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Endothelial cell growth factors in embryonic and adult chick brain are related to human acidic fibroblast growth factor

Werner Risau, Peter Gautschi-Sova[1] and Peter Böhlen[1]

Max-Planck-Institut für Entwicklungsbiologie, Spemannstr. 35, 7400 Tübingen, FRG and [1]Biochemisches Institut, Universität Zürich, Winterthurerstr. 190, 8057 Zürich, Switzerland

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We have investigated the nature of endothelial cell growth factors in 14-day embryonic and adult chick brain extracts. Mitogenic activity was isolated by a combination of cation-exchange, heparin-Sepharose affinity, and reverse-phase HPLC. Two major mitogenic fractions eluted from heparin-Sepharose at 0.8-1.3 M and 1.5-2 M. Biologically active proteins eluting at 0.8-1.3 M NaCl, after purification to homogeneity from embryonic and adult brain, were found to possess the same amino-terminal sequence as human acidic fibroblast growth factor (aFGF). The notion that the isolated mitogens represent chick aFGF is further supported by the findings that their affinity for heparin and their retention behavior in highly resolutive HPLC are indistinguishable from those of genuine aFGF. Mitogenic activities eluting at 1.5-2 M NaCl were also present in embryonic and adult brain, but in quantities insufficient for preliminary characterization. The high specific mitogenic activity for endothelial cells, high affinity for heparin and cross-reactivity with antibodies against bovine basic FGF (bFGF) suggest a relationship of those materials with basic FGF. Our data also suggest that the sequence of aFGF is highly conserved among vertebrates. While angiogenesis occurs predominantly in the embryonic brain, the absence of notable differences in the contents of the potent angiogenic factors aFGF and bFGF in embryonic versus adult chick brain is interesting.

Key words: angiogenesis/amino acid sequence/basic FGF/heparin-binding growth factors

Introduction

Acidic and basic fibroblast growth factors (aFGF and bFGF) were first isolated from brain and pituitary by conventional techniques (Thomas *et al.*, 1984; Böhlen *et al.*, 1984), and then from other tissues by taking advantage of their high affinity for heparin (for review, see Thomas and Gimenez-Gallego, 1986; Gospodarowicz *et al.*, 1986; Folkman and Klagsbrun, 1987). aFGF has been reported to be present only in neural tissue, but recent results indicate that it might be more widely distributed (Gautschi-Sova *et al.*, 1987). Mitogenic activity of FGFs is most prominent for mesoderm-derived cells, but stimulation of epithelial cells has also been reported (Crabb *et al.*, 1986). Their activity as angiogenesis factors *in vivo* may represent one of the major biological activities of these growth factors (for review, see Folkman and Klagsbrun, 1987). However, since the turnover of endothelium in adult tissues is very low (Denekamp, 1984), the presence of angiogenesis factors in the adult is puzzling. It has been suggested that they are stored in an active form and released, e.g. in tissue repair (Vlodavsky *et al.*, 1987). Inhibitors might also play a role (Folkman and Klagsbrun, 1987). Since we have recently characterized a heparin-binding angiogenesis factor from embryonic chick brain (Risau, 1986), we determined whether this factor is a FGF-like factor. Furthermore, we investigated whether mitogenic activities for endothelial cells in embryonic and adult brain are distinguishable.

Results

Embryonic chick brain

We have previously used heparin-Sepharose affinity chromatography to purify an endothelial cell growth factor from embryonic chick brain (Risau, 1986). Since the affinity for heparin suggested that this factor might be related to the FGFs we subsequently purified the major biological activity to homogeneity according to published procedures (Böhlen *et al.*, 1985; Gautschi-Sova *et al.*, 1987). The results are summarized in Figure 1. Mitogenic activity eluted from CM-Sephadex with 0.6 M NaCl (not shown) separated into two peaks of activity on a heparin-Sepharose column eluted with a gradient from 0.6 to 3 M NaCl (Figure 1a). The first peak eluted at 0.9-1.3 M, the second peak with approximately equal activity eluted at 1.5-2 M NaCl. This elution pattern from heparin-Sepharose has been consistently observed using extracts from >8000 embryonic chick brains (telencephalon and mesencephalon pooled from 14-day embryos; six separate experiments). We used 14-day embryos, because at this time growth factor activity reached a plateau during embryonic brain development (Risau, 1986) suggesting that possibly inactivation or modification of growth factor molecules could occur afterwards.

