Vol. 119, No. 1, 1984 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 29, 1984 Pages 311-318

BRAIN AND PITUITARY FIBROBLAST GROWTH FACTOR ACTIVITIES BEHAVE IDENTICALLY ON THREE INDEPENDENT HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SYSTEMS

John A. Smith, Derek P. Winslow, Michael J. O'Hare and Philip S. Rudland

Ludwig Institute for Cancer Research (London Branch),
Royal Marsden Hospital, Sutton, Surrey. U.K.

Received November 1, 1983

Fibroblast Growth Factors obtained from bovine brain and pituitary glands were compared. They were shown to behave identically on three high performance liquid chromatographic systems, which separate proteins by the independent criteria of size, charge and hydrophobicity. They also migrated similarly on electrophoresis in polyacrylamide gels in the presence of sodium dodecyl sulphate with an apparent molecular weight of 12,000. It was concluded that the two factors were very similar, or identical. They were active at promoting DNA synthesis in rat mammary fibroblasts (Rama 27) at a concentration of 0.1 ng/ml.

In 1974, Gospodarowicz described the presence of a potent mitogen for mouse 3T3 fibroblasts in extracts of brain and pituitary glands (1). The activity was named fibroblast growth factor (FGF) and was subsequently purified from each of these bovine tissues (2, 3). Both activities were characterised as basic molecules of about 13,000 M_r that were acid and heat labile, and as having common growth-promoting activities for a variety of cultured cells derived from embryonic mesoderm (2, 4, 5). It was suggested originally that these two activities might be either related or identical (6), until it was shown that brain FGF could be separated into three forms (2, 7) that corresponded to different peptide fragments of MBP, a structural protein of the central nervous system. Because brain and pituitary FGF preparations had different amino acid compositions and antigenic properties (7) it was concluded that they were distinct entities.

Subsequently, Thomas et al, (8) reported that the brain FGF activity could be separated from the MBP fragments on an affinity column of

Abbreviations: FGF - Fibroblast Growth Factor; EGF - Epidermal Growth Factor; MBP - Myelin Basic Protein

0006-291X/84 $1.50

insolubilised anti-MBP serum. They showed (8) that 3T3 growth-promoting activity could also be found associated with an acidic molecule. The basic activity of brain FGF was found to have properties similar to those of pituitary FGF. Gambarini and Armelin (9) have also found an acidic component of pituitary FGF. However, Gospodarowicz et al., (10) have prepared brain FGF by isoelectric focussing methods and reaffirm the identity of brain FGF with the MBP fragments. Moreover, they find essentially no activity in proteins with acidic pI's in their extracts, and suggest that such findings may be due to contamination of the brain activity with 'myoblast' growth factors (11). In this paper, we re-examine the possible identity of brain and pituitary FGF, and their relationship to MBP, using the higher resolving power and different criteria of separation of three different high performance liquid chromatographic systems.

MATERIALS AND METHODS

Materials Freshly excised cow pituitaries and brains, frozen quickly by submersion in liquid nitrogen and transported within a period of two weeks packed in solid CO_2, were supplied by Imperial Laboratories Ltd., Salisbury, England. All reagents used were of analytical grade. Carboxy methyl trisacryl was from LKB. Cytochrome C and lysozyme were from Sigma. Bovine Serum Albumin was FrV Reagent Grade from Miles Laboratories Ltd., Stoke Poges, Slough, England. Acetonitrile was HPLC S grade from Rathburn Chemicals Ltd., Walkerburn, Peebleshire, Scotland. Pituitary derived FGF (batch 934 54) was from Collaborative Research Inc., Waltham, Mass. Radio-isotopes were from Amersham International.

Preparation of bovine FGF FGF was purified from frozen bovine brain or pituitary glands by a method similar to that of Gospodarowicz et al, (3). The tissues were homogenised at 4°C in 50 mM phosphate-citrate buffer pH 4, and the homogenate was centrifuged for 60 min at 8000 rpm in a Sorvall GSA rotor. The precipitate was re-extracted in 50 mM phosphate-citrate buffer and the supernatants combined. $(NH_4)_2 SO_4$ (290 g/litre) was added, and the solution left for 2-3 hours. The precipitate which formed was removed by centrifuging at 8000 xg for 60 mins. More $(NH_4)_2 SO_4$ (200 g/litre) was added and the precipitate which formed, and which contained most of the mitogenic activity for fibroblastic cells was collected by centrifuging at 8000 xg for 60 mins. The precipitate was dissolved in water, dialysed extensively against water, and finally against 0.1 M sodium phosphate buffer pH 6. The solution was applied to a column 20 cm x 5 cm of carboxymethyl trisacryl which had been equilibrated with 0.1 M sodium phosphate buffer pH 6. After extensive washing with 0.1 M sodium phosphate buffer, pH 6, the column was eluted sequentially by the stepwise addition of 0.15 M and 0.5 M NaCl in 0.1 M sodium phosphate buffer pH 6. The material eluting with 0.5 M NaCl was used as crude FGF, and contained about 80% of the mitogenic activity present in the original extract.

