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PITUITARY FOLLICULAR CELLS SECRETE A NOVEL HEPARIN-BINDING GROWTH FACTOR SPECIFIC FOR VASCULAR ENDOTHELIAL CELLS

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A growth factor for vascular endothelial cells was identified in the media conditioned by bovine pituitary follicular cells and purified to homogeneity by a combination of ammonium sulfate precipitation, heparin-sepharose affinity chromatography and two reversed phase HPLC steps. The growth factor was a cationic, heat stable and relatively acid stable protein and had a molecular weight, as assessed by silver-stained SDS-PAGE gel, of ~45,000 under non reducing conditions and ~23,000 under reducing conditions. The purified growth factor had a maximal mitogenic effect on adrenal cortex-derived capillary endothelial cells at the concentration of 1-1.2 ng/ml (22-26 pM). Further characterization of the bioactivity of the growth factor reveals that it exerts mitogenic effects also on vascular endothelial cells isolated from several districts but not on adrenal cortex cells, lens epithelial cells, corneal endothelial cells, keratynocytes or BHK-21 fibroblasts, indicating that its target cells specificity is unlike that of any previously characterized growth factor. Microsequencing reveals a unique N-terminal amino acid sequence. On the basis of its apparent target cell selectivity, we propose to name this factor vascular endothelial growth factor (VEGF).

We have previously described the culture of homogeneous populations of bovine pituitary follicular or folliculo-stellate cells (FC) (1) and subsequently characterized them as ion transport elements, possibly involved in the regulation of ion composition and osmolarity of the interstitial fluid in the adenohypophysial cell cords (2,3). We have also reported that FC produce the angiogenic mitogen basic fibroblast growth factor (bFGF) (4).

The gene for bFGF (5), similarly to the gene for acidic fibroblast growth factor (aFGF) (6), does not code for a conventional signal peptide, required for the extracellular transport of proteins according to classic secretory pathways (7). Accordingly, the growth factor is not appreciably secreted in the medium (8,9) and responsive cell types are dependent on exogenous bFGF for optimal proliferation in culture, even though they may contain significant intracellular concentrations of the mitogen (10,11,12,).

We observed, however, that the medium conditioned by bovine pituitary FC is mitogenic for adrenal-cortex-derived capillary endothelial cells. Interestingly, these cells are responsive either to bFGF or aFGF but are not stimulated to proliferate by EGF, TGF alfa, TGF beta, PDGF, insulin or TNF (13). These observations led us to consider the

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possibility that an endothelial cell growth factor distinct from FGF and possibly any other known growth factor may be secreted by cultured FC.

In this communication we report on the purification and biological characterization of such a growth factor. Its unique N-terminal amino acid sequence, as well as its apparent specificity for vascular endothelial cells, distinguishes it from any previously described growth factor.

MATERIALS AND METHODS

Reagents Tissue culture media and reagents were obtained from Gibco (Grand island, N.Y.) through the Genentech Media Prep Facility or the UCSF Cell Culture Facility. Acetonitrile and 2-propanol were purchased from Fisher Sci. (Fair Lawn, NJ). Heparin-sepharose (H-S) was obtained from Pharmacia (Piscataway, N.J.). Vydac HPLC columns were from The Separation Group (Hesperia, Ca). Molecular weight markers for PAGE and protein determination kit were from Bio Rad Labs (Richmond, Ca). Tissue culture plates were purchased from Costar, except for large scale Nunc plates (24.5 x 24.5 cm), which were from Applied Sci. (San Francisco, Ca). All other reagents were from Sigma Chemical Co (St. Louis, MO) or Applied Biosystems (Foster City, Ca).

Culture of follicular cells and media collection Primary cultures of bovine pituitary FC were established as previously described (1,2). At confluency, cells were passaged into large scale tissue culture plates in the presence of low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine and antibiotics. Shortly after reaching confluency the cultures were extensively washed with PBS in order to remove serum components. The cells were then incubated in a serum-free medium consisting of DMEM plus transferrin (10 ug/ml), insulin (5 ug/ml), selenium (10^{-8} M), 2 mM glutamine and antibiotics. After three or four days, the medium was collected and replaced with fresh serum-free medium. The collected medium was centrifuged (1000 xg, 15 min at 4^{o} C) and stored at -70^{o} C. The conditioned medium was then collected every three or four days for up to six weeks.