The first peak of activity eluting from heparin-Sepharose was subjected to cation-exchange chromatography on a Mono S column (Figure 1b). Two major peaks of activity eluted from the column between 0.3 and 0.4 M NaCl. The major biological activity (fractions 19-21, indicated by the bar in Figure 1b) was further purified by reverse-phase HPLC on a Vydac C4 column (Figure 1c). Minor activities present in fractions 17-18 and 22-24 of the Mono S column were not further analyzed. The Mono-S-purified mitogen peak was subjected to reverse-phase HPLC on a C4 column and found to be apparently homogeneous. Sequence analysis of the HPLC-purified protein (indicated by the bar in Figure 1c) showed amino-terminal sequences identical to that of human aFGF (Table I; Figure 4).

Although growth factor material eluting from heparin-Sepharose at 1.5-2 M NaCl was considerably pure on SDS gels (Figure 2, lane 1) we have not obtained sufficient material for sequencing. In Western blots, however, the ma-

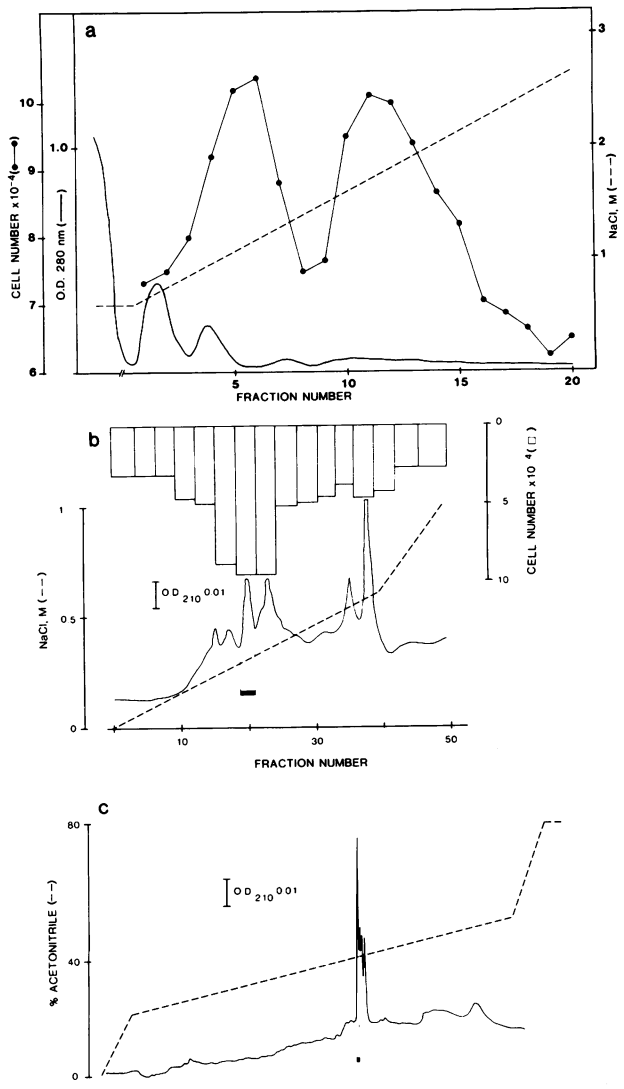

Fig. 1. Purification of embryonic chick brain endothelial cell growth factors. Fractions containing mitogenic activity from the CM-Sephadex C50 column were loaded onto a heparin–Sepharose column. The column was eluted using a linear NaCl gradient from 0.6 to 3 M (**a**). Fractions were collected and tested for mitogenic activity on BAEC. Growth factor activity eluting between 0.9 and 1.3 M NaCl (first peak) was pooled, applied to a Mono S column (**b**) and eluted using a NaCl gradient from 0 to 0.6 M. Fractions were collected and tested for growth factor activity on BAEC. Fractions 19–21 (indicated by bar) were pooled and chromatographed on a Vydac C4 column (**c**) using a linear gradient of 20–55% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid as the mobile phase. The fraction indicated by the bar was used for sequencing.

jor protein present in this material (mol. wt 16 kd) reacted with antibodies against bovine bFGF (Figure 2, lane 2).