Radio-iodination of Proteins Proteins were labelled with [^{125}I] NaI by the method of Hunter and Greenwood (12).

HPLC Methods Gradient elution reversed phase HPLC, size exclusion and ion exchange HPLC were performed as previously described (13).

SDS Polyacrylamide Gel Electrophoresis 15% SDS polyacrylamide gels were used following the method of Laemmli (14). Autoradiography was performed using Kodak X Omat 5 film and an exposure time of 24 h.
Assay for FGF activity The ability of FGF to increase rates of DNA synthesis was assayed using a clonal line of cells from the stroma of normal rat mammary glands, Rama 27, as previously described (13).

RESULTS

Chromatography of FGF preparations

Crude pituitary and brain FGF, prepared as described in Materials and Methods, were subjected to size exclusion chromatography (Fig. 1b, c). The profiles of mitogenic activity on Rama 27 fibroblastic cells were similar, showing a peak of activity at an elution volume corresponding to a molecular weight of 15-25,000.

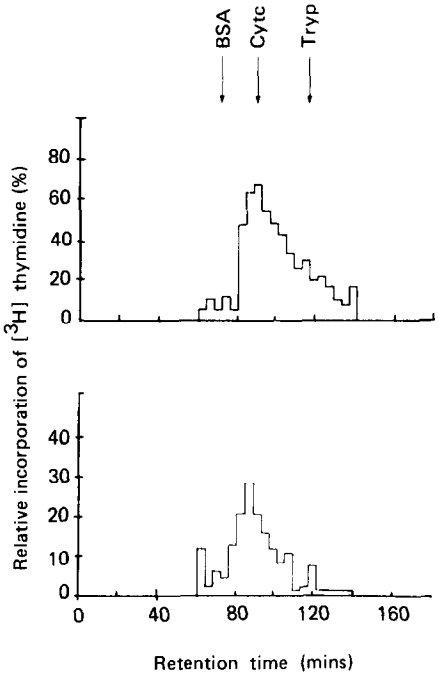

Fig. 1 Size Exclusion High Performance Liquid Chromatography of Fibroblast Growth Factor Preparations. a) A 20 mg sample of crude pituitary FGF preparation, prepared as described in "Materials and Methods" was loaded onto a LKB TSK-G 3000 SWG column, 60 cm x 21.5 mm I.D. and chromatographed at a flow rate of 2 ml/min in 0.1 M sodium phosphate buffer pH 6.5 containing 0.3 M NaCl. 8 ml fractions were collected. 10 µl samples were used for assay. b) A 6 mg sample of crude brain FGF preparation prepared as described in "Materials and Methods" was loaded onto a LKB TSK-G 3000 SWG column, 60 cm x 21.5 mm I.D. and chromatographed as described for b). 8 ml fractions were collected. 100 µl samples were used for assay.
Molecular weight markers used were Bovine Serum Albumin (68 kd), Cytochrome C (11.7 kd) and L-Tryptophan (0.2 kd).

