Vol. 180, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1991 Pages 145-151

MITOGENIC PROPERTIES OF A NEW ENDOTHELIAL CELL GROWTH FACTOR RELATED TO PLEIOTROPHIN

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Received August 7, 1991

A growth factor was isolated from a neutral pH extract of adult bovine brain. Purification of this polypeptide was achieved by a three step procedure including cationic exchange, heparin-Sepharose affinity and Mono S chromatography. This heparin binding protein had a molecular weight of 18,000 as assessed by silver-stained SDS-PAGE and was not immunologically and structurally related to acidic or basic FGF. Freshly purified protein had a maximal mitogenic effect on bovine brain capillary cells at a concentration of 100 pM. Microsequencing revealed an unique amino-terminal sequence homologous to heparin-binding growth-associated molecule (HB-GAM), a neuronal maturation protein, to pleiotrophin (PTN), a fibroblast cell growth factor and to one form of the putative protein product of the MK gene, a retinoic acid induced-gene.

In recent years, numerous factors that induce angiogenesis have been isolated and characterized. The acidic and basic forms of fibroblast growth factor (acidic and basic FGF) (1,2), transforming growth factor ß (TGF ß) (3, 4), transforming growth factor α (TGFα) (5), tumor necrosis factor α (TNFα) (6, 7), angiogenin (8), platelet-derived endothelial cell growth factor (PD-ECGF) (9) and more recently folliculo stellate-derived growth factor named vasculotropin (10) can be can be included in this class.

In a course of purification of FGFs from adult bovine brain, we detected a heparin binding polypeptide immunologically distinct from FGF related molecules which displayed a mitogenic effect on capillary endothelial cells . In

Abbreviations: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide electrophoresis; HB-GAM, heparin-binding growth-associated molecule; PTN, pleiotrophin; MK, midkine; FGF, fibroblast growth factor; NCS, new calf serum; BBC, bovine brain capillary; BSA, bovine serum albumin.

0006-291X/91 $1.50

this report, we describe the purification and biological properties of this mitogenic molecule.

Materials and Methods

Materials - Adult bovine brains were obtained from a local slaughterhouse and transported on ice. S-Sepharose Fast Flow, Mono S HR 5/5 and heparin-Sepharose CL-6B were from Pharmacia LKB Biotechnology. Dulbecco's modified Eagle's medium (DMEM), new calf serum (NCS), streptomycin, penicillin and fungizone were obtained from Gibco. Human recombinant acidic FGF was a kind gift of Dr. Jaye (Rhône Poulenc Rorer) and human basic FGF a kind gift from Dr. Mazue (Farmitalia, Milano). All other reagents were of analytical grade and were purchased from Prolabo.

Purification procedures - Batches of 3 kg of bovine brains were dissected free of blood clots and meninges, homogenized for 3 min using a Philips blender (type HR 1375/A) in two volumes of cold PBS (140 mM NaCl, 3 mM KCl, 1.46 mM KH_2PO_4, 6.5 mM Na_2HPO_4 pH 7.4). The homogenate was stirred for 2 hr and there
after clarified by centrifugation at 14,400 g for 60 min at 4°C. The resulting supernatant was then filtered through three layers of cheesecloth and directly applied to a S Sepharose Fast Flow column (200 ml) at a flow rate of 42 ml/min. The column was preequilibrated with 100 mM sodium phosphate buffer pH 6.00 (washing buffer) until the absorbance at 280 nm of the eluate became lower than 0.1 absorbance unit. Bound material was eluted in 500 ml of washing buffer containing 0.6 M NaCl at a flow rate of 8 ml/min. Eluted fractions were subsequently loaded onto heparin-Sepharose CL-6B gel (7.5 ml) that had been preequilibrated in PBS buffer containing 0.5 M NaCl (equilibration buffer). In order to eliminate the unadsorbed material, the resin was extensively washed with the equilibration buffer until the absorbance of the washing eluate reached the baseline. After readjustment of flow rate at 0.5 ml/min, the bound material was then eluted by stepwises increasing NaCl concentration to 1 M. Fractions of 2 ml were collected and individually analyzed for growth promoting activity and protein concentration. The active eluted fractions from heparin-Sepharose affinity chromatography were pooled, diluted 10-fold with 50 mM sodium phosphate buffer pH 7.0 (dilution buffer) and loaded onto a Mono S HR 5/5 column equilibrated at room temperature in dilution buffer. Elution was performed at a flow rate of 0.5 ml/min with a linear gradient ranging from 0 to 1 M NaCl in dilution buffer. Absorbance was monitored at 280 nm. Fractions of 1 ml were collected and tested for mitogenic activity and protein concentration.

