Human brain fibroblast growth factor

Isolation and partial chemical characterization

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Fibroblast growth factor (FGF) has been purified to homogeneity from human brain by a procedure involving salt precipitation, cation-exchange chromatography, Heparin-Sepharose affinity chromatography and reverse-phase HPLC. Isolation was monitored by radioimmunoassay and/or by testing column fractions for their capacity to stimulate the proliferation of vascular endothelial cells in vitro. The amino-terminal sequence of human brain FGF was determined as Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-. This sequence is identical to that of the amino-terminal region of bovine FGF. Additional evidence, including amino acid composition, chromatographic retention behavior, and immunoreactivity suggest that the human and bovine mitogens are very similar in structure.

Heparin-Sepharose affinity chromatography *HPLC* *Microsequencing* *Endothelial cell proliferation* *Radioimmunoassay*

1. INTRODUCTION

Fibroblast growth factor (FGF) has been isolated from bovine pituitary tissue and shown to be a basic polypeptide (p*I* 9.6) with an approximate molecular mass of 16 kDa that possesses the amino-terminal sequence Pro-Ala-Leu-Pro-Glu-Asp-Gly-Gly-Ser-Gly-Ala-Phe-Pro-Pro-Gly [1–3]. A mitogen indistinguishable from pituitary FGF has also been isolated from bovine brain tissue [3]. FGF is mitogenic for a variety of neuroectoderm- and mesoderm-derived cell types [2,4–6] and is angiogenic in vivo [2,7]. Owing to the novel and highly effective Heparin-Sepharose affinity chromatography procedure for the purification of FGF-like growth factors described by Shing et al. [8] the isolation of pure FGF from brain [3] and other tissues of bovine origin (in preparation) and the partial chemical characterization of these mitogens has been achieved using relatively small quantities of tissue. We now report the purification to homogeneity of two forms of basic FGF from human brain and show that they are structurally closely related to basic FGF from bovine pituitary or brain.

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2. MATERIALS AND METHODS

2.1. *Brain tissue*

Brains (24–48 h post-mortem) were provided by Dr X. Trujillo, UCSD Medical School, San Diego). Tissue was stored at −80°C until used.

2.2. *Isolation of FGF*

For the preparation of partially purified FGF a previously published procedure was used [3,9]. Briefly, brain tissue (2.3 kg) was extracted in 0.15 M ammonium sulfate at pH 4.5. The major mitogenic activity was enriched by ammonium

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sulfate precipitation and by batch adsorption/elution using a Carboxymethyl-Sephadex C-50 (Pharmacia) cation-exchange column (3 × 20 cm). Mitogenic activity eluting in the 0.6 M sodium chloride fraction was further purified by Heparin-Sepharose (Pharmacia) affinity chromatography at room temperature using a linear salt gradient for elution. Fractions with biological activity were subjected to final purification in two reverse-phase HPLC systems. Further details are contained in the figure legends.

2.3. *Assays*

Column fractions were either tested for their ability to stimulate the proliferation of adult bovine aortic arch endothelial (ABAE) cells as in [2,3] and/or by radioimmunoassay (RIA). The RIA was developed [1,10] by raising antibodies against the synthetic peptide Tyr10-FGF(1–9) the sequence of which corresponds to that of the first 9 residues of bovine FGF. Additional details are indicated in the figure legends.

2.4. *Structural characterization*

Amino acid analyses were performed on a Liquimat III analyzer (Kontron, Zürich) as in [11]. Amino acid sequence analysis of the unmodified HPLC-purified growth factor was carried out using the Applied Biosystems gas/liquid phase microsequenator [12]. Phenylthiohydantoin derivatives of amino acids were analyzed by HPLC [13].

3. RESULTS AND DISCUSSION

The purification to homogeneity of human brain FGF is shown in fig.1. Heparin-Sepharose affinity chromatography yielded a highly purified protein fraction eluting at 1.5–1.6 M NaCl that contained the majority of the immunoreactivity. Further purification by reverse-phase HPLC revealed the presence of multiple forms of FGF (fig.1B) of which two immunoreactive (not shown) and bioactive forms (both derived from the major protein peak shown in fig.1B) were isolated (fig.1C) and shown to be pure by sequence analysis (see below). As determined by amino acid analysis, only 200–300 pmol pure mitogens were isolated from 2.3 kg brain tissue. This in contrast with the considerably larger amounts of FGF that can be ob-