Concentration of conditioned medium Four to six liter batches of conditioned medium (CM) were subjected to ammonium sulfate precipitation. Ammonium sulfate (500 g/L) was added under constant stirring, until the salt was completely in solution. After 8-12 hours in the cold room, the material was centrifuged (20,000 xg, 45 min at 4^{o} C). The supernatant was discarded and the pellet was resuspended with 10 mM Tris/Cl, pH 7.2, 50 mM NaCl and dialyzed at 4^{o} C against the same buffer for 8-12 h. The final volume was 50-60 fold less than the original.

Heparin-sepharose affinity chromatography The concentrated CM was applied to a H-S column (14) (10 ml) preequilibrated with 10 mM Tris/Cl, pH 7.2, 50 mM NaCl. The column was then washed with the same buffer until the absorbance at 280 nm was negligible and then eluted stepwise with 10 mM Tris/Cl, pH 7.2 containing 0.15, 0.9 and 3 M NaCl. The flow rate was 1.5 ml/min. Fractions of 1.5 ml were collected and aliquots, diluted with 0.2% gelatin in PBS, were tested for mitogenic activity on endothelial cells.

Reversed phase HPLC The most bioactive H-S fractions (0.9 M NaCl pool) were diluted fourfold with 0.1% trifluoroacetic acid (TFA) in water and applied to a Vydac C4 HPLC column (10 x 250 mm) preequilibrated in 0.1% TFA/20% acetonitrile. The column was eluted with a linear gradient of acetonitrile (20-45% in 115 min) at a flow rate of 2 ml/min. The absorbance was monitored at 210 nm. Fractions of 2 ml were diluted in 0.2% gelatin in PBS for assay on endothelial cells. The most biactive fractions were pooled, diluted two fold in 0.1% TFA water and applied to a Vydac C4 HPLC column (4.6 x 250 mm) preequilibrated in 0.1% TFA/20% 2-propanol. The column was eluted with a linear gradient of 2-propanol (20-45% in 113 min). The flow rate was 0.6 ml/min. Aliquots of fractions were diluted for bioassays. The remainder of fractions was dried in a Speed-Vac for SDS/PAGE (15) and structural analysis.

Bioassays Bovine adrenal cortex and brain-derived capillary endothelial cells and bovine adrenal cortex cells were obtained from Dr. D. Gospodarowicz (San Francisco, Ca) and were maintained as previously described (4, 11,). Adult or fetal bovine aortic endothelial cells, human umbilical vein endothelial cells, bovine corneal endothelial cells, lens epithelial cells, BHK-21 fibroblasts and human keratynocytes were cultured and maintained as previously described (12,16,17,18,19,20). For bioassay, cells were seeded in the presence of their respective growth media at the density of 2 x 10^4 /35 mm dish or 1 x 10^4/well in 12 multiwell plates. Fractions were added to cells in 5 ul/ml aliquots. After 4 or 5 days, cells were dissociated by exposure to trypsin and counted in a Coulter counter.

Protein microsequencing Approximately 20 pmol of protein from the most bioactive fractions obtained from the second C4 step were applied directly to a gas phase protein sequenator Model 470A (Applied Biosystems). Edman degradation cycles were carried out and identification of amino acid derivatives was made by an on line HPLC column (21).

RESULTS

The media conditioned by FC was found to stimulate the proliferation rate of low-density microvascular endothelial cells. Table 1 summarizes the steps for the purification of the growth promoting activity and the corresponding yield in bioactivity. The mitogenic activity was precipitated by 50% ammonium sulfate and resuspended to a volume suitable for subsequent purification. The H-S step provided an efficient way of further concentrating such activity and also provided a ten fold purification. Approximately 90% of the biological activity was eluted in the presence of 0.9 M NaCl (Fig 1). The bioactivity was not affected by heating the fractions at 65° C for 5 min and was decreased 25-30% following the exposure to 0.1% TFA (pH 2) for two hours. Chromatofocusing using a Mono P column (data not shown) indicated that the p.i. of the growth factor is ~8.5.