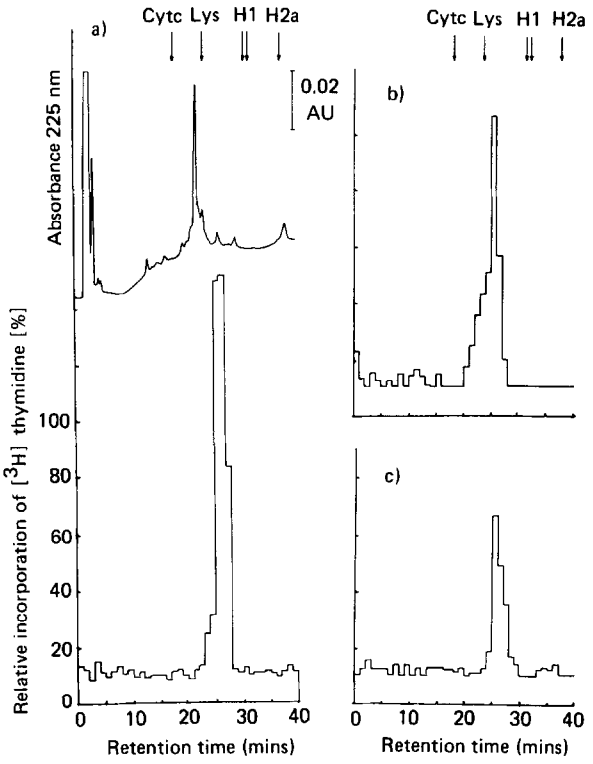

Fig. 2 Ion Exchange High Performance Liquid Chromatography of Fibroblast Growth Factor Preparation. a) A 10 µg sample of commercial pituitary FGF was loaded onto an Altex Spherogel TSK IEX 535 CM column, 15 cm x 6.0 I.D. and chromatographed at a flow rate of 1 ml/min in the same buffer with a gradient of 25 mM/min of NaCl in 0.1 M sodium phosphate pH 6. 1 ml fractions were collected. 10 µl samples were used for assay.
b) A 50 µg sample of the initial extract was dialysed against 0.1 M sodium phosphate buffer pH 6 and chromatographed on an Altex Spherogel TSK IEX 535 CM column as described for a). 1 ml fractions were collected, 10 µl samples were used for assay.
c) A 1 mg sample of crude brain FGF preparation prepared as described in "Materials and Methods" was chromatographed on an Altex Spherogel TSK IEX 535 CM column as described for a). 1 ml fractions were collected. 20 µl samples were used for assay.
Standard proteins used were cytochrome C, lysozyme, rat thymus histone H1 (two peaks) and rat thymus histone H2a.

High resolution ion exchange chromatography was performed on a column with carboxy-methyl charged groups (Fig. 2). Mitogenic activity from the initial pituitary extract (Fig. 2b) or from crude brain FGF prepared as described in Materials and Methods (Fig. 2c) was found at the same elution time. The growth factor activity present in a preparation of Thyroid Stimulating hormone, the original source of pituitary-derived fibroblast stimulating activity, (15) also eluted at the corresponding time and was well separated from the major TSH-related components.

When a commercial preparation of pituitary FGF was chromatographed by ion exchange HPLC, the activity eluted at the same time as that in our preparations (Fig. 2a). The peak of U.V. absorbance (215 nm) which accompanied activity in the commercial preparation was equivalent to approximately 0.25 µg protein. Since the nominal content of protein was 10 µg and recovery of activity was about 100%, the commercial preparation had a maximum purity of 2.5%. The FGF activity in the commercial preparation also eluted at the same time as our preparations on the size exclusion and reversed phase systems.

High resolution reversed phase chromatography was performed on a column with a C_3 bonded phase (Fig. 3). Mitogenic activity from crude

