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EVIDENCE FOR FGF-LIKE GROWTH FACTOR IN ADULT BOVINE RETINA : ANALOGIES WITH EDGF I

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Eye Derived Growth Factor (EDGF) is the genus name for growth factor activities found in several ocular tissues. Purification from bovine retina by Cibacron blue affinity chromatography has previously given a fraction which can induce target cell proliferation at doses of 30 ng per ml of culture medium. Radioimmunoassay using a labelled synthetic decapeptide [Tyr 10] - FGF (1-10) including the 9 N terminal aminoacids of brain Fibroblast Growth Factor (FGF) indicated that EDGF contained a FGF-like material. Further purification of Cibacron blue purified EDGF with heparin sepharose chromatography yielded two active fractions after elution with a sodium chloride gradient. One fraction named EDGF I eluted between 1.3 and 1.5 M NaCl and accounted for over 50% of the input biological activity and comigrated with purified FGF on SDS PAGE at a molecular weight of 16,000 d as a single band. FGF competed with EDGF I for binding to specific receptors on bovine epithelial lens cells. We conclude that retina contains a growth factor activity (EDGF I) similar if not identical to FGF.

Several ocular tissues have been shown to contain growth factor activity over a broad range of responsive target cells (1,2,3). Adult bovine retina has been used as a source of purification of such growth promoting activity to which we applied the genus name of Eye Derived Growth Factors (2) (EDGF). In 1982 (4) EDGF was purified over 5,000 times and a fraction obtained after acetic acid precipitation, Cibacron blue affinity chromatography and gel permeation chromatography with an apparent molecular weight of 17,500 d ± 2,500 d and an isoelectrical point of 4.5 ± 0.5 could stimulate cell proliferation at doses of 5 ng x ml^{-1}. It was clear from SDS PAGE that this growth factor activity needed to be further purified and characterized.

However at this step of purification we compared EDGF from retina with growth factor activity derived from brain (called Brain Derived Growth Factor or BDGF) and we concluded that both brain and retina tissues, contained growth factor activities with analogous biochemical and biological behaviour (5). From biochemical and biological data

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obtained in previous studies we also excluded an identity with Epidermal Growth Factor (EGF) and Fibroblast Growth Factor (FGF) (2,5).

The recent purification of FGF from pituitary and brain to homogeneity (6) as well as that of other mitogens from the brain and other tissues (7,8,9,10) including retina (11), all based on heparin sepharose affinity chromatography, prompted us to use this technique to further purify EDGF. We showed that EDGF could be fractionated into two or three different biological active fractions during heparin chromatography (12). A crude homogenate of retina obtained at a neutral pH applied to a heparin sepharose affinity chromatography could be separated into three biologically active fractions : a non-retained acid labile fraction, which we did not further characterize but named EDGF'III ; a fraction eluted with 1 M Nacl named EDGF II which cross-reacted with poly and monoclonal antibodies against ECGF

and competed with this molecule in a radioreceptor assay (13). Elution at 1.4 M NaCl yielded another fraction named EDGF I. Heparin sepharose chromatography of EDGF AB only produced EDGF I and II. In this report we compared EDGF I to basic FGF.

MATERIALS AND METHODS

1. Preparation of FGF and EDGF I

FGF was prepared and purified to homogeneity as published (6). EDGF was purified by acetic acid treatment (EDGF 0.1N) or by Cibacron blue affinity chromatography (EDGF AB) as previously described (4) and applied to heparin sepharose chromatography in a PBS buffer where NaCl concentration had been increased to 0.65 M to diminish non specific interactions. EDGF I was the fraction eluted between 1.3 and 1.5 M Nacl (12).

2. Bioassays

The mitogenic activity was measured in bovine epithelial lens cells as described (14). Other cells were also used, such as Rat 3T3 or human fibroblasts.

A stimulation unit was defined as the amount of growth factor required to give half maximal tritiated thymidine incorporation into target cells. Each measurement was performed at four different dilutions and in triplicates.

FGF was also tested for its ability to stimulate the proliferation of adult bovine aortic endothelial cells (ABAE) as described (7). Half maximal stimulation was obtained with values between 25 and 50 pg of purified FGF and measured in bioassays based on 3H thymidine incorporation into BEL cells, and on ABAE cell proliferation. This half maximal dose was defined as the stimulation unit.