Table 1

Summary of purification of VEGF from 6 liters of conditioned medium

Purification step	Protein (ug)	Maximal stimulation (ng/ml)	Purification (fold)	Yield (%)
C.M. *	190,000	2500	1	100
A.S. *	175,000	2500	1	92
H-S *	13,000	250	10	68
R-P 1 ^	25	5	500	6
R-P 2 ^#	4	1.2	2000	4

C.M., conditioned medium; A.S, ammonium sulfate precipitate; H-S, heparin-sepharose; R-P 1, reversed phase HPLC step 1; R-P 2, reversed phase HPLC step 2.

Protein concentration was determined by Bio Rad Kit (*), by comparing the relative intensities of bands with standards in silver-stained PAGE (^) or by sequencing (#).

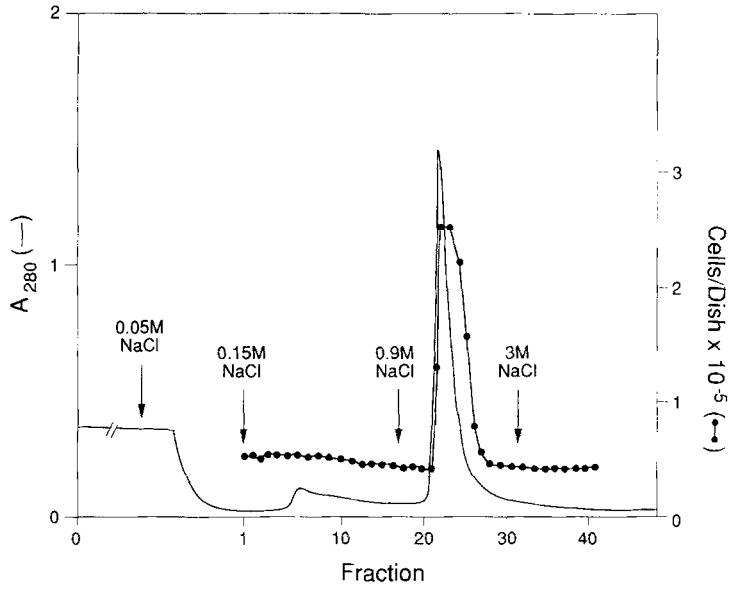

Figure 1

Heparin-sepharose (H-S) bioactivity profile of FC conditioned medium. The medium (6 liters) was concentrated and applied to a H-S which had been preequilibrated in 10 mM Tris/Cl, pH 7.2 containing 50 mM NaCl. The column was washed with the same buffer and then eluted sequentially with 10 mM Tris/Cl, pH 7.2, containing 0.15, 0.9 and 3 M NaCl. Aliquots of the collected fractions were diluted 100 fold in 0.2 % gelatin in PBS. 5 ul/ml was applied to capillary endothelial cells for bioassay.

