or pituitary and variously termed acidic FGF (Lemmon *et al.*, 1982; Gambarini *et al.*, 1982), endothelial cell growth factor (ECGF) (Maciag *et al.*, 1979), brain-derived growth factor (Barritault *et al.*, 1982) or otherwise (Brockes *et al.*, 1980; Kellett *et al.*, 1981; Kasper *et al.*, 1982), in order to distinguish them from basic FGF. Although those mitogens resemble each other with respect to their *in vitro* mitogenic activity on cell types such as fibroblasts and endothelial cells and with respect to certain chemical characteristics (e.g., mol. wts.), it has not been clear, in the absence of structural data, whether they are chemically related to each other. Only recently have new data become available allowing further insight into this question. Thomas *et al.* (1984) reported the isolation of bovine brain acidic FGF. Our group isolated basic FGF from bovine pituitry and brain and determined the amino terminal sequence of this mitogen as Pro-Alu-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly (Böhle *et al.*, 1984; Gospodarowicz *et al.*, 1984, 1985c). Basic FGF is strongly mitogenic *in vitro* (half-maximal stimulation of cell proliferation at 30–50 pg/ml) for a variety of normal diploid cells derived from either the neuroectoderm or the mesoderm (Gospodarowicz *et al.*, 1985c) and is also angiogenic *in vivo* (Gospodarowicz *et al.*, 1979, 1985b). Following the demonstration that heparin-Sepharose affinity chromatography is highly effective in the purification of an FGF-like endothelial cell growth factor from a transplantable chondrosarcoma (Shing *et al.*, 1984), this technique was rapidly applied to the isolation of basic FGF from brain and pituitary (Gospodarowicz *et al.*, 1984; Lobb and Fett, 1984; Klagsbrun and Shing, 1985) and other tissues (in preparation). Moreover, it was found that acidic FGF (Lobb and Fett, 1984; Klagsbrun and Shing, 1985) and ECGF (Maciag *et al.*, 1984; Conn and Hatcher, 1984) also possess strong affinity for heparin, allowing their rapid purification. Most significantly, Lobb and Fett (1984) were able to show that the acidic growth factor for endothelial cells which they had purified has an amino acid composition virtually identical to that of acidic FGF isolated by Thomas *et al.* (1984). Based on this evidence we have isolated acidic FGF from bovine brain and now report the amino terminal sequence of this mitogen, as well as its preliminary biological characterization and comparison with basic FGF.

Results

Results from the purification of bovine brain acidic and basic FGFs are summarized in Table I. Most of the bioactivity present in the acid (pH 4.5) extract (>90%) was recovered from the ammonium sulfate precipitate. Virtually all of this mitogenic activity was found in the 0.6 M NaCl eluate from the carboxymethyl Sephadex C-50 chromatography, resulting in a 75-fold purification of growth factor activity. Heparin-Sepharose affinity chromatography is shown in Figure 1. Most of the protein (>99%) did not bind to the column and the unabsorbed material had little bioactivity (<0.1% of the input, Figure 1B). Stepwise elution of the heparin-Sepharose column with 1 M NaCl and 2 M NaCl solutions yielded two fractions with mitogenic activity for adult bovine aortic arch endothelial (ABAE) cells (Figure 1A). The mitogenic activity present in the 1 M NaCl fraction was identified (see below) as acidic FGF and represents 8.7% of the original activity (Table I). The active material in this fraction was purified 2500-fold from the crude brain FGF preparation and stimulates half-maximal proliferation of ABAE cells at a dose of 6 ng/ml (Figure 1B). Mitogenic activity in the 2 M salt fraction was purified 300 000-fold as compared with the crude FGF preparation and stimulated half-maximal ABAE cell proliferation at 60 pg/ml. It was ascertained that this material corresponds to basic FGF (data not shown) as previously characterized by us (Böhlen *et al.*, 1984; Gospodarowicz *et al.*, 1984, 1985c). The yields of acidic and basic FGFs from three different purifications ranged from 620–710 μg/kg brain and 37–45 μg/kg for acidic and basic FGF, respectively.

