Partial molecular characterization of endothelial cell mitogens from human brain: acidic and basic fibroblast growth factors

Peter Gautschi, Marijke Fràter-Schröder and Peter Böhlen*

Biochemisches Institut, Universität Zürich, Winterthurerstr. 190, CH-8057 Zürich, Switzerland

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Two endothelial cell growth factors have been isolated from human brain extracts by a procedure involving heparin-Sepharose affinity chromatography and reverse-phase HPLC. On the basis of molecular size, amino acid composition and extended amino-terminal sequences, the two proteins are structurally closely related to bovine acidic and basic fibroblast growth factors (aFGF, bFGF). The N-terminal sequences of human and bovine aFGF differ in two positions while those for bFGF are indistinguishable. Human aFGF is microheterogenous: in addition to aFGF two N-terminally truncated forms lacking the first and the seven first amino acids, respectively, were characterized. Human and bovine mitogens possess similar mitogenic activities.

Fibroblast growth factor *Heparin-Sepharose affinity chromatography* *HPLC* *Microsequencing* *Microheterogeneity*

1. INTRODUCTION

Two potent mitogens, acidic fibroblast growth factor (aFGF) and basic FGF (bFGF), have recently been isolated from bovine brain and pituitary and characterized by complete sequence analysis [1–6]. The two factors are potent mitogens for a wide variety of neuroectoderm- and mesoderm-derived cells in vitro and are angiogenic in vivo. Earlier data have also demonstrated the existence of human acidic and basic FGFs. The available data (affinity for heparin [7], amino acid compositions [8–10], a short N-terminal sequence of human bFGF [8]) suggest that the human and bovine bFGFs are structurally closely related. We now report the further chemical (N-terminal sequence) and biological characterization of human brain aFGF and demonstrate that this protein is isolated as several microheterogenous forms. Since aFGF and bFGF can be purified to homogeneity from the same batch of brain tissue, we also show an extended amino-terminal sequence of human bFGF.

* To whom correspondence should be addressed

2. MATERIALS AND METHODS

2.1. *Isolation*

Human brains (12–18 h post-mortem) were stored at −80°C until use. FGFs were isolated as in [4]. Briefly, human brains were processed (2 batches of 1.4 and 3.6 kg) by extraction in 0.15 M ammonium sulfate, pH 4.5, ammonium sulfate precipitation, cation-exchange chromatography, heparin-Sepharose affinity chromatography, and reverse-phase HPLC.

2.2. *Structural characterization*

M_r values of the growth factors were determined by SDS polyacrylamide gel electrophoresis as described [11]. Proteins were visualized by silver staining (Bio-Rad reagent kit and procedure).

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Amino acid analyses were performed with 10–20 pmol protein on a Chromakon 500 amino acid analyzer (Kontron, Zürich) equipped with an ophthalaldehyde fluorescence detection system [12]. Results were not corrected for hydrolysis losses.

Heparin-Sepharose fractions (1–5 nmol FGF) were reduced and alkylated as follows: samples were deaerated with argon, reduced with dithiothreitol (5-fold molar excess over protein cysteine content) in 0.5 M Tris-Cl, pH 8.6/10 mM EDTA/6 M guanidine hydrochloride (final concentrations) under argon at room temperature for 2 h, and alkylated by adding iodo[2-^{14}C]acetic acid (3-fold molar excess over total SH in the reduced sample solution) under argon for 30 min at room temperature in the dark. Alkylated proteins were purified by reverse-phase HPLC (see fig.1 for conditions). Aliquots of chromatography fractions were counted in a Beta-Matic beta-counter (Kontron).

Sequence analyses of native and/or alkylated FGFs were carried out using an Applied Biosystems gas/liquid phase microsequenator with experimental protocols for sequencing and HPLC identification of phenylthiohydantoin (PTH) amino acids as supplied by the manufacturer.

2.3. *Bioassay*

The mitogenic activity of column fractions was determined using cultured bovine aortic endothelial (ABAE) cells as described [1,11]. Cells were seeded at low density (20000 cells/35 mm dish) in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (Hyclone Sterile Systems, Logan, UT). Cells were grown for 5–6 days in the presence of sample aliquots (added on days 0 and 2), trypsinized, and counted in a Coulter particle counter.