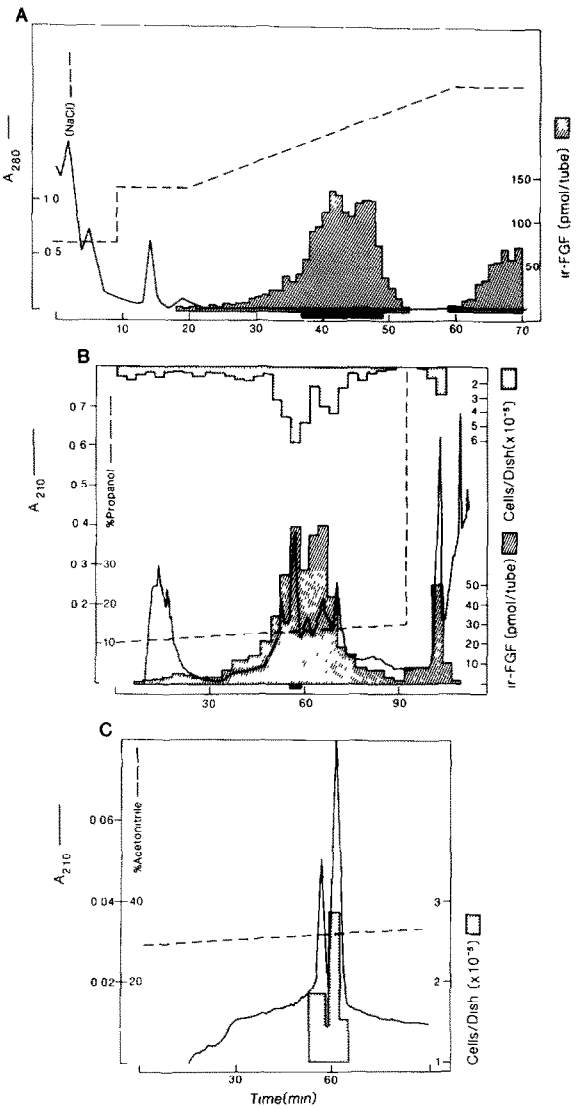

Fig.1. Isolation of human brain FGF. (A) Heparin-Sepharose affinity chromatography. The active fraction from Carboxymethyl-Sephadex C-50 (110 ml) was directly loaded onto a Heparin-Sepharose column (1.6 × 5 cm) that had been equilibrated with 0.6 M sodium chloride in 0.01 M Tris–Cl (pH 7.0). The column was first washed with the same buffer (flow rate 90 ml/h) until the absorbance at 280 nm returned to the baseline value and then with 1.1 M sodium chloride in 0.01 M Tris–Cl (pH 7). Mitogenic activity was eluted with a 60-min linear salt gradient of 1.1 M to 2.0 M sodium chloride in 10 mM Tris–Cl (pH 7). The flow rate was 1.5 ml/min, fractions of 1.5 ml were collected. Aliquots of 20 μl of each fraction were used for RIA. ir-FGF, immunoreactive FGF. The black horizontal bar under the chromatogram indicates fractions used for further

tained from similar quantities of bovine brain [3]. It is conceivable that the lower yields of human mitogen are a result of post-mortem degradation of FGF in human tissue. Amino acid compositions of the two isolated FGFs (fig.1C) are shown in table 1. Both the quantitatively major (peak II) and minor (peak I) forms possess amino acid compositions that are virtually indistinguishable and are also very similar to the composition of bovine FGF [1]. Amino-terminal sequences of the human FGFs are given in table 2. Although some amino acid residues could not be identified unequivocally due to low amounts of proteins sequenced, the data nevertheless show clearly that the amino-terminal sequences of the two human FGF forms are identical. The sequences are also identical to that of bovine brain (table 2) and pituitary FGF [1]. The sequencing data for bovine FGF are included in table 2 because they have not been previously published. For the sequence analysis we used a preparation of bovine brain FGF, the purification of which by Heparin-Sepharose affinity chromatography and reverse-phase HPLC was described in [3]. The conclusion that various forms of human and bovine FGFs have identical amino-terminal sequences is also corroborated by the fact that these mitogens crossreact (parallel displacement curves in the RIA) with antibodies specifically recognizing the amino-terminal sequence of bovine pituitary FGF [1,10].

The combined evidence (amino-terminal sequence, amino acid composition, chromatographic retention behavior, immunoreactivity, bioactivity) strongly suggests that human brain FGF is structurally closely related to bovine brain or pituitary basic FGF. Although molecular mass and isoelectric point of human brain FGF have not been determined due to the small quantities of mitogen

Table 1

Amino acid compositions of HPLC-purified human brain FGFs[a]

Amino acid	Residues/molecule[b]		
	Peak I	Peak II	Bovine pituitary FGF[c]
Asx	12.7	11.5	10
Thr	5.0	5.4	4
Ser	9.6	9.1	9
Glx	12.4	13.1	12
Pro	9.0	8.5	9
Gly	16.6	15.0	16
Ala	9.1	9.1	9
Cys	5.3	6.2	6
Val	4.5	5.1	5
Met	1.9	1.7	2
Ile	3.2	3.9	3
Leu	12.7	13.0	11
Tyr	5.9	5.4	7
Phe	6.5	6.6	7
His	2.6	2.3	3
Lys	11.6	11.7	13
Trp	0.3	0.4	1
Arg	9.7	10.3	11

[a] Values are means from 2–3 determinations (18 h hydrolyses at 110°C) using 5 pmol protein per analysis. Results are not corrected for hydrolysis-related losses