The homogeneity of acidic FGF in the 1 M NaCl heparin-Sepharose column eluate was analyzed by SDS-polyacrylamide gel electrophoresis (Figure 2) and by reverse phase h.p.l.c. (Figure 3). As shown in Figure 2, the major proteins migrate as a doublet with apparent mol. wt. of 15–16 000 (Figure 2,

Table I. Purification of acidic and basic FGF from 1 kg of bovine brain

Purification step	Protein recovered (mg)	Maximal mitogenic effect (ng/ml)	ED_{50}[a] (ng/ml)	Total activity[b] (units x 10^5)	Recovery of biological activity (%)	Purification factor
Ammonium sulfate precipitate	18 000	150 x 10^3	15 x 10^3	12.0	100	1
Carboxymethyl Sephadex C-50	190	2 x 10^3	200	9.5	80	75
HS 1 M NaCl (fraction 55–65)	0.630	30–40	6	1.05	8.7	2500
HS 2 M NaCl (fraction 73–77)	0.043	0.5	0.05	8.6	72	300 000

HS: heparin-Sepharose.
[a]Concentration of FGF preparation required to give a 50% maximal response in the assay system.
[b]One unit of activity is defined as the quantity of FGF required to give half-maximal stimulation of cell proliferation in the assay system described.