3. RESULTS AND DISCUSSION

Human FGFs were purified to near homogeneity by heparin-Sepharose affinity chromatography with results similar to those in [4,8]. Mitogenic material subsequently identified as aFGF and bFGF (see below) eluted from the column at 1.0–1.2 and 1.4–1.6 M NaCl, respectively. Reverse-phase HPLC of aliquots of heparin-Sepharose column fractions containing either

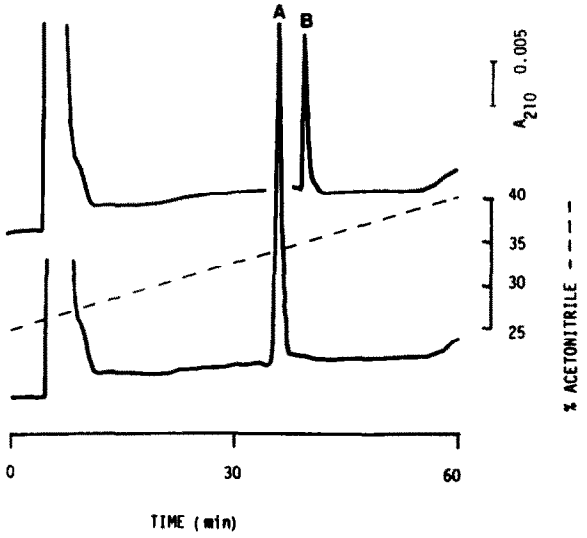

Fig.1. Reverse-phase HPLC of heparin-Sepharose-purified FGFs. Chromatography conditions: Vydac C4 column (25 × 0.46 cm, 5 μm particles, 300 Å pores, The Separation Group, Hesperia, CA), 60 min gradient of 25–40% acetonitrile in 0.1% trifluoroacetic acid, flow 0.6 ml/min. (A) aFGF, aliquot of pool of fractions eluting at 1.0–1.2 M NaCl. (B) bFGF, aliquot of pool of fractions eluting at 1.4–1.6 M NaCl.

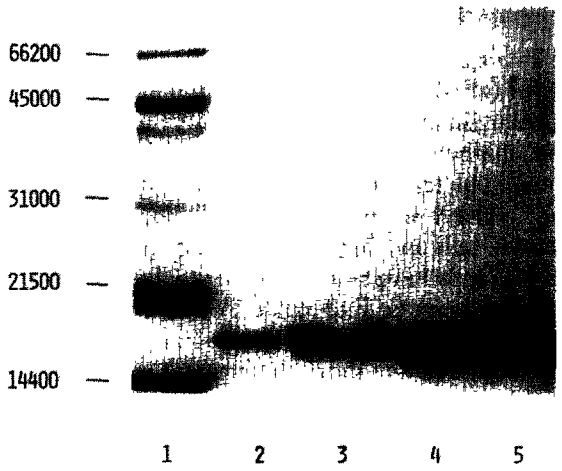

Fig.2. SDS gel electrophoresis of heparin-Sepharose-purified FGFs. Lanes: (1) M_r markers (bovine serum albumin, M_r 66200; ovalbumin, M_r 45000; carbonic anhydrase, M_r 31000; trypsin inhibitor, M_r 21500; lysozyme, M_r 14400); (2) bovine pituitary bFGF; (3) human bFGF; (4) human aFGF; (5) bovine brain aFGF. 40–80 ng protein was applied to each lane.

Table 1

N-terminal amino acid sequences of human acidic and basic FGFs

			1	10	20	30	
			•	•	•	•	
aFGF:	human A	(batch 1)[a]	F	NLPPGNYKKP	KLLYxSNGGH	FLRILPDGTV	DGT–
	human B	(batch 2)[b]		NLPPGNYKKP	KLLYCSNGGH	FLRILPDGxV	D–
	human C	(batch 2)[b]		YKKP	KLLYCSNGGH	FLRILPDGTV	D–
	bovine		F	NLPLGNYKKP	KLLYCSNGGY	FLRILPDGTV	DGT–
				*	*		
bFGF:	human[c]		PALPEDGGSG	AFPPGHFKDP	KRLYxKNGGF	FLRIxP–	
	bovine		PALPEDGGSG	AFPPGHFKDP	KRLYCKNGGF	FLRIHPDGRV	D–
			•	•	•	•	•
			1	10	20	30	40

[a]500 pmol HPLC-purified native aFGF from batch 1

[b]700 pmol HPLC-purified alkylated aFGF from batch 2. The sequenced material consisted of two proteins, human B (main sequence) and human C

[c]500 pmol HPLC-purified bFGF from batch 2

(x) PTH-amino acid not identified. *Established differences between human and bovine FGF sequences. For all analyses initial yields were 49–60%, average repetitive yields: 93.3–94.9%. Bovine sequences from [2,6]

aFGF or bFGF is shown in fig.1. Approx. 16 and 4 nmol homogeneous aFGF and bFGF, respectively, were isolated from 1 kg brain tissue (as determined by amino acid analysis).

M_r values of heparin-Sepharose-purified protein fractions were established by gel electrophoresis (fig.2). Human and bovine aFGFs (lanes 4,5) migrated as doublets. In the case of bovine aFGF the doublet bands are known to correspond to aFGF (upper band, M_r 16000, 140 residues) and to an N-terminally truncated des(1–6)-aFGF (unpublished and [5,6]). Therefore, comparison of the migration of human aFGF to the bovine FGFs of known sizes rather accurately indicates that human aFGF (lane 4, upper band) has an M_r of approx. 16500 and consists of 144–145 amino acid residues. Although no direct proof is available, it is likely on the basis of sequencing results (see below) that the lower band of the human aFGF doublet also corresponds to a truncated aFGF form. Heparin-Sepharose-purified human bFGF, on the other hand, shows one major band (with a minor contaminating band of unknown nature) on gel electrophoresis. Comparison of human and bovine bFGFs suggests that the human protein is very similar in size to the known bovine mitogen (M_r 16500, 146 residues).

