Acidic Fibroblast Growth Factor Is Present in Nonneural Tissue: Isolation and Chemical Characterization from Bovine Kidney†

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ABSTRACT: Endothelial cell growth factor activity purified from bovine kidney by heparin–Sepharose affinity chromatography was previously identified as basic fibroblast growth factor [Baird, A., Esch, F., Böhlen, P., Ling, N., & Gospodarowicz, D. (1985) *Regul. Pept. 12*, 202–213]. We now show that a major mitogenic fraction, isolated from heparin–Sepharose-purified material by Mono-S cation-exchange chromatography and reverse-phase high-performance liquid chromatography, is related to acidic fibroblast growth factor (aFGF). Sequence analysis showed the amino-terminal sequence to be Tyr-Lys-Lys-Pro-Lys-Leu-Leu-Tyr-X-Ser-Asn-Gly-Gly-Tyr-Phe-Leu-Arg-Ile-Leu-Pro-Asp-Gly-Thr-Val-Asp-. The molecular mass of the protein, as determined by polyacrylamide gel electrophoresis, was 15.5 kDa. In combination, those data strongly suggest that this mitogen is amino terminally truncated acidic fibroblast growth factor. So far, aFGF has only been found in neural tissues, i.e., in the brain and retina. Our results strongly suggest that this mitogen also occurs in extraneural tissue.

Over the last 3 years two mitogens for vascular endothelial cells, acidic and basic fibroblast growth factors (aFGF and bFGF),[1] have been purified to homogeneity (Thomas et al., 1984; Böhlen et al., 1984), their amino acid sequences determined (Gimenez-Gallego et al., 1985; Esch et al., 1985), and their cDNAs cloned and sequenced (Jaye et al., 1986; Abraham et al., 1986). In addition to their mitogenic activity for endothelial and many other cell types, both growth factors display a wide spectrum of biological activities in vitro and are angiogenic in vivo (Baird et al., 1986; Gospodarowicz et al., 1986; Folkman & Klagsbrun, 1987). Those properties suggest that they may be involved in neovascularization and could act as local regulators of tissue growth and repair. Basic FGF was first isolated from the pituitary (Böhlen et al., 1984) and the brain (Gospodarowicz et al., 1984), but it is now recognized that this protein is present in many tissues (Gospodarowicz et al., 1986; Baird et al., 1986). In contrast, acidic FGF has so far only been identified in neural tissues such as brain (Thomas et al., 1984; Böhlen et al., 1985a; Burgess et al., 1986) and retina (Baird et al., 1985a), where it occurs in high concentrations. On the basis of this difference in tissue distribution, it was thought that aFGF might fulfill a neural tissue specific physiological function. We now report the purification to homogeneity and chemical characterization of a FGF from a nonneural tissue, the bovine kidney.

Experimental Procedures

Growth Factor Isolation. Five kilograms of bovine kidney from freshly slaughtered animals was cleaned from fat and connective tissue, cut into small pieces, and immediately extracted, following a previously described method (Böhlen et al., 1985a). Briefly, kidney tissue was homogenized and extracted at pH 4.5. The biological activity was precipitated with ammonium sulfate, dialyzed against water, and purified by carboxymethyl-Sephadex (Pharmacia) cation-exchange chromatography and heparin–Sepharose (Pharmacia) affinity chromatography. Heparin–Sepharose fractions were further purified by Mono-S (Pharmacia cation-exchange chromatography and reverse-phase HPLC (experimental details are contained in the legends to Figures 1 and 2).

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

Polyacrylamide Gel Electrophoresis. Molecular weights of isolated proteins were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis as described (Gospodarowicz et al., 1984).

Radioimmunoassay. Aliquots of fractions from heparin–Sepharose affinity chromatography were tested in a radioimmunoassay (RIA) with an antiserum raised against the synthetic peptide corresponding to the amino-terminal sequence PALPEDGGS of bFGF-(1–146) (Baird et al., 1985c).