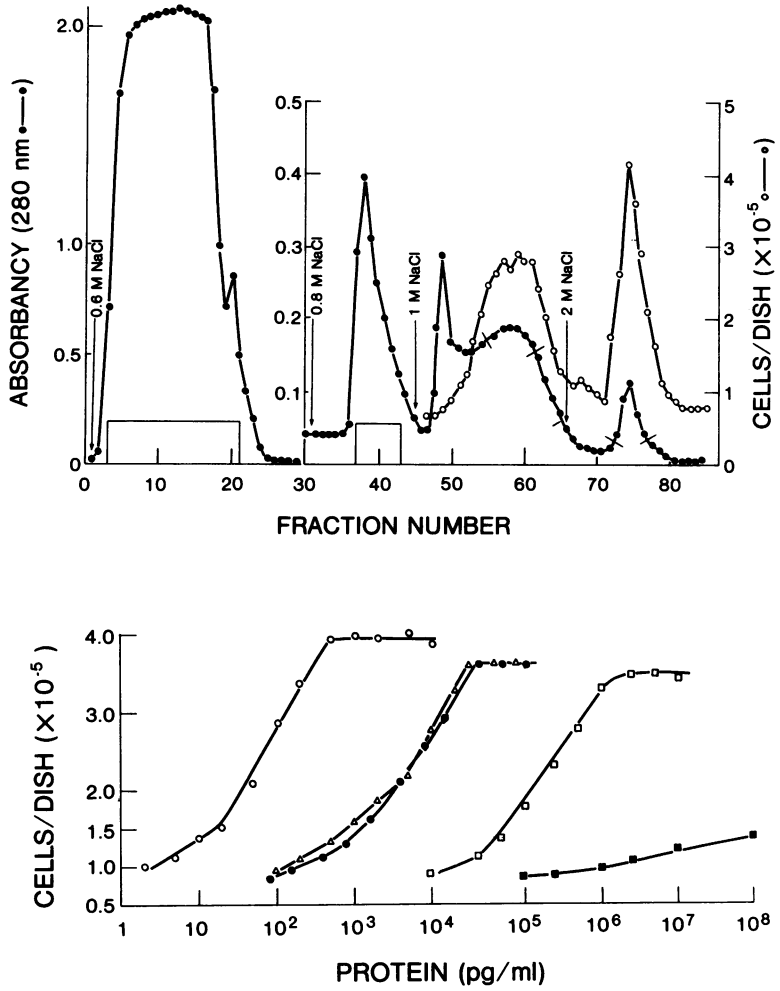

Fig. 1. Top panel: Heparin-Sepharose affinity chromatography of bovine brain FGF. Fractions containing mitogenic activity from the carboxymethyl Sephadex C-50 chromatography (110 ml, 7.6 mg/ml) were loaded onto a heparin-Sepharose column (1.6 x 8 cm) that had been equilibrated at room temperature with 0.6 M NaCl/10 mM Tris-HCl, pH 7.0. Chromatography was performed at room temperature by washing the column, after sample loading, with the equilibration buffer until the absorbance of the eluate at 280 nm became negligible, followed by stepwise elution with 0.8, 1.0 and 2.0 M NaCl in 10 mM Tris-HCl, pH 7.0. The flow-rate was 35 ml/h. Fractions of 9 ml were collected during loading and washing of the column; afterwards the fraction size was 2 ml. For fractions 3–21, protein concentration was determined by weighing a 2-ml aliquot after dialysis and lyophilization and bioassays were performed by diluting aliquots with DMEM/0.5% BSA to a concentration of 0.7 mg/ml and adding 10 μl aliquots of the dilute samples to low density ABAE cell cultures. For the fractions containing 0.8–2.0 M NaCl, mitogenic activity was determined by adding 10-μl aliquots (after dilution 1:10, 1:50 or 1:500 with DMEM/0.5% BSA for fractions containing 0.8 M, 1.0 M and 2.0 M NaCl, respectively) to low density ABAE cultures. The histogram shows final cell densities of pooled fractions 3–21 and 37–43 after 4 days in culture. Densities of control cultures after 4 days were 1.65 x 10^5 cells/35 mm dish. After use the heparin-Sepharose column was stripped with 3 M NaCl/10 mM Tris-HCl, pH 7.0. **Bottom panel:** Mitogenic activities of fractions taken from various purification steps. Carboxymethyl Sepharose C-50 0.6 M NaCl fractions (■—■), heparin-Sepharose fractions: unabsorbed (□—□), 1 M NaCl [pooled fractions 55–61 (△—△), and 62–65 (●—●)], and 2 M NaCl [fractions 73–75, (○—○)].

lanes C and D). A quantitatively minor component with somewhat higher mol. wt. is also present in this fraction. In contrast, basic FGF present in the 2 M NaCl fraction migrates as a single band with apparent mol. wt. of 16–17 000 (Figure 2, lane E) and is indistinguishable in this respect from bovine pituitary basic FGF (lane B). Similar migration patterns were observed regard-