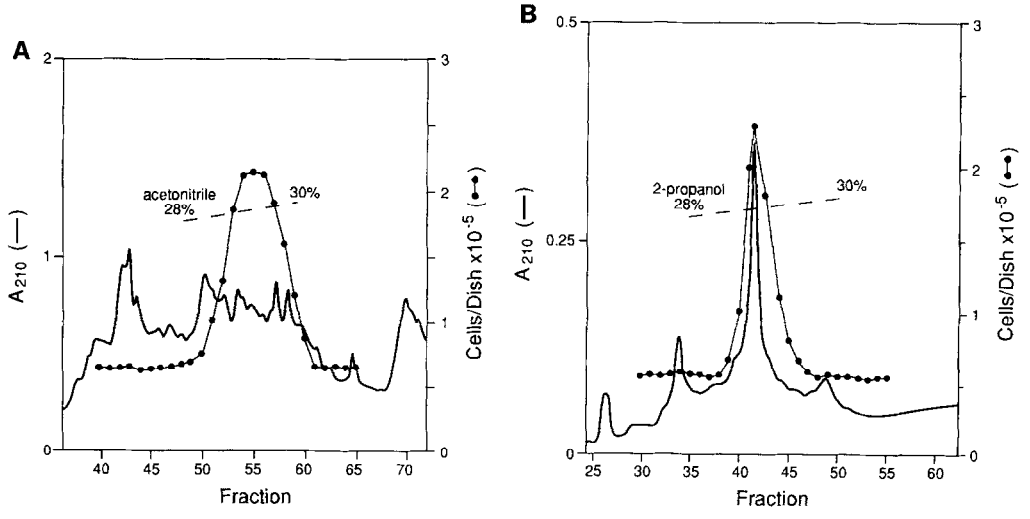

Figure 2 A and B

Sequential reversed phase HPLC profiles of endothelial cell mitogenic activity. The most bioactive H-S fractions were applied to a C4 column (10 x 250 mm) preequilibrated with 0.1% TFA/20% acetonitrile (panel A). After the column was washed with 10 ml of equilibration buffer, the sample was eluted with a linear gradient of acetonitrile. Aliquots of each fraction were diluted tenfold with 0.2% gelatin in PBS and 5 ul/ml were applied to capillary endothelial cells for bioassay. The most biactive fractions were pooled and applied to a C4 column (4.6 x 250 mm) which had been preequilibrated with 0.1% TFA/20% 2-propanol (panel B). After washing the column with 3 ml of equilibration buffer, the sample was eluted with a linear gradient of 2-propanol. Aliquots of fractions were tested for bioactivity.

The most bioactive H-S fractions were applied to a semi preparative C4 reversed phase HPLC column, a method suitable for rapid purification of proteins and peptides. The bioactivity was eluted as a single peak in the presence of ~29 % acetonitrile (Fig. 2 A). A silver-stained (22) SDS/PAGE gel on the most bioactive fractions revealed the presence of three or four bands. These fractions were further purified by a second reversed phase HPLC step, using an analytical C4 column which was eluted with a gradient of 2-propanol, instead of acetonitrile. A single peak of bioactivity corresponding to a distinct peak in the absorption profile was obtained (Fig. 2 B).

The peak fractions from the second reversed phase step displayed a single band on a silver stained SDS/PAGE, with an apparent M_r of ~23,000 under reducing conditions (Fig. 3). The intensity of staining of the band was highly correlated to the mitogenic activity across the bioactivity profile. Because previous experiments, using a molecular

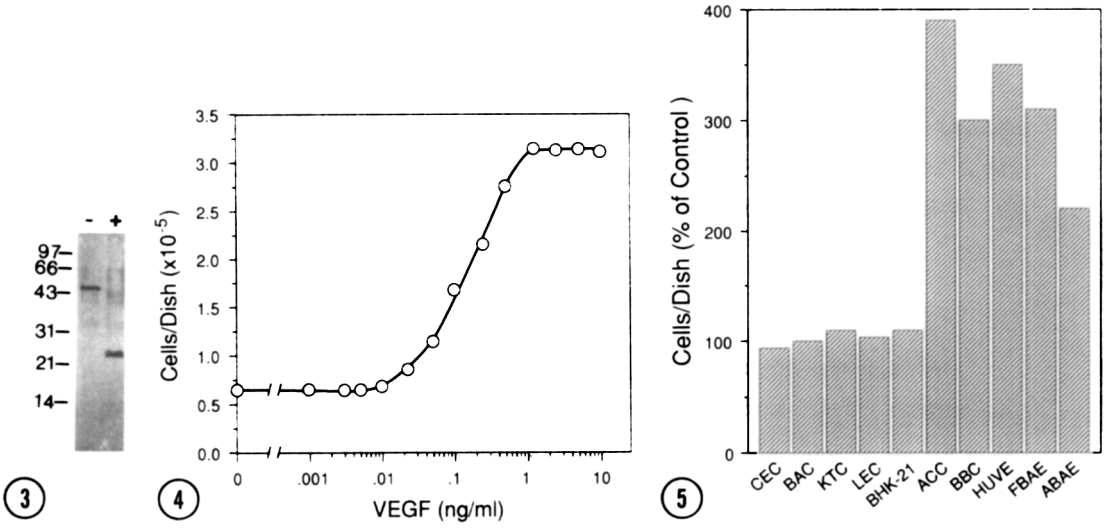

Figure 3

NaDodSO4/PAGE analysis of most bioactive fraction from chromatogram shown in Fig. 2 B . Two 50 ul aliquots of such fraction were dried in a speed vac and redissolved in sample buffer containing (+) or not (-) 2.5% 2-mercaptoethanol. The samples were heat-denatured and electrophoresed in a 12.5% PAGE which was subsequently silver stained. The molecular weight markers are: phosphorylase B, 97,400; bovine serum albumin, 66,200; ovalbumin, 43,000; carbonic anhydrase, 31,000