Protein concentration was measured using the Biorad protein assay with bovine serum albumin as a standard. For NaCl gradient elution fractions of the heparin chromatography, protein concentration was measured either by amino acid analysis or comparative density on silver stained SDS PAGE.

3. Radioimmunoassay

Antibodies against bovine serum albumin conjugated synthetic decapeptide Pro Ala Leu Pro Glu Asp Gly Gly Ser Tyr :

[Tyr] - FGF (1-10) which represents the sequence of the nine terminal aminoacids of FGF were obtained as described (15). The presence of cross-reacting materials in acetic acid and Cibacron blue purified EDGF was detected and measured using the radioactivity decapeptide as a tracer (16).

4. Radioreceptor assay

- Purified EDGF I was labelled with the chloramine T technique and repurified on heparin sepharose chromatography.
- Preparations with specific activities between 10,000 and 20,000 cpm / ng were used.
- Human fibroblasts AG 1523 were grown in 24-well culture plates (2 cm^2 per well) up to the end of the exponential phase and rinced twice with PBS-BSA (1 mg x ml) binding buffer at 4°C. Displacement of ^{125}I-EDGF I (0.7 nM) was obtained by incubation for 2 hours at 4°C with increasing amounts of cold FGF. Cells were washed 6 times with the binding buffer, solubilized with 1% triton and counted.

RESULTS

I. Immunological evidence for the presence of FGF cross-reactivity material in retina

Radioimmunoassays were performed against EDGF 0.I N and EDGF AB. Fig. 1 illustrates

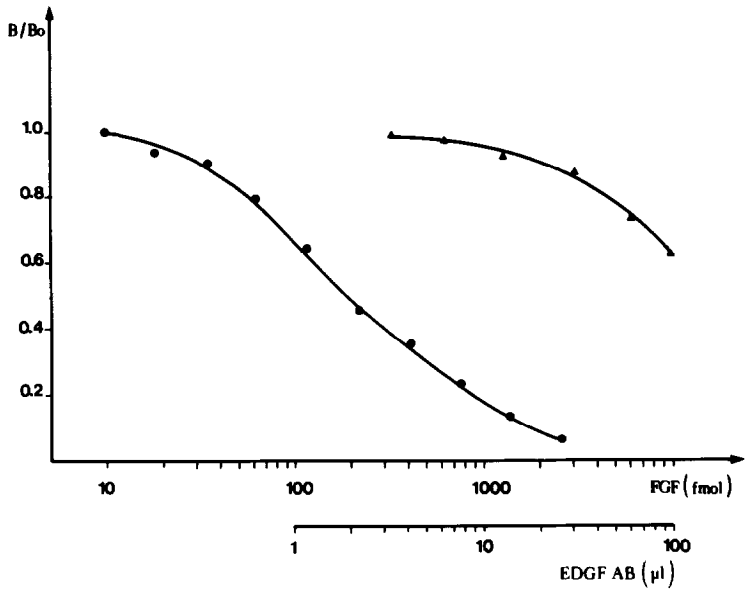

Fig. 1: Radioimmunoassay for FGF in partially purified EDGF AB by Cibacron blue chromatography.

Antibodies raised against the Tyr 10 -FGF (1-10) N terminal peptide is used in this assay. ^{125}I labeled peptide is displaced by increasing amounts of FGF (•••) or EDGF AB(▲▲)

the displacement of ^{125}I-labeled N terminal peptide by purified FGF and by various volumes of EDGF AB. In this example 100 ul (22 µg) of EDGF AB displace 130 f mole of FGF (1-9) peptide. For an estimated molecular weight of 16,000 d for FGF this translates into 21 ng of intact FGF per ml of EDGF AB or 95 ng of intact FGF per mg of EDGF AB.

The same measurement was done with EDGF 0.1N and indicated a content of 11.4 ng of intact FGF per ng EDGF 0.1N (dose curve not shown).

2. Analogous behaviour of FGF and EDGF I on heparin sepharose and SDS PAGE electrophoresis

Since heparin is a very heterogeneous family of molecules, binding of FGF or EDGF I to heparin sepharose could vary from one batch to another, and elution at 1.4 M NaCl of biological activity for both factors might lead to premature and perhaps artefactual conclusions if this property is not tested with the same batch of heparin sepharose. Fig. 2 illustrates the elution profiles and biological activity of Cibacron blue purified EDGF and FGF which were purified using the same heparin sepharose batch.