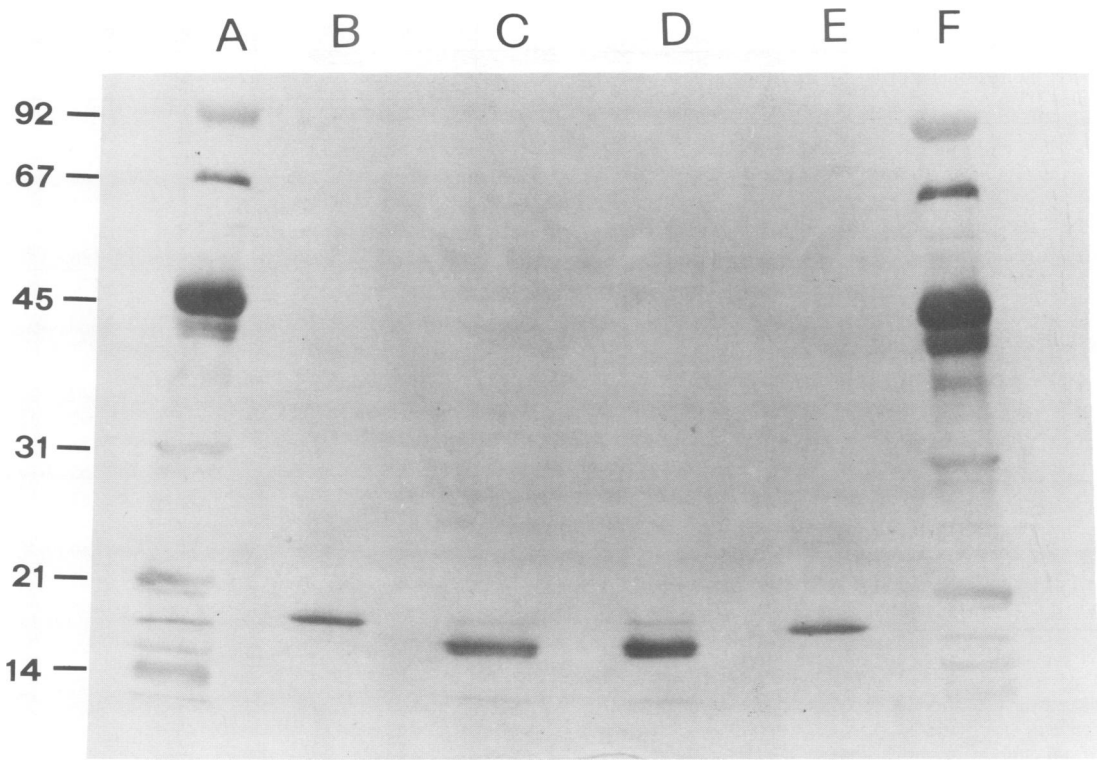

Fig. 2. SDS-polyacrylamide gel electrophoresis of heparin-Sepharose purified FGFs. Samples of 10 μl (0.9 μg protein) were applied to the gel. **Lanes A** and **F:** protein standard mixture, including phosphorylase b (92.5 kd), BSA (67 kd), ovalbumin (45 kd), carbonic anhydrase (31 kd), soybean trypsin inhibitor (21.5 kd) and lysozyme (14.4 kd). **Lane B:** bovine pituitary basic FGF. **Lanes C** and **D:** heparin-Sepharose 1 M NaCl eluate, pool of fractions 55–61 and 62–65, respectively. **Lane E:** heparin-Sepharose 2 M NaCl eluate, pool of fractions 73–77.

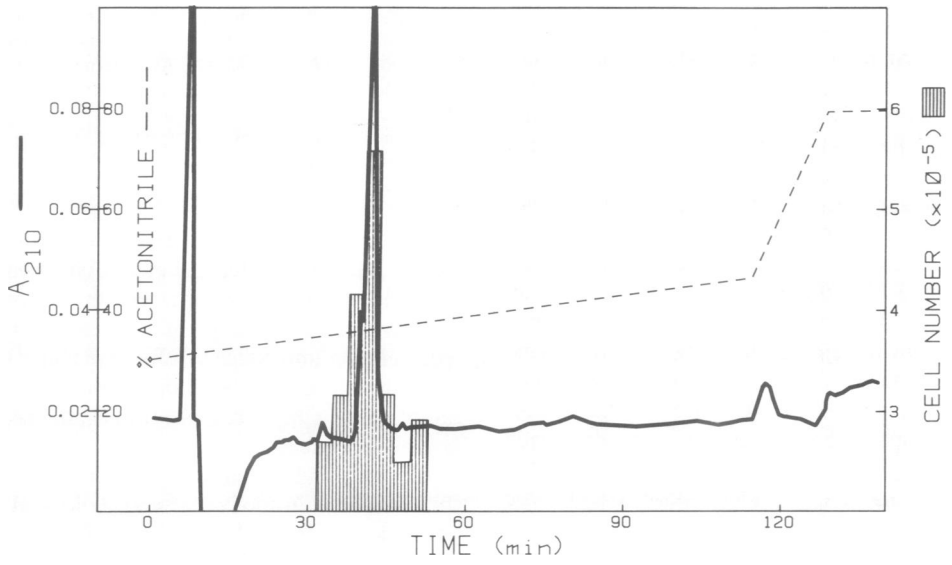

Fig. 3. Reverse phase h.p.l.c. of bovine acidic FGF. Batches of 0.5–1.5 ml of heparin-Sepharose purified FGF were chromatographed on a Vydac C4 column (0.46 x 25 cm, 300 Å pore size, 5 μm particle size, The Separations Group, Hesperia, CA), using a linear 2-h gradient of 30–45% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid as the mobile phase. The flow-rate was 0.6 ml/min and fractions of 3 ml were collected. Bioassays were performed as described in Figure 1.