Bioassay. Bovine aortic cells were prepared from the aortic arch and cultured in Dulbecco's modified Eagle's medium (Gibco) with 10% calf serum (Hyclone, Logan, UT) in the presence of bFGF or aFGF as described (Gospodarowicz et al., 1984). Human endothelial cells were prepared from saphenous veins by collagenase digestion as described (Mueller et al., 1980). Human cells were cultured on gelatin-coated (0.2% gelatin in phosphate-buffered saline) 24-well plates (Nunc) in medium 199 (Gibco) supplemented with 20% human serum, bovine aFGF [1 ng/mL, prepared as described

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[1] Abbreviations: aFGF, acidic fibroblast growth factor; bFGF, basic fibroblast growth factor; ECGF, endothelial cell growth factor; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin; AUFS, absorbance units full scale; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride.

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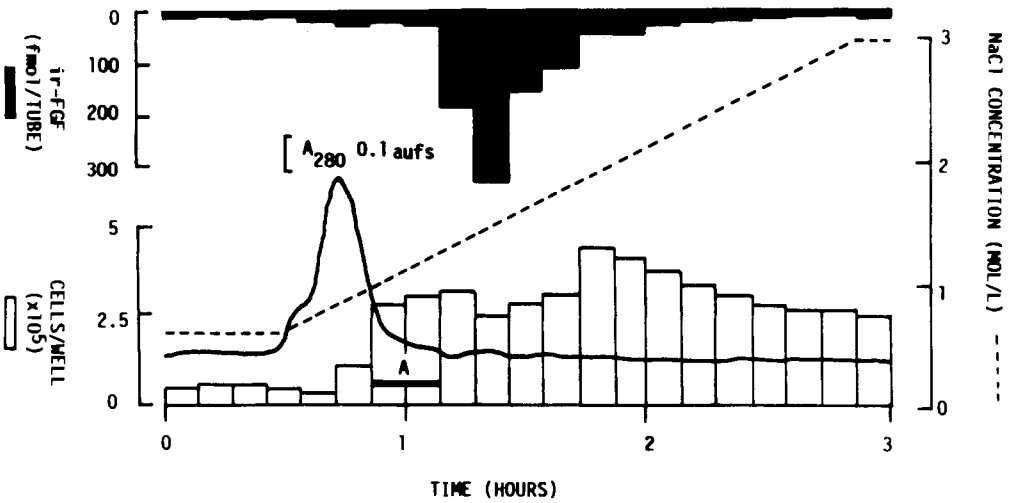

FIGURE 1: Heparin–Sepharose affinity chromatography. Fractions eluted with 0.6 M NaCl from a carboxymethyl-Sephadex column were pooled and loaded onto a heparin–Sepharose affinity column (1.5 × 20 cm) at a flow rate of 495 mL/h. The column was equilibrated with 10 mM Tris-HCl/0.6 M NaCl, pH 7. After being loaded, the column was washed with the equilibration buffer until the absorbance at 280 nm reached a minimum value. Elution was performed with a 2-h linear gradient from 0.6 to 3.0 M NaCl in 10 mM Tris-HCl, pH 7. Aliquots of fractions were pooled for bioassay and radioimmunoassay. Fractions used for further purification with Mono-S cation-exchange chromatography are indicated by a horizontal bar (designated with "A").

(Böhlen et al., 1985a)], heparin (45 ng/mL), and antibiotics (gentamycine, 50 μg/mL; fungizone, 2.5 μg/mL). Endothelial cells were identified by immunofluorescence detection of factor VIII related antigen (Risau et al., 1986). For experiments, confluent cultures were trypsinized, and cells were reseeded at 8000 cells/well (bovine endothelial cells) or 15 000 cells/well (human endothelial cells) in 0.5 mL of culture medium. For bioassay, cells were grown in the presence of aliquots of chromatographic fractions (added on days 0 and 2) for 5 days. Fractions from heparin–Sepharose affinity chromatography were tested for their ability to stimulate the proliferation of adult bovine aortic endothelial cells, while HPLC-purified proteins were tested for mitogenic activity on human saphenous vein endothelial cells in the presence of heparin (45 μg/mL), an assay providing higher sensitivity (Gospodarowicz & Cheng, 1986). After 5 days of culture, cells were trypsinized and counted in a Coulter particle counter.