Adult chick brain

Extracts from 2 kg of adult chick brains were prepared using the same procedure as for embryonic chick brains. Heparin–Sepharose chromatography revealed a pattern of growth factor activities eluting at 0.8–1.2 M and 1.5–2 M NaCl (Figure 3a), which is similar to that found with endothelial cell growth factors from embryonic brain. A total of 16 kg of adult chick brains was analyzed in eight separate experiments, yielding similar results. Equal amounts of biological activities were present in both peaks. The activity

Table I. Amino-terminal sequence analyses of chick FGFs

Cycle no.	Chick embryo		Chick adult	
	Amino acid	Amount (pmol)	Amino acid	Amount (pmol)
1	N	26.0	N	100.0
2	Y	21.0	Y	114.0
3	K	15.0	K	*
4	K	15.5	K	*
5	P	20.0	P	81.0
6	K	10.0	K	*
7	L	13.0	L	68.0
8	L	15.0	L	86.0
9	Y	11.0	Y	58.0
10	X	–	X	–
11	S	6.8	S	70.0
12	N	6.7	N	35.0
13	G	7.7	G	40.0
14	G	10.0	G	46.0
15	H	0.7	H	7.2
16	F	3.3	F	22.0
17	L	3.8	L	28.0
18	R	6.5	R	*
19	I	2.7	I	17.0
20	L	4.6	L	28.0
21	P	3.6	P	17.0
22	D	1.6	D	12.0
23	G	4.2	G	17.0
24	X	–	X	–
25	V	1.0	V	7.2
26			D	12.0
27			G	17.0

The amount of adult chick FGF applied to the sequenator was 200 pmol (initial yield was 50%, average repetitive yield was 88%); the amount of embryonic chick FGF applied to the sequenator was 50 pmol (initial yield was 50%, average repetitive yield was 92.7%). Sequence results indicate substantially pure proteins.
– no amino acid residue identified.
* no quantitative determination possible.

Fig. 2. Western blot analysis of embryonic chick brain endothelial cell growth factor eluting at 1.5–2 M NaCl from heparin–Sepharose. **Lane 1**: silver staining of proteins; **lane 2**: blot using antibodies against a synthetic peptide of bovine bFGF (Knörzer *et al.*, unpublished) and an avidin–biotin–peroxidase detection system (Amersham). Mol. wt markers are indicated at the left (kd).

eluting at lower salt concentrations (0.8–1.2 M) was further analyzed by reverse-phase HPLC (Figure 3b). The main peak of activity (indicated by the bar in Figure 3b) was rechromatographed on the same column and yielded two peaks (not shown), which were subjected to sequencing. The amino-terminal sequence of both proteins were identical to each other and to human aFGF (Table I, Figure 4). It is unclear whether heterogeneity of aFGF as observed in

reverse-phase HPLC is due to microheterogeneity of aFGF *in vivo* or to partial degradation (amino-terminal truncation) during isolation.

Discussion

FGFs have been isolated predominantly from bovine and human tissues. Amino acid and nucleotide sequences of both aFGF and bFGF differ little between bovine and human (Gimenez-Gallego *et al.*, 1986; Gautschi-Sova *et al.*, 1986; Jaye *et al.*, 1986; Abraham *et al.*, 1986b). We show here that the amino-terminal sequences of 25 amino acids of chick embryonic and adult aFGF are identical to human aFGF. This suggests that the growth factor is highly conserved among vertebrates and therefore probably plays an important biological role. Evidence in support of this notion is that the chick brain mitogen is indistinguishable from bovine or human aFGF on heparin–Sepharose affinity chromatography, cation-exchange and reverse-phase HPLC. On the basis of amino-terminal sequence and chromatographic retention behavior (affinity chromatography, reverse-phase HPLC) no difference was observed between the adult and embryonic mitogen.