less of whether the samples were in their native or reduced forms, indicating the absence of disulfide-linked subunits in those proteins. Analysis of the 1 M NaCl eluate from the heparin-Sepharose column by reverse phase h.p.l.c. (Figure 3) revealed the presence of a major, biologically active peak, together with two minor peaks that indicate slight impurity of the heparin-Sepharose-purified acidic FGF. H.p.l.c.-purified acidic FGF has similar potency to stimulate ABAE cell proliferation as heparin-Sepharose-purified FGF, indicating that this mitogen is stable under acidic conditions (pH 2).

The amino acid analysis of heparin-Sepharose/h.p.l.c.-purified acidic FGF (Figure 3, fraction with the highest bioactivity) is

Table II. Amino acid composition of bovine acidic FGF[a]

Residue	H.p.l.c. purified acidic FGF (Figure 3)[b]	Data from Thomas *et al.* (1984)
Asx	15.6	14.1
Thr	8.6	8.6
Ser	9.9	9.8
Glx	15.5	16.4
Pro	6.0	6.8
Gly	13.3	14.2
Ala	4.7	4.6
Cys	3.6	4.1
Val	4.6	4.7
Met	1.0	0.9
Ile	5.4	5.6
Leu	20.0	18.8
Tyr	7.1	7.4
Phe	8.1	7.0
His	5.7	5.4
Lys	12.9	13.0
Trp	1.1	1.2
Arg	5.4	5.8

[a]Values represent residues/molecule determined from 24-h hydrolysates of 10 pmol of protein and are not corrected for hydrolysis losses. The composition is calculated for a 149-residue protein to facilitate comparison with the results of Thomas *et al.* (1984).
[b]Results are means from duplicate determinations.

shown in Table II. The composition is strikingly similar to that of acidic FGF as reported previously (Lobb and Fett, 1984; Thomas *et al.*, 1984). Microsequencing of this material established the amino terminal sequence as Phe-Asn-Leu-Pro-Leu-Gly-Asn-Tyr-Lys-Lys-Pro-Lys-Leu-X-Tyr-X-Ser-Asn-Gly-X-Tyr-Phe-Leu-Arg-Ile-Leu-Pro-Asp-Gly (Table III).

Acidic FGF does not crossreact with antibodies to basic FGF (data not shown). This corroborates the results obtained by sequence analysis. The antibodies against basic FGF are highly specific in recognizing the amino terminal sequence of that mitogen, a lack of crossreactivity with acidic FGF therefore suggests the absence of that sequence in the acidic FGF molecule.

Results shown in Figure 1B indicate that both acidic and basic FGFs are mitogenic for vascular endothelial cells derived from large vessels. However, acidic FGF is ~100 times less potent than basic FGF. When tested *in vitro* on other types of endothelial cells, i.e., human umbilical vein, bovine brain- and adrenal cortex-derived capillary endothelial cells, acidic FGF is similarly active but less potent than basic FGF (Figure 4). Qualitatively and quantitatively comparable observations were also made when the mitogenic activity of the two growth factors was tested on adrenal cortex cells, another mesoderm-derived cell type (Figure 4).