Table 1 indicates that most biological activity of EDGF was recovered in two fractions, one eluted between 0.8 and 1 M NaCl EDGF II corresponding to 33 % of the input biological activity, and a second one eluted between 1.3 and 1.5 M Nacl which contained 80 % of the biological activity of the starting material. All the biological activity of FGF was eluted between 1.3 and 1.5 M NaCl, as expected.

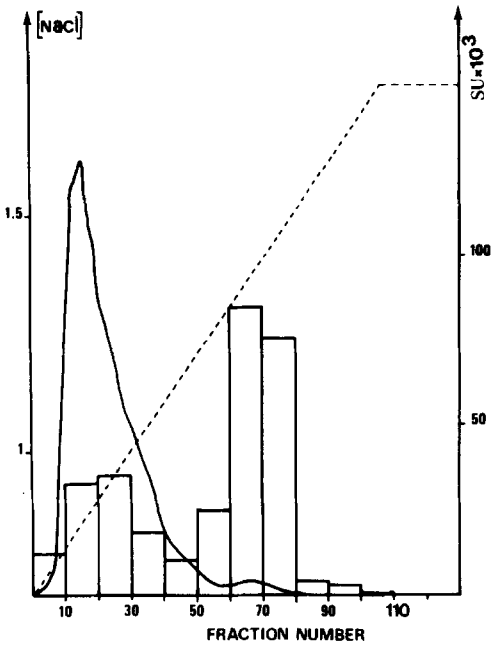

Fig. 2: Heparin sepharose chromatography of EDGF AB.

A230 absorption profile of chromatographed EDGF is presented (continuous line) before and during NaCl gradient elution (dotted line). Biological activity has been determined for each fraction. The number of stimulation unit (SU) per fraction is represented by rectangles. EDGF I elutes between fraction 60 and 80.

Dose response curves of 3H-thymidine incorporation are presented in Fig. 3, and results summarized in table 1. Fractions have stimulation units below 50 pg per ml of culture medium.

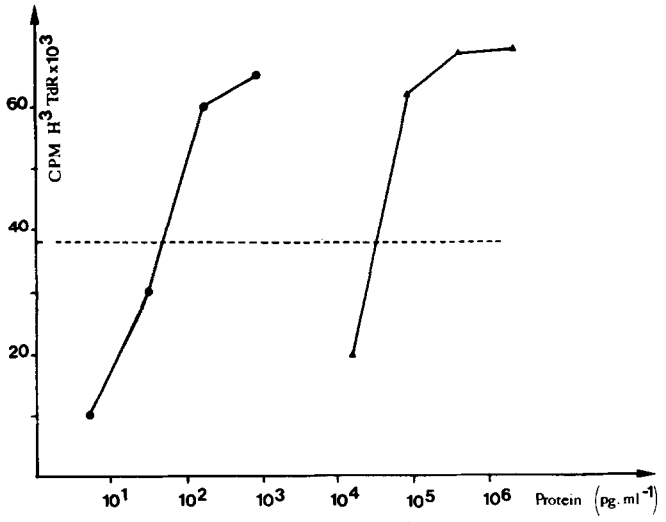

Fig. 3: Dose response curves of EDGF I.

starting material (EDGF AB)
(SU is at 30 ng x ml⁻¹)

pool of the fractions 60 to 80 : elution at 1.3-1.5 M NaCl
(SU is at 50 pg x ml⁻¹)

Table I : Biological activity yields of heparin sepharose chromatography of EDGF AB

	SU $pg.ml^{-1}$	Total SU	Activity yield
Starting Material	30.000	203.000	100%
Non Retained Fraction	ND	ND	ND
EDGF II Elution 0.8-1.5M NaCl	1000	68.000	33%
EDGF I Elution 1.3-1.5 M NaCl	50	162.000	80%
Pool of Fractions Elution 0.65-1.6 M NaCl	4000	300.000	150%

Values are for 300 retinas.

Electrophoresis on SDS PAGE, stained with silver nitrate, shows a single band for EDFG I and FGF with the same apparent molecular weight, 16,000 (Fig.4).

^{125}I-EDGF I is able to bind specifically to human fibroblastic cells (AG 1523). This binding is saturable and reversible (Moenner et al, manuscript in preparation). Competition experiments show that this binding can be deplaced by adding increasing amounts of cold FGF (Fig.5).