Protein determination - Protein concentration were determinated using BCA protein assay (11) with bovine serum albumin as a standard.

Mitogenic Assays - The growth promoting activity of the various purification fractions was determinated using bovine brain capillary (BBC) as target cells routinely cultivated and subcultured in DMEM and supplemented with 10 % of new calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml fungizone (DMEM-10). For bioassay, cells were plated at a initial density of 5 x 10^2 per cm^2 in 6 multiwell plates in 2 ml of DMEM-10. Four hours later, batches of 2 wells were trypsinized and resulting cells were counted to define cell density on day 0. Aliquots of 10 µl from purification fractions diluted 1/2 with PBS containing 0.1% BSA were then added in duplicate on days 0 and 2. Cells were grown until day 4, and final cellular density was determinated using a Coulter counter. For [^{3}H] thymidine incorporation into DNA, BBC were seeded in 24-well plates in 0.5 ml of DMEM-10 at a final density of 20,000 cells per well and grown for 48 h. Medium was then replaced with DMEM containing 5 µg/ml insuline, 5 µg/ml transferrin, 5 ng/ml sodium selenite and 5 mg/ml of bovine serum albumin (BSA). Cells were further incubated for 24 hours. Ten-µl of fractions from each purification step were then added. After incubation at 37°C for 20 h, the cells were pulsed with 1 µCi methyl [^{3}H] thymidine per well for 4h, and cell monolayers were treated with 0.5 ml of 10 % trichloracetic acid for 15 min at 4°C, washed with tap water, and dissolved in 0.5 ml of 0.3 M NaOH for counting in scintillation counter. Assays were performed in triplicate.

Results

Table 1 summarizes the steps for the purification of PTN-like molecule. Clarified neutral extract from adult bovine brain was loaded onto a column of S Sepharose Fast Flow. About 61% of the protein exhibiting mitogenic activity was eluted at 0.6 M NaCl. This step provides a 62.5-fold purification. The resulting bioactive fractions were collected and subsequently passed through a column of heparin-Sepharose. More than 99% of the total protein loaded was not retained by the column. Fractions eluted with 1 M NaCl were tested and yielded a further 1333 fold increase in specific activity (Table 1). These fractions were further purified by Mono S cationic-exchange chromatography. 82% of the biological activity injected was recovered from the Mono S column in a single peak which eluted at an ionic strength of 0.5 to 0.65 M NaCl (Fig. 1). Analysis by SDS-PAGE of the mitogenic material present in Mono S peak, revealed a 18 kDa band (insert of Fig. 1). N-terminal amino-acid sequence analysis of this material revealed a single amino-acid terminal attesting to the homogeneity of this molecule. The resulting N-terminal amino-acid sequence: Gly-Lys-Lys-Glu-Lys-Pro-Glu-Lys-Lys-Val-Lys-Lys-Ser-Asp-Cys-Gly-Glu-Trp-Gln-Trp-Ser-Val-Cys-Val-Pro, is identical to heparin-binding growth-associated molecule (HB-GAM) (12),

Table 1. The purification of PTN-like from 1 kg of adult bovine brain

Purification step	Proteins (mg)*	ED_{50} (ng/ml)**	Specific Activity (Unit/mg)	Purification factor
Brain homogenization	18 000	25 000	40	1
S Sepharose fast flow	175	400	2 500	62.5
Heparin Sepharose	0.45	0.5	2 000 000	50 000
Mono S FPLC	0.37	0.3	3 332 000	83 300

* Protein concentration was estimated by using the BCA Protein Assay Reagent with bovine serum albumin as standard.