Discussion

The isolation and partial characterization of an endothelial cell growth factor from bovine brain as reported here confirms and extends previous results by Thomas *et al.* (1984) who first isolated an acidic growth factor for fibroblasts (acidic FGF) having the same amino acid composition and by Lobb and Fett (1984) who showed that an endothelial cell mitogen isolated by heparin-Sepharose affinity chromatography is indistinguishable from acidic FGF. Thomas *et al.* (1984) isolated two microheterogenous forms of acidic FGF (mol. wt. 16.6–16.8 d). Although we used similar chromatography conditions in the last h.p.l.c. step we

Table III. Amino terminal sequence analysis of bovine brain acidic FGF

Cycle	<PhNCS-AA	Quantity (pmol)
1	Phe	270
2	Asn	159
3	Leu	202
4	Pro	134
5	Leu	165
6	Gly	186
7	Asn	120
8	Tyr	109
9	Lys	132
10	Lys	25
11	Pro	130
12	Lys	102
13	Leu	125
14	Leu*	65
15	Tyr	81
16	X	–
17	Ser	49
18	Asn	57
19	Gly	103
20	X	–
21	Tyr	27
22	Phe	43
23	Leu	65
24	Arg	44
25	Ile	52
26	Leu	62
27	Pro	44
28	Asp	10
29	Gly	64

<PhNCS-AA, phenylthiohydantoin amino acid. Analysis of phenylthiohydantoin amino acids showed no evidence for protein contamination of the sample. Amount of protein applied to sequenator: 500 pmol. Initial yield: 42.0%. Average repetitive yield: 94.1%.
*Identification tentative (bad h.p.l.c. injection).
X: Residue not identified – residues No. 16 and 20 could be Cys because no other amino acid residues were identified and Cys residues are not identifiable under the experimental conditions used (sequencing of the unmodified protein).

do not have conclusive evidence for the presence of two mitogen forms. However, SDS-gel electrophoresis (Figure 2, Lobb and Fett, 1984) also shows the presence of two proteins with very similar mol. wt. The possibility exists therefore that the small peak eluting immediately before the major bioactive peak (Figure 3) represents one of these forms or that the two forms were inseparable in our h.p.l.c. system and possess the same amino terminal sequences.

The two endothelial cell growth factors of the brain can now be compared in molecular and biological terms. The available data clearly show that acid and basic FGFs are two different molecular entities. The possibility that acidic FGF is a fragment of basic FGF (a notion consistent with the mol. wt. and bioactivities of the two mitogens) can be ruled out on the basis of amino acid analyses and the known sequences [complete sequence of basic FGF (in preparation) *versus* amino terminal sequence of acidic FGF]. Despite their obvious differences in chemical structure the acidic and basic FGFs display a remarkable similarity in their biological activity towards endothelial and some other mesoderm-derived cells. Most notably the *in vitro* intrinsic activities as determined with those cell types are the same for both growth factors. The two mitogens may therefore bind to the same cellular receptor. Preliminary results suggest that this