Results and Discussion

Evidence for the presence of aFGF-like material in bovine kidney was obtained when prepurified tissue extracts were subjected to heparin–Sepharose affinity chromatography as shown in Figure 1. On the basis of bioassay and RIA, three different regions were distinguished: (a) bioactive fractions eluting between 1.1 and 1.5 M NaCl, (b) bioactive and immunoreactive fractions eluting between 1.6 and 2 M NaCl, and (c) bioactive fractions eluting at higher salt concentrations. It is well established (Böhlen et al., 1985a; Gautschi et al., 1985, 1986) that, with similarly prepared brain extracts, heparin–Sepharose fractions eluting at approximately 1–1.2 M NaCl contain aFGF. Therefore, the corresponding fractions from kidney (marked by a horizontal bar and designated "A" in Figure 1) were pooled and subjected to further analysis. Reverse-phase HPLC of an aliquot from the heparin–Sepharose pool A indicated substantial heterogeneity of the sample (data not shown). For this reason a further purification step on a Mono-S cation-exchange column was performed (Figure 2A). A fraction was collected that had an identical retention time as authentic aFGF from bovine brain. This fraction, when further analyzed on reverse-phase HPLC (Figure 2B), consisted of two polypeptides. Sequence analyses of both polypeptides showed identical amino-terminal sequences (Table I), which are in complete agreement with the sequence of bovine aFGF beginning at residue 8. Molecular weights (Figure 3) of both substances, as determined by $NaDodSO_4$–polyacrylamide gel electrophoresis, were approximately 15–15.5 kDa and indistinguishable from each other and very similar to that of bovine brain des-(1–6)-aFGF isolated previously (Gautschi et al., 1985). The combined data from amino-terminal sequence analysis and gel electrophoresis strongly suggest that both isolated proteins are different mo-

Table I: Amino-Terminal Sequence Analyses[a]

	HPLC peak 1		HPLC peak 2	
	PTH-amino acid	amount (pmol)	PTH-amino acid	amount (pmol)
1	Tyr	87	Tyr	200
2	Lys	78	Lys	155
3	Lys	135	Lys	225
4	Pro	67	Pro	130
5	Lys	74	Lys	139
6	Leu	61	Leu	104
7	Leu	102	Leu	175
8	Tyr	49	Tyr	97
9	X		X	
10	Ser	19	Ser	46
11	Asn	35	Asn	61
12	Gly	39	Gly	61
13	Gly	61	Gly	100
14	Tyr	28	Tyr	48
15	Phe	30	Phe	47
16	Leu	33	Leu	57
17	Arg	37	Arg	51
18	Ile	27	Ile	37
19			Leu	63
20			Pro	34
21			Asp	37
22			Gly	35
23			Thr	15
24			Val	9
25			Asp	35

[a] For sequencing, 200 and 400 pmol of protein from peaks 1 and 2 (Figure 2B), respectively, were applied to the sequenator. Initial yields were about 50% (based on protein quantitation by peak height measurement in HPLC). Average repetitive yields were 89.8% and 90.8% for peaks 1 and 2, respectively. X indicated the absence of an identifiable amino acid derivative. This is compatible with the known presence of cysteine in the corresponding position at the aFGF sequence (Gimenez-Gallego et al., 1985). Analysis of phenylthiohydantoin derivatives showed no detectable contamination of the samples applied to the sequenator.