** The ED_{50} was determined as the protein concentration (ng/ml of culture medium) that induced a half maximal stimulation in the BBC cell assay. One stimulation unit corresponds to this concentration.

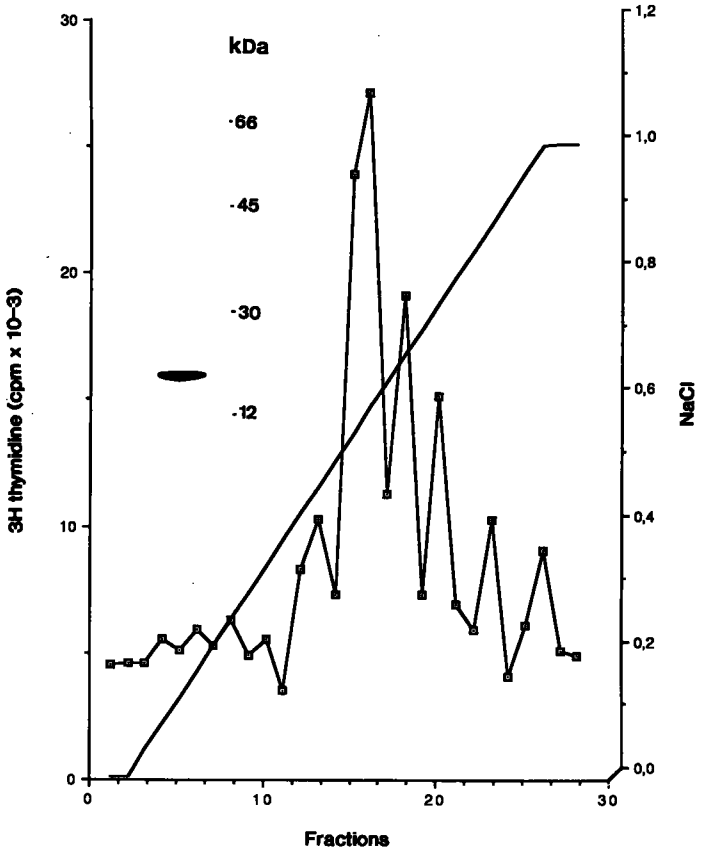

Figure 1. Mono S ion-exchange chromatography. The bioactive fractions derived from heparin-Sepharose affinity chromatography were pooled and diluted 10-fold with 50 mM sodium phosphate buffer, pH 7.00. The resulting sample was then directly applied onto a Mono S HR 5/5 column. The sample was eluted with a gradient of NaCl (0-1 M). Aliquots of each fraction were tested for mitogenic activity and protein concentration.
The insert shows a silver stained SDS-PAGE analysis of the fractions eluted from Mono S column at a concentration 0.55-0.6 M NaCl.

heparin-binding neurotrophic factor (HBNF) (13), PTN (14). Furthermore, the sequence displayed 48.3% identity in a 89-amino acid overlap with predicted sequence of the putative protein product of the MK gene (15). As illustrated in Figure 2, Mono S purified material had a dose-dependent mitogenic effect on bovine capillary endothelial cells. The dose response curve for a purified growth factor revealed a half maximal effect on bovine brain capillary endothelial cell proliferation at 10 pM (0.2 ng/ml) and a maximal effect at 100 pM (2 ng/ml).