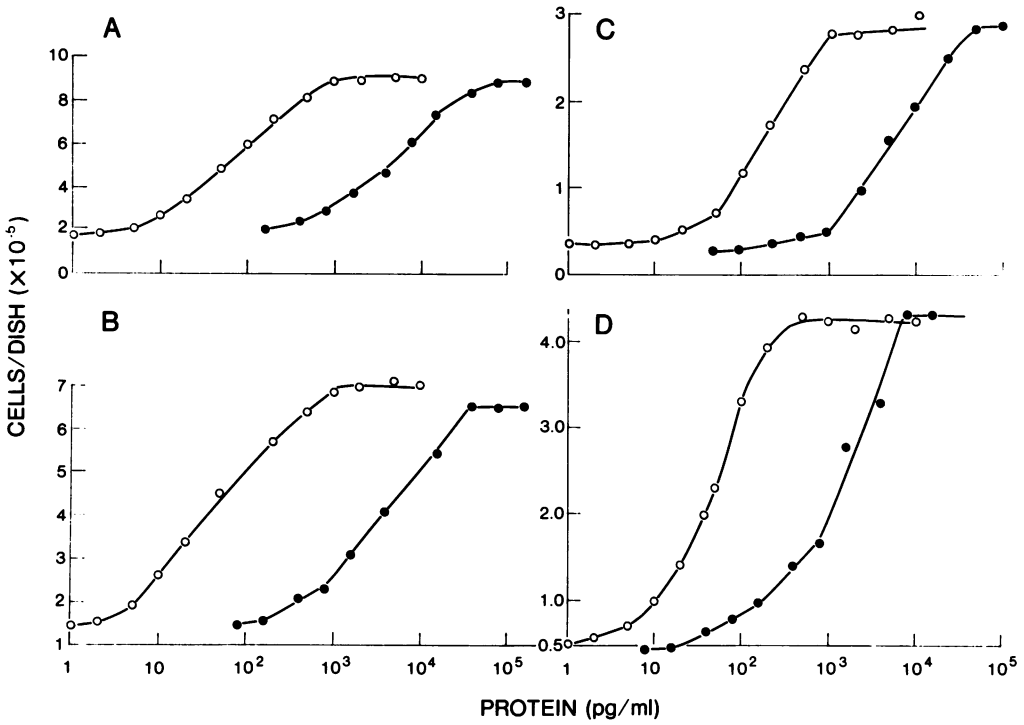

Fig. 4. Effect of acidic and basic FGF on the proliferation of various mesoderm-derived cell types *in vitro*. **A:** capillary endothelial cells from bovine adrenal cortex. **B:** capillary endothelial cells from bovine brain. **C:** human umbilical vein endothelial cells. **D:** bovine adrenal cortex cells. Cells were seeded in triplicate at low density (1–2 x 10^4 cells/35 mm dish) and maintained in the presence of various concentrations of either acidic or basic FGF (10-μl aliquots added on days 2 and 4) for 5–6 days after which they were trypsinized and counted in a Coulter counter. Culture conditions for all cell types except umbilical endothelial cells were those used for ABAE cells described in Materials and methods. Human umbilical endothelial cells (used after second passage) were cultured on gelatin-coated dishes in Hepes (25 mM) buffered medium 199 supplemented with 10^{-8} M selenium, and 20% fetal calf serum (Gospodarowicz *et al.*, 1983).

is indeed the case (G.Neufeld, personal communication). Moreover, both proteins bind strongly and very selectively to heparin. Based on this evidence, the possibility can be considered that acidic FGF contains within its sequence a part that is similar or even identical to a sequence segment of basic FGF that carries biological activity (receptor binding site) and heparin binding site. These intriguing similarities lend credibility to the speculation (Klagsbrun and Shing, 1985) that FGFs may bind to heparin-like molecules located on the cell surface and that this binding is a physiologically relevant event in growth factor action. However, despite such qualitative equality, quantitative differences exist with respect to the binding affinity to heparin and the biological potencies. Basic FGF is bound more strongly to heparin and it is also 30–100 times more potent than acidic FGF in stimulating the proliferation of normal diploid cells as tested in this study. Similar potency differences exist, but appear to be less pronounced, when the stimulation of the proliferation of some established cell lines, e.g., BALB/c 3T3, Swiss 3T3 and baby hamster kidney (BHK) cells (unpublished data) is tested. Furthermore, differences exist with respect to growth factor distribution and tissue concentration; so far acidic FGF has only been identified in neural tissue where it appears to occur in relatively high concentration. In contrast, basic FGF is present in brain in much lower concentration (this report; Gospodarowicz *et al.*, 1984), and the mitogen is present in a variety of non-neural tissues, e.g., pituitary (Böhlen *et al.*, 1984; Gospodarowicz *et al.*, 1984), kidney, adrenal cortex, corpus luteum (in preparation) and placenta (Gospodarowicz *et al.*, 1985a), sometimes at considerably higher concentration than in the brain. Obviously more data are needed before a thorough biological comparison between acidic and basic FGF is possible and before an understanding of the physiological function(s) of these mitogens will emerge. In this context, it will be interesting to see whether certain other growth factors, e.g., retina derived growth factor (D'Amore *et al.*, 1981), brain derived growth factor (Barritault *et al.*, 1982), or cartilage derived growth factor (Bekoff and Klagsburn, 1982) are structurally related to either acidic or basic FGF as one might expect on the basis of some of their reported characteristics. It should be of particular importance to determine if acidic FGF, like its basic counterpart (Gospodarowicz *et al.*, 1985c), is angiogenic *in vivo* and whether the two growth factors are physiological mediators of angiogenesis and angiogenesis-dependent processes such as tumor growth, wound healing, or development of the corpus luteum and the placenta.