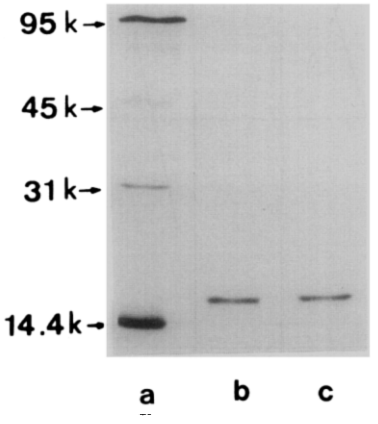

Fig. 4: SDS PAGE electrophoresis.

SDS gel electrophoresis of EDGF I (b) and brain FGF (c) (20% polyacrylamide gel) after heparin sepharose purification. Molecular weight was calculated using markers (a) and least square regression linearisation.

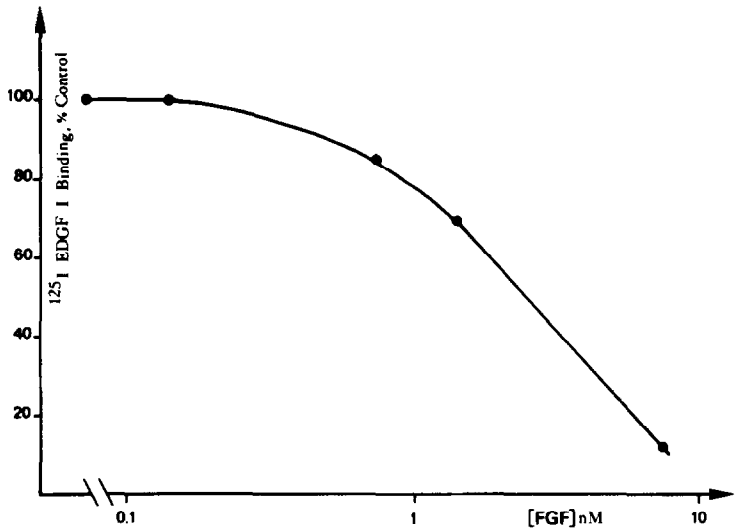

Fig. 5: Radioreceptor assay.

Radioreceptor competition assay between ^{125}I EDGF I and cold FGF on human foreskin fibroblasts AG1523.

DISCUSSION

Radioimmunoassay with the ^{125}I-Tyr FGF (1-9) N terminal decapeptide and Cibacron blue purified EDGF indicates that FGF cross-reacting material is present in adult bovine retina.

At this step of purification the stimulation unit of EDGF is at 30 ng per ml of culture medium (4). The cross-reacting material represents 0.095 % of the protein content.

The stimulation unit of FGF or EDGF I is below 50 pg/ml of culture medium after heparin sepharose chromatography. This value reflects a 600 fold purification and is in agreement with the estimation given by radioimmunoassay. The accuracy of this estimation (mainly due to variability of a factor of two in the measurement of the stimulation unit) is consistent with the value of 33 % of the input biological activity recovered in EDGF II as indicated in Table 1.

The presence of FGF or FGF-like material in retina has not previously been reported. We previously found that isoelectrofocalisation yielded over 90 % of the recovered biological activity at pH 4.5 ± 0.5, although we could not account for an overall loss of 70% of biological activity (3). Since FGF has an isoelectrical point at 9.6 it may be that EDGF I was lost as our pH gradient ranged from 3 to 9.

Our results are also partially in contradiction with a recent publication asserting that adult bovine retina contains a single growth factor named Retinal Derived Growth Factor (RDGF) (9) eluted from a heparin sepharose chromatography. Based on this property as well as on the measurement of the isoelectrical point, the authors suggested that RDGF might be identical to a growth factor purified from brain (acidic FGF) or hypothalamus (ECGF). Our recent comparative studies led us to conclude that EDGF II was analogous to those growth factors (13).

However, as opposed to our results, RDGF was the only growth promoting activity found in the retina. This discrepancy may reflect differences in our preparations since RDGF was purified after an overnight incubation of undissociated retinas while EDGF was purified from a total disruption of tissue by homogenization.

During the preparation of this manuscript, A.Baird et al. (17) demonstrated that retina contained basic FGF on the basis of identical N-terminal aminoacid sequences. These results, added to those presented in this manuscript, strongly support that EDGF 1 and basic FGF are the same molecule.

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