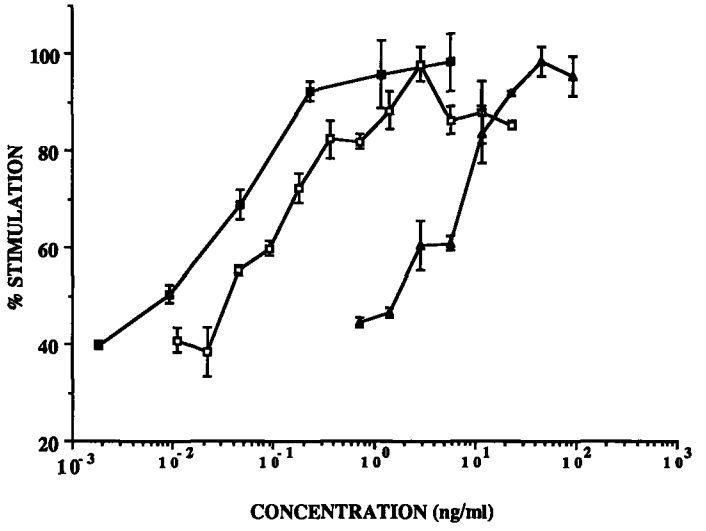

Figure 2. Graph showing the mitogenic effect of PTN-like molecule on BBC cells.
Bovine brain capillary endothelial cells ($5x10^3$ cells in 1 ml) were plated onto 6-well tissue culture dishes (Falcon) on day 0. On day 0 and 2 various concentrations of freshly PTN-like (□), acidic (▲) or basic (■) FGF were added in duplicate as indicated. After 5 days in culture, duplicate dishes were trypsinized and cells were counted. The values shown are means +/- SD for two wells in two independent experiments.

Discussion

In this report, we described the purification and the biological characterization of an endothelial cell growth factor derived from adult bovine brain. The combination of S Sepharose Fast Flow chromatography, heparin affinity chromatography and Mono S FPLC chromatography allowed us to isolate this heparin binding molecule yielding 370 µg per kg of adult bovine brain tissue with an overall 83,300 fold increase in specific activity (Table1). On the basis of growth-stimulating properties, molecular weight and affinity for heparin-Sepharose, this mitogenic molecule ressembles FGFs. However, several lines of evidence indicated that this polypeptide differs significantly in structure from FGF related molecules. These include: i) failure to react with highly specific antibodies directed against acidic and basic FGF using Western blot analysis (not shown), ii) chromatographic retention times differing clearly in Mono S chromatography and iii) an amino terminal sequence different from those previously described for FGFs or FGF-related molecules. Furthermore, by N-

amino acid sequence homology, this protein seems to be closely related if not identical to PTN (12) recently purified from bovine uterus. Contradictory results have been reported by P. Böhlen (13) indicating that both mitogenic and neurite outgrowth promoting activity could be a intrinsic property of this heparin binding molecule. However, our results are more consistent with those reported by Milner (12) and Li (14). Our purified preparations of PTN-like displayed both mitogenic activity and neurite outgrowth activity tested on PC 12 clone cells (not shown). However, using an enzyme immunoassay that we have previously described, we are not able to detect acidic or basic FGF in these preparations (results not shown). Minimal detection, arbitrarily defined as 80 % B/B_0 (16,17) was 0.2-0.5 ng/ml and 0.5-0.8 ng/ml for acidic and basic FGF respectively. Hence, when PTN-like is added at 2 ng/ml of culture medium, less than 2-5 pg acidic FGF or 5-8 pg per ml of culture medium basic basic FGF could be present. As illustrated in Fig.2, the dose response curve revealed a half maximal effect ranging from 8 to 10 ng/ml and from 45 to 50 pg/ml for acidic and basic FGF respectively. These data exclude the possibility that the mitogenic activity (maximal effect at 2 ng/ml) observed was due to acidic or basic FGF contaminating our final preparation. Furthermore, the absence of acidic or basic FGF in our PTN-like preparation can be ruled out by the thermal and pH lability of this molecule as regard to its mitogenic activity. Extraction at pH 4.5 yields inactive mitogenic forms of PTN-like molecules and storage at 4°C for ten days reduces the activity by 90% while the neurite outgrowth activity is maintained (Courty et al., unpublished results). The precise relationship between neurite outgrowth and mitogenic effect remains to be studied. The *in vitro* endothelial cell growth promoting activity of PTN-like molecules were recently confirmed by experiments indicating that this factor was angiogenic in the rabbit corneal pocket assay (Courty et al. in preparation).

Acknowledgments

This work was supported by grants from "Ministére de l'éducation nationale", from INSERM C.E. n° 872002 and n° 892003, from "Association pour la recherche sur le cancer (ARC)". José Courty is "Chargé de recherche au CNRS".

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