Materials and methods

Isolation of FGF

Bovine brains were collected at the slaughterhouse and kept at −80°C for up to 2 weeks until use. For extraction of FGF 4 kg of tissue was defrosted overnight at 4°C and homogenized in a Waring blender in 10 l of 0.15 M ammonium sulfate at 4°C. The pH was adjusted immediately to 4.5 with 10 M HCl and the suspension stirred for 2 h at 4°C. After centrifugation the supernatant was adjusted to pH 7.0, ammonium sulfate (230 g/l, final concentration 1.89 M) was added and the precipitate removed by centrifugation. Further addition of ammonium sulfate (300 g/l, final concentration 4.16 M) to the supernatant gave a precipitate that was collected by centrifugation, redissolved in water and dialyzed against water overnight in the cold. From this material, referred to as crude FGF preparation, acidic FGF was further purified by cation-exchange chromatography on a carboxymethyl Sephadex C-50 column (3 x 20 cm) as described previously. (Gospodarowicz *et al.*, 1978, 1984, 1985c; Böhlen *et al.*, 1984). Bioactive material eluting from the ion exchange column with 0.6 M NaCl/0.1 M Na phosphate, pH 6.0, was subjected to heparin-Sepharose (Pharmacia) affinity chromatography as described in the legend to Figure 1A. Fractions with biological activity were pooled and stored at −80°C until further use. Unless otherwise

stated protein was determined by the dye-fixation assay (Bradford, 1976) using bovine serum albumin (BSA) as standard. Reverse phase h.p.l.c. of heparin-Sepharose-purified acidic FGF was performed on a Vydac C4 column as described in the legend to Figure 2.

Structural characterization

Mol. wt. determination was performed by SDS-polyacrylamide gel electrophoresis as previously described (Gospodarowicz *et al.*, 1984). Gels were fixed and stained using the BioRad silver nitrate staining kit according to the manufacturer's recommendations. Amino acid analyses were done on a Liquimat III amino acid analyzer (Kontron, Zürich, Switzerland) equipped with o-phthalaldehyde fluorescence detection system and proline conversion accessory, as described (Böhlen and Schroeder, 1982). Amino acid sequence analysis of the unmodified h.p.l.c.-purified growth factor was carried out using the Applied Biosystems gas/liquid phase microsequenator (Esch, 1984). Phenylthiohydantoin derivatives of amino acids were analyzed by h.p.l.c. (Hunkapiller and Hood, 1983).

Bioassays

Column fractions were tested for their ability to stimulate the proliferation of adult bovine aortic arch endothelial (ABAE) cells as described (Böhlen *et al.*, 1984; Gospodarowicz *et al.*, 1984, 1985c). Briefly, cells were seeded at low density (20 000 cells/35 mm dish) in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (Hyclone, Sterile Systems, Logan, UT). Cultures were grown for 4 days in the presence of chromatography fraction aliquots (added on days 0 and 2) and then counted in a Coulter particle counter. Mitogenic activity of purified FGF was also determined using cultures of bovine brain and adrenal cortex derived capillary endothelial cells, human umbilical vein endothelial cells and adrenal cortex cells. Culture and assay conditions were similar to those used for ABAE cells except that culture media were adapted for optimal results (Gospodarowicz *et al.*, 1985b, 1985c). Additional details are given in the figure legends.

Radioimmunoassay (RIA)

Acidic FGF was tested for crossreactivity in an RIA using antiserum raised against the BSA-coupled synthetic peptide Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Tyr, the first nine residues of which represent the amino terminal sequence of bovine basic FGF. The antibodies recognize synthetic antigen and native bovine basic FGF on an equimolar basis (Böhlen *et al.*, 1984; Baird *et al.*, 1985).

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