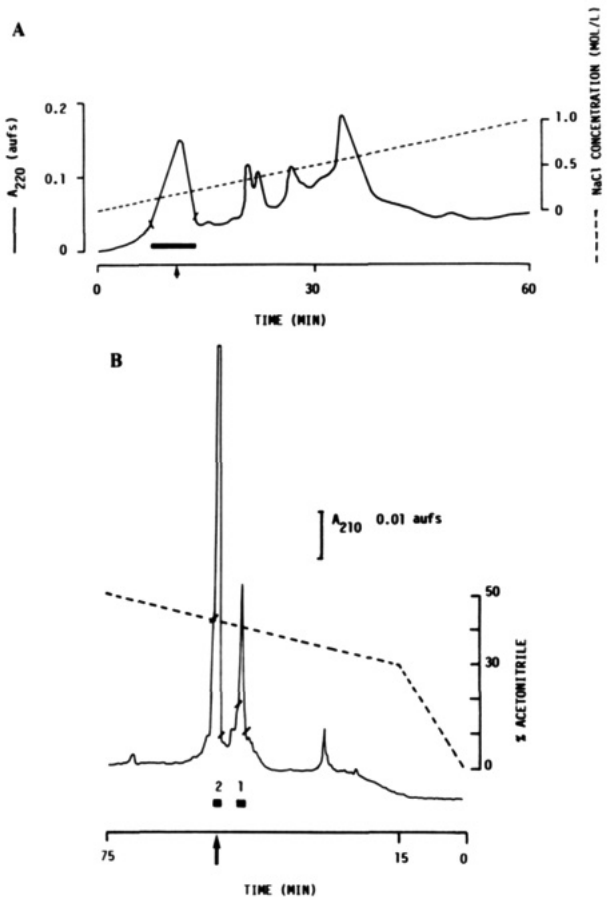

FIGURE 2: (A) Mono-S cation-exchange chromatography. The pool of bioactive fractions from heparin–Sepharose affinity chromatography, marked with "A" in Figure 1, was diluted 5 times and loaded onto a Mono-S column (HR 5/5, Pharmacia) equilibrated with 50 mM sodium phosphate, pH 6.8 (equilibration buffer). After being loaded, the column was washed with the equilibration buffer until the absorbance at 220 nm reached a minimum value. Elution was performed with a 1-h linear gradient of 0–1 M NaCl in the equilibration buffer at a flow rate of 1 mL/min. Due to the low concentration of bioactivity in the collected fractions, a bioassay was not performed. Therefore, those fractions were pooled that had the same retention behavior as authentic aFGF from bovine brain (indicated in the figure by an arrow). (B) Reverse-phase HPLC. Pooled fractions from Mono-S cation-exchange chromatography [marked by a horizontal bar in (A)] were loaded on a Vydac C4 column (25 × 0.46 cm, 5-μm particles, 300-Å pore size, The Separations Group, Hesperia, CA). Elution was performed with a linear 1-h gradient from 30 to 50% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.7 mL/min. Peak fractions were collected as indicated by the horizontal bars. An aliquot of 200 μL of HPLC fraction 2 was dried together with 100 μg of bovine serum albumin in a Speedvac centrifuge (Savant). The dried protein was redissolved in 200 μL of culture medium, and aliquots of 10 μL (corresponding to 9 ng of mitogen in the major peak fraction) were used for bioassay using saphenous vein endothelial cells (see Experimental Procedures).

lecular forms of amino terminally truncated aFGF, des-(1–7)-aFGF. Identical amino-terminal sequences and indistinguishable molecular weights suggest that the two aFGF forms are similar in overall structure. The structural difference responsible for the slight difference in the hydrophobicity of the two molecules (different retention times in reverse-phase HPLC) is not known. Since aFGF contains three cysteine residues (one of them may be present in the sulfhydryl form), it is possible that the two aFGF derivatives arise from disulfide scrambling, which might take place under mild denaturing conditions. Disulfide scrambling is known to occur in interleukin 2 (Browning et al., 1986), which also possesses three cysteine residues (one presumably as free sulfhydryl). Aliquots of HPLC peak 2 (Figure 2B) were tested for bioactivity in