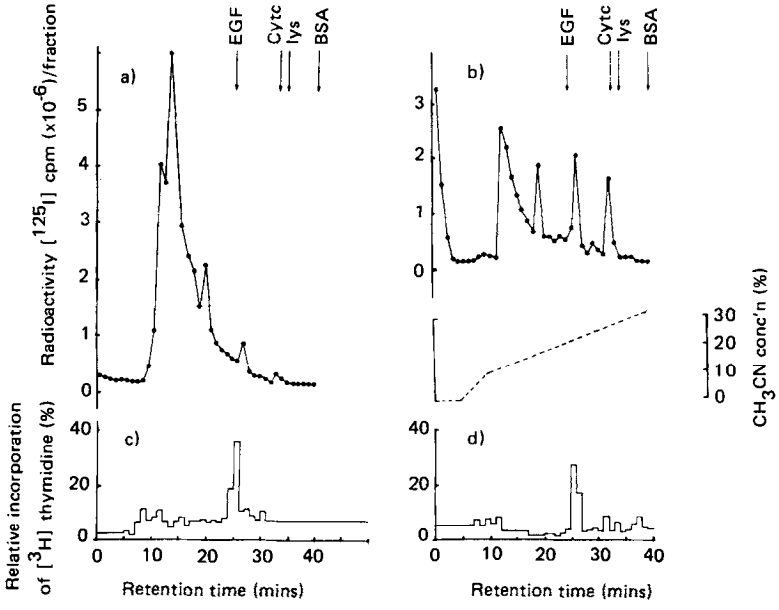

Fig. 3 Reversed Phase High Performance Liquid Chromatography of FGF Preparations. a,b) Chromatography of ^{125}[I] radiolabelled brain and pituitary FGF preparations, respectively on an Ultrapore RPSC column. In each case the crude FGF preparations were subject to further purification by size exclusion and ion exchange HPLC prior to iodination. Standard proteins used were mouse EGF cytochrome C, lysozyme and bovine serum albumin.
c) A 500 µg sample of crude brain FGF preparation and d) A 1 mg sample of crude pituitary FGF preparation prepared as described in "Materials and Methods" were chromatographed on an Ultrapore RPSC column, on a column 15 cm x 4.6 mm I.D. using 0.45 M NaCl in 0.1% (w/v) Trifluoroacetic acid as primary solvent and 0.1% (w/v) Trifluoroacetic acid in CH_3CN as secondary solvent, with the CH_3CN gradient shown, and a flow rate of 1 ml/min. 1 ml fractions were collected. 100 µl samples were used for assay.

pituitary and brain FGF prepared as described in Materials and Methods was found at the same elution time.

Analysis of Purified Material

Since the resolution of the molecular filtration HPLC system is not as great as that achieved by ion exchange or reversed phase HPLC, an alternative method of assessment of molecular weight was used, namely, SDS polyacrylamide gel electrophoresis was used. The presence of SDS in the samples is however, toxic for the cells in the bioassay, even at a concentration of 0.5 µg/ml. Therefore crude brain and pituitary FGF prepared as described in "Materials and Methods" was subjected to size exclusion HPLC, followed by carboxymethyl ion exchange HPLC, and then iodinated in the presence of 5 µg BSA and re-purified by ion exchange HPLC. This material was then chromatographed by reversed phase HPLC (Fig. 3), and fractions from this chromatogram were subjected to SDS polyacrylamide gel electrophoresis, (Fig. 4). The major radiolabelled products from the brain

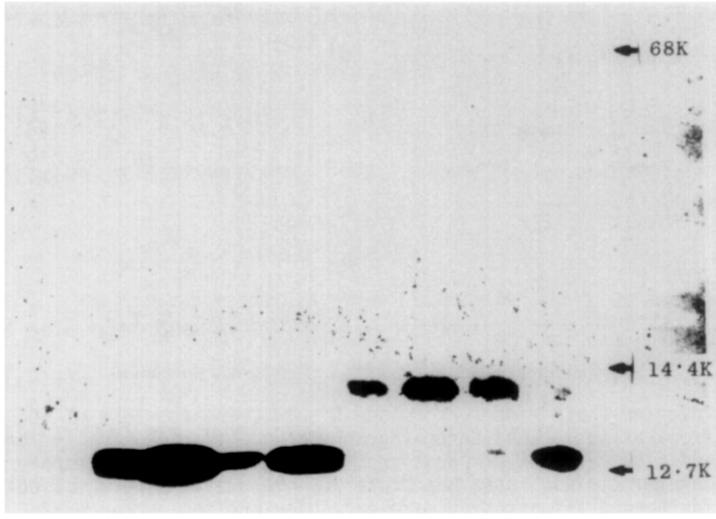

Fig. 4 SDS Polyacrylamide Gel Electrophoresis of Fibroblast Growth Factor Preparations. Autoradiogram of iodinated materials from the Ultrapore RPSC chromatograms shown in Fig. 3. a,b) pituitary preparation fraction 27 c,d, brain preparation fraction 27 e) brain preparation fraction 13 f) brain preparation, fraction 15 g) brain preparation fraction 17 h) brain preparation, fraction 20. Loadings were 100,000 cpm except channels a,c, 20,000 cpm.