[b] Compositions are based on 140 amino acid residues per molecule

[c] From [1]

purification. (B) Reverse-phase HPLC of immunoreactive Heparin-Sepharose fractions on a Vydac C4 column (25 × 0.46 cm, 300 Å pore size, 5 μm particle size, the Separations Group, Hesperia, CA). The sample (18 ml) was loaded by pumping it directly onto the column. FGF-like material was eluted with a 90-min gradient of *n*-propanol (10–16%) in 30 mM phosphoric acid. After completion of the gradient the column was washed with 30 mM phosphoric acid in *n*-propanol. The flow rate was 0.6 ml/min, fractions of 1.8 ml were collected; 20-μl aliquots of each fraction were used for RIA. For bioassay 10-μl aliquots were diluted 5-fold in Dulbecco's modified Eagle's medium containing 0.5% bovine serum albumin, and 10-μl portions were added to ABAE cell cultures seeded at an initial density of 20000 cells/35 mm dish. Addition of diluted column fractions to cell cultures was repeated after 48 h and cells were counted after 4 days. Densities of control cultures were 1.5×10^5 cells/dish after 4 days. The black horizontal bar under the chromatogram denotes the fraction used for final purification. (C) Reverse-phase HPLC of the major bioactive FGF fraction from the first HPLC purification step (fig.1B). A Vydac C4 column was used in conjunction with an acetonitrile gradient (28–34% in 90 min) in 0.1% (v/v) trifluoroacetic acid as the mobile phase. Flow rate, 0.6 ml/min. Fractions were collected manually as appropriate for the separation of the two peaks. Aliquots of column fractions (1 μl per 100-μl fraction volume) were used for bioassay as described in (B).

Table 2

Amino-terminal sequences of two forms of human brain FGF and the major form of bovine brain FGF

Cycle	Human brain FGF				Bovine brain FGF	
	Peak I		Peak II			
	PTH-AA[a]	pmol	PTH-AA	pmol	PTH-AA	pmol
1	Pro	405	[b]	–	Pro	38
2	Ala	118	Ala	14	Ala	31
3	Leu	177	Leu	38	Leu	27
4	Pro	165	Pro	67	Pro	24
5	Glu	77	Gen	22	Glu	13
6	Asp	67	Asp	14	Asp	4.2
7	Gly	106	Gly	39	Gly	17
8	Gly	26	Gly	12	Gly	3.0
9	Ser	22	Ser	4	Ser	2.0
10	Gly	39	–	–	[b]	–
11	Ala	27	–	–	Ala	12
12	Phe	33	–	–	Phe	8.0
13	Pro	91	–	–	Pro	6.2
14	[b]	–	–	–	Pro	4.7
15	Gly	25	–	–	Gly	7.7
pmol sequenced [c]			150		200	
Initial yield (%) [c]			30		16	
Repetitive yield (%)	79.6		86.7		88.9	

[a] PTH-AA, phenylthiohydantoin amino acid
[b] PTH-AA identification uncertain
[c] Values obtained are unreliable

Analysis of phenylthiohydantoin amino acids showed no evidence for >10% protein contamination of samples

available, it is reasonable to assume, based on the amino acid compositions, that human and bovine FGFs are also closely related with respect to size and isoelectric point.

Heparin-Sepharose affinity chromatography is a powerful technique for the isolation of pituitary and brain FGFs. We have previously shown that this purification step can afford a 3000–4000-fold enrichment of the mitogen, yielding essentially pure bovine pituitary FGF [3], as well as highly purified bovine or human brain FGF preparations, the heterogeneity of which can be ascribed for the most part to the presence of multiple molecular forms of the same mitogen ([3], fig.1B,C of this report). Interestingly, Heparin-Sepharose also displays affinity for brain-derived acidic mitogens (p*I* 5–6) for endothelial cells. Using Heparin-Sepharose affinity chromatography, Conn and Hatcher [14] purified two endothelial cell growth factors (p*I* = 5.2) from human brain, Maciag et al. [15] purified an endothelial cell mitogen, with similar p*I* (5.5) and molecular mass, from bovine brain, and most recently, Klagsbrun and Shing [16] demonstrated the presence in bovine brain and hypothalamus of both acidic and basic growth factors for capillary endothelial cells. Based on available evidence it may reasonably be assumed that the basic mitogen of Klagsbrun and Shing is identical to basic FGF as reported by us [1–3]. Furthermore, it is conceivable that the above-mentioned acidic endothelial cell growth factors are structurally related to each other. The possibility even exists that those mitogens are related to bovine brain acidic FGF isolated and partially characterized by Thomas et al. [17]. In this context it is most intriguing to speculate that other mitogens, previously described to occur in many tissues and characterized with various bioassays (e.g., chondrosarcoma-derived growth factor [8], cartilage-derived growth factor [18], retina-derived growth factor [19], eye-derived growth factor [20]), are structurally related to either basic or acidic FGF. Evidence is already available that at least basic FGF does occur in many tissues, e.g., macrophages [21], placenta [22], adrenals, corpus luteum, kidney (in preparation). However, definite conclusions will only be possible once the primary structures of these growth factors are known.

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