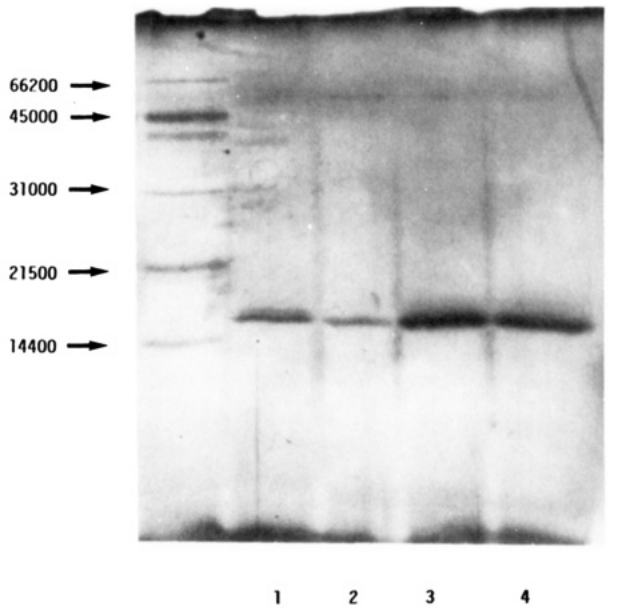

FIGURE 3: Polyacrylamide gel electrophoresis of HPLC-purified aFGF. Aliquots of 40–80 ng of protein were applied to each lane. The gel was stained according to the Bio-Rad silver staining procedure. (Lane 1) aFGFs from bovine brain [(upper band) aFGF-(1–140); (lower band) des-(1–6)-aFGF [see Gautschi et al. (1986)]]; (lane 2) HPLC peak 1 (from Figure 2B); (lane 3) HPLC peak 2 (from Figure 2B); (lane 4) HPlC peaks 1 and 2 (equimolar mixture). Molecular weight markers (left lane) were bovine serum albumin (45 000 Da), ovalbumin (45 000 Da), carbonic anhydrase (31 000 Da), trypsin inhibitor (21 500 Da), and lysozyme (14 400 Da).

a sensitive assay using human endothelial cells in the presence of heparin. In this assay, heparin–Sepharose-purified brain aFGF (our standard preparation) produced maximal response at a concentration of 1 ng/mL. HPLC-purified aFGF from bovine kidney, at a concentration of 9 ng/mL, also elicited maximal response (no dose–response analysis was performed). The combined yield of aFGF forms from kidney amounted to approximately 5 μg/kg of wet tissue. This yield is considerably lower than that obtained with aFGF from brain (Böhlen et al., 1985a) but is similar to that reported for bFGF from tissues such as brain, placenta, and thymus (Gospodarowicz et al., 1986).

As discussed above, kidney tissue extracts also contain mitogenic materials eluting from the heparin–Sepharose column at 1.6–2 M NaCl and 2–3.0 M NaCl (Figure 1). Bioactivity and immunoreactivity profiles show clearly that at least two different mitogens occur in those heparin–Sepharose fractions. Immunoreactivity eluting at 1.6–2 M NaCl is strongly indicative of the presence of bFGF in those fractions. This finding is in general agreement with previous results by Baird et al. (1985b), who isolated and partially sequenced kidney bFGF. However, in contrast to their report which describes the isolation and sequence analysis of two amino terminally truncated forms of bFGF lacking the first 15 amino acid residues, our data strongly suggest that the amino terminally extended form bFGF-(1–146), originally isolated from bovine pituitary (Böhlen et al., 1984; Esch et al., 1985), can also be present in kidney extracts. This conclusion is based on the specificity of the antiserum used in the RIA, which only recognizes the amino-terminal sequence PALPEDGGS of bFGF-(1–146) (Baird et al., 1985c) but not the truncated form des-(1–15)-bFGF. It remains unclear whether the generation of truncated bFGF and, likewise, of several forms of aFGF including des-(1–7)-aFGF (this report), des-(1)-aFGF, des-(1–6)-aFGF (Gautschi et al., 1986; Thomas et al., 1985) or α-ECGF (Burgess et al., 1986) arises from protein processing

in vivo or from enzymatic degradation during tissue extraction and/or protein purification.

The bioactive region eluting from the heparin–Sepharose column at higher salt concentration (>2 M NaCl, Figure 1) has so far not been the subject of further investigations. However, similar activity was also seen previously in preparations of bovine and human brain (Gautschi et al., 1985; Böhlen et al., 1985b).

The presence of aFGF in bovine kidney, as described here, is the first demonstration of the existence of aFGF in an extraneural tissue. At present, we cannot definitely exclude the possibility that aFGF is derived from the local nerve supply. This possibility is considered unlikely, however, because the content of aFGF in kidney tissue seems very high in relation to the limited mass of nervous tissue in this organ. Finally, it is possible that previously aFGF in kidney (and perhaps in other tissues) were overlooked due to the presence of similar concentrations of the much more potent bFGF. Results from this study show that Mono-S cation-exchange chromatography provides an efficient technique for further purifying and unambiguously discerning FGFs obtained by heparin–Sepharose affinity chromatography.

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