Amino acid compositions were determined for human aFGF as Asx(14.3), Thr(7.3), Ser(8.9), Glx(15.8), Gly(15.3), Ala(4.6), Val(5.2), Met(0.8), Ile(4.9), Leu(18.3), Tyr(7.1), Phe(4.9), His(5.2), Lys(9.6), Arg(7.1) and for human bFGF as Asx(13.9), Thr(6.7), Ser(8.3), Glx(11.7), Gly(15.1), Ala(8.6), Val(7.0), Met(2.0), Ile(4.6), Leu(14.0), Tyr(6.4), Phe(7.1), His(3.3), Lys(11.7), Arg(9.7) (Pro was not determined, Cys and Trp only qualitatively determined). These amino acid compositions are very similar to those of the bovine mitogens [8–10]. The results from N-terminal sequence analyses of acidic and basic FGFs are shown in table 1. Sequence analysis of aFGF obtained from two isolation batches indicated that isolated aFGF can occur in several molecular forms: one isolation (batch 1) produced the N-terminal sequence aFGF human A (table 1) which aligns exactly with the known bovine sequence. In another isolation (batch 2) the purified aFGF actually consisted of a mixture of two related FGF proteins which could not be separated by HPLC in either their native or alkylated forms under the conditions used. Although two PTH-amino acid derivatives were identified in each sequencer cycle, unambiguous assignment of both sequences was possible because of the presence of a 2-fold excess of the amino acid derivatives arising from the main constituent in the mixture and because of previous knowledge of the N-terminal sequence of human aFGF (sequencing of batch 1).

Sequencing indicated that the main product (human B, table 2) is an aFGF that lacks the N-terminal Phe residue (des-Phe[1]-aFGF). The minor sequence (human C) was also found to correspond to an N-terminally truncated aFGF (des(1–7)-aFGF). The N-terminal sequence of human aFGF differs from that of the bovine mitogen by the replacement of two amino acids (table 2). Human brain bFGF, on the other hand, does not differ from bovine bFGF in the extended N-terminal region.

Thus, human and bovine aFGFs clearly differ in structure with respect to size and at least the N-terminal sequence. In contrast, human and bovine bFGFs are indistinguishable with respect to all structural criteria employed. In this context is it noteworthy that bFGFs purified from other human tissues (cartilage, hepatoma) possess higher M_r values of 18000–19000 [10].

Heparin-Sepharose-purified human aFGF and bFGF are capable of stimulating the proliferation

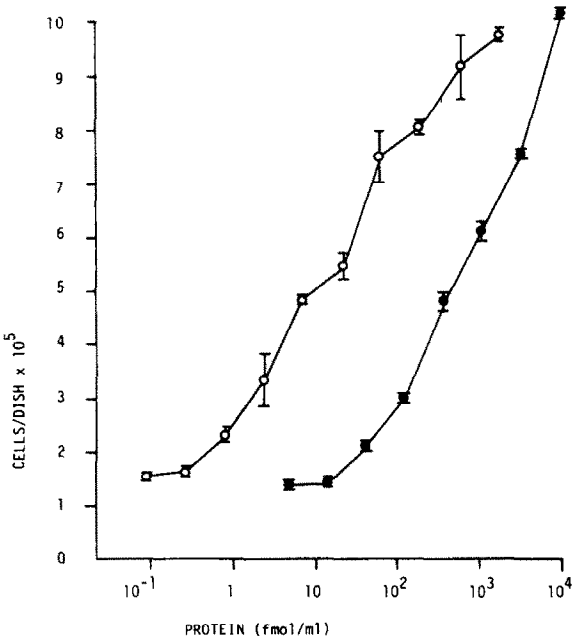

Fig.3. Mitogenic activity of human FGFs. 10-μl aliquots containing increasing amounts of heparin-Sepharose-purified aFGF (●) and bFGF (○), diluted in DMEM/0.5% bovine serum albumin, were added to ABAE and growth assayed as described in section 2. Maximal stimulation of cells with bovine bFGF (1 ng/ml given twice) results in cell counts of 1×10^6 cells/35 mm dish. Error bars indicate range of duplicate values.

of endothelial cells in vitro. Dose-response analysis (fig.3) showed that half-maximal stimulation of ABAE cells in vitro occurs at approx.1000 and 20 fmol/ml for aFGF and bFGF, respectively. Thus, the relative potencies of human aFGF and bFGF are similar to those of the bovine mitogens. However, human FGFs appear to be somewhat less potent than bovine FGFs, at least on bovine target cells.

After completion of this work a report describing the amino-terminal sequences of human brain aFGF and bFGF appeared [13]. Our sequencing data fully confirm those results.

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