The previously published heparin–Sepharose elution profile for the embryonic factor (Risau, 1986) is slightly different from those obtained when the present isolation procedure was used. Specifically the first peak eluted earlier when the acid extraction method was used. The second peak eluting between 1.6 and 2 M salt was not detected previously. These differences might be due to the different extraction method uses [phosphate-buffered saline (PBS) versus 0.15 M ammonium sulfate and subsequent acid extraction] and the higher salt concentration that is needed to elute the second peak (the salt gradient used here was from 0.6 to 3 M salt, whereas previously it was 0.1–2 M). Nevertheless, this second peak is indicative of the presence of bFGF in embryonic and adult chick brain. We have tried several times to further purify growth factor material from the second peak from embryonic and adult brains using HPLC methods. We have indeed observed protein peaks at retention times in reverse-phase HPLC that are suggestive of bFGF but have not yet obtained sufficient material for sequencing. This may be explained by the differences in specific activities between bFGF and aFGF, because bFGF has been reported to be 30–100 times more active than aFGF (Gospodarowicz *et al.*, 1986). Antibodies against bovine bFGF cross-reacted with this material indicating that

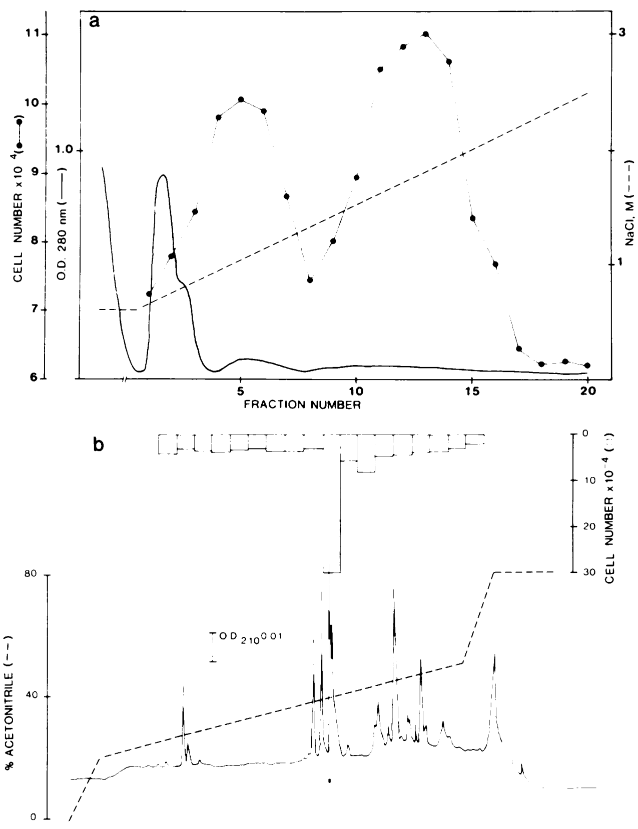

Fig. 3. Purification of adult chick brain endothelial cell growth factors. Fractions containing mitogenic activity from the CM-Sephadex C50 column were applied to heparin–Sepharose and eluted using a linear 0.6–3 M NaCl gradient (**a**). Fractions containing growth factor activity for BAEC eluting between 0.8 and 1.2 M NaCl (first peak) were chromatographed on a Vydac C4 column (**b**) as described in the legend of Figure 1. Growth factor activity was tested on human saphenous vein endothelial cells. The major mitogenic activity (indicated by bar) was used for sequencing.

bFGF is indeed present in chick brain and probably also highly conserved.

We also observed that the target cell specificity for endothelial cells of the embryonic brain-derived angiogenesis factor differs dependent on the assay conditions. As previously reported (Risau, 1986), serum deprivation [0.5% fetal calf serum (FCS)] kept smooth muscle cells (and fibroblasts),

bovine aFGF		F NLPLGNYKKP KLLYCSNGGY FLRILPDGTV DG
human aFGF		F NLPPGNYKKP KLLYCSNGGH FLRILPDGTV DG
chick embryo		NYKKP KLLYXSNGGH FLRILPDGXV
chick adult		NYKKP KLLYXSNGGH FLRILPDGXV DG
bovine bFGF	PALPEDGGSG	AFPPGHFKDP KRLYCKNGGF FLRIHPDGRV DG
human bFGF	PALPEDGGSG	AFPPGHFKDP KRLYCKNGGF FLRIHPDG