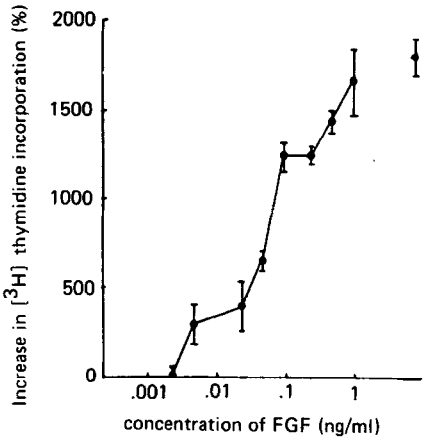

Fig. 5 Effect of Pituitary-Derived Fibroblast Growth Factor on a Rat Mammary Fibroblast Cell Line. The stimulation of incorporation of ^{3}[H] radiolabelled thymidine into DNA was measured in Rama 27 cells. FGF was a commercial preparation further purified by ion exchange HPLC.The stimulation achieved by addition of 5% foetal calf serum is indicated at the right.

preparations have the same molecular weight as the proteins labelled FGF-1 and FGF-2 and identified as fragments of MBP by Gospodarowicz et al, (10). These radio-labelled proteins, however, elute earlier than the growth promoting activity on the reversed phase system. The radio-labelled protein which elutes at the position which contains mitogenic activity migrates on electrophoresis with an apparent molecular weight of 12,000. FGF was highly active at stimulating DNA synthesis in a fibroblast cell line up to the level obtained with 5% FCS. Half-maximal stimulation was achieved at a concentration of 0.1 ng/ml (Fig. 5).

DISCUSSION

In contrast with EGF, the structure, function and molecular biology of FGF are essentially unknown, despite the fact that its purification was published shortly afterwards. This is because there has been a lack of availability of pure FGF, as exemplified by the large amount of non-FGF material in at least one commercial preparation (Fig. 1). This lack of homogeneous material, and of suitable chromatographic systems to establish its purity, has also meant that it is still unclear whether or not the pituitary- and brain-derived FGFs are the same molecule. Here we have shown that the FGF activity from both sources behaves identically on a new

system of size exclusion, on high resolution ion exchange separation and on the reversed phase column, which separates by the independent criterion of hydrophobicity (16). While it has not yet been possible to recover growth promoting activity from SDS polyacrylamide gels, the similar mobilities of the radioactive bands of the radio-iodinated materials derived from pituitary and brain suggests that the factors are also virtually identical in molecular weight. Thus there can now be little doubt of the common identity of these two mitogenic activities and no major activity other than that of the basic FGF was present in our extracts. The brain FGF activity was separated from MBP on the reversed phase system. Thus our establishment of chromatographic parameters for FGF should make possible its final purification from pituitary and brain.

ACKOWLEDGEMENTS

We thank Mr. G. Cowley for assistance with radiolabelling.

REFERENCES

1. Gospodarowicz, D. (1974) Nature 249, 123-127.
2. Gospodarowicz, D., Rudland, P.S., Lindstrom, J. and Benirschke, H. (1975a) Adv. Metab. Disord. 8, 301-335.
3. Gospodarowicz, D., Bialecki, H. and Greenberg, G. (1978) J. Biol. Chem. 253, 3736-43.
4. Rudland, P.S., Seifert, W. and Gospodarowicz, D. (1974) P.N.A.S. 71, 2600-2604.
5. Gospodarowicz, D., Greenberg, G., Bialecki, H and Zetter, B.R. (1978) In Vitro 14, 85-118.
6. Gospodarowicz, D. (1975) J. Biol. Chem. 250, 2515-1520.
7. Westall, F.C., Lennon, V.A. and Gospodarowicz, D. (1978) P.N.A.S. 75, 4675-8.
8. Thomas, K.A., Riley, M.C., Lemmon, S.K., Baglan, N.C. and Bradshaw, R.A. (1980) J. Biol. Chem. 255, 5517-20.
9. Gambarini, A.G. and Armelin, H.A. (1982) J. Biol. Chem. 257, 9692-9697.
10. Gospodarowicz, D., Lui, G.M. and Chang, J. (1982) J. Biol. Chem. 257, 1266-12276.
11. Gospodarowicz, D., Weseman, J. and Moran, J. (1975) J. Biol. Chem. 256, 216-219.
12. Hunter, W.M. and Greenwood, R.C. (1962) Nature 194, 495-496.
13. Smith, J.A., Ham, J., Winslow, D.P., O'Hare, M.J. and Rudland, P.S. (1984) J. Chromatog. 305, 295-308.
14. Laemmli, U.K. (1970) Nature 227, 680-685.
15. Holley, R.W. and Kiernan, J.A. (1968) Proc. Natl. Acad. Sci. 60, 300-304.
16. O'Hare, M.J. and Nice, E.C. (1979) J. Chromatog. 171, 209-226.