Fig. 4. Amino-terminal sequences of embryonic and adult chick brain endothelial cell growth factors and comparison with human and bovine aFGF and bFGF. Underlined amino acids are identical with human aFGF.

but not endothelial cells, unresponsive. However, if we used 5% bovine platelet-poor plasma-derived serum [devoid of platelet-derived growth factor (PDGF)] instead of serum deprivation, smooth muscle cells were as responsive to the embryonic brain-derived factor as endothelial cells, consistent with its characterization as aFGF. aFGF purified from bovine brain had the same effects (Risau, 1986; data not shown). Thus, factors present in serum are limiting for the full mitogenic response of smooth muscle cells but not endothelial cells.

Our results show that FGFs are present in embryonic tissues and might play a role during embryonic angiogenesis which involves actively growing endothelial cells. Other growth or chemotactic factors might also be involved either synergistic with or independent of the FGFs in the regulation of embryonic angiogenesis. It is in this respect interesting that new oncogenes coding for FGF-homologous proteins have been recently identified (Taira *et al.*, 1987; Dickson and Peters, 1987; Delli Bovi *et al.*, 1987), and thus have defined a new family of growth factors. It will be important to determine the biological activities of the encoded proteins especially as far as angiogenesis is concerned.

Materials and methods

Growth factor isoaltion

Two thousand embryonic chick brains (14-day old telencephali and mesencephali pooled and stored at −80°C; ~500 g wet weight) were thawed overnight at 4°C and homogenized in a solution of 0.15 M ammonium sulfate using a Waring Blendor. All subsequent steps including acid extraction, ammonium sulfate precipitation, CM-Sephadex C50 (Pharmacia) and heparin−Sepharose (Pharmacia) chromatography were performed as described previously for the isolation of bovine brain FGF (Böhlen *et al.*, 1985). Briefly, the 0.6 M NaCl eluate of the CM-Sephadex column was directly applied to a heparin−Sepharose column (1 × 4 cm). The column was washed and then eluted using a 100 ml gradient from 0.6 to 3 M NaCl containing 10 mM Tris−HCl. Fractions (5 ml) were collected and aliquots (10 μl) were tested for their ability to stimulate the proliferation of bovine aortic endothelial cells (BAEC; see below). Fractions containing growth factor activity which eluted between 0.9 and 1.3 M NaCl were pooled, appropriately diluted with 50 mM phosphate buffer, pH 6.8, and applied to a Mono S (Pharmacia) cation-exchange column. Mitogenic activity was eluted using a gradient from 0 to 0.6 M NaCl in 50 mM phosphate buffer, pH 6.8. Fractions containing mitogenic activity for BAEC were pooled and subjected to reverse-phase HPLC on a Vydac C4 (The Separations Group, Hesperia, CA) column.

Adult chick brains were prepared from chick heads obtained from a slaughterhouse and stored frozen at −80°C. Growth factor isolation was performed with 2 kg batches as described for embryonic brains. Mitogenic activity eluting from heparin−Sepharose between 0.8 and 1.2 M NaCl was further purified by reverse-phase HPLC on a Vydac C4 column.

Bioassays

Bovine aortic endothelial cells were seeded at 8000 cells/well in 24 well plates (Costar). Aliquots of fractions from heparin−Sepharose chromatography were added the next day and cells were grown for 3 days. HPLC-purified proteins were tested for mitogenic activity on human saphenous vein endothelial cells in the presence of heparin, an assay providing higher sensitivity (Gautschi-Sova *et al.*, 1987). At the end of incubation cells were trypsinized and counted in a Coulter particle counter.

Amino-terminal sequence analysis

Amino-terminal sequencing of HPLC-purified proteins was performed on an Applied Biosystems 470A gas/liquid phase microsequenator. Phenylthiohydantoin (PTH) derivatives of amino acids were identified by reverse-phase HPLC using a model 120A On-line PTH analyzer (Applied Biosystems). Experimental protocols for both procedures were as supplied by the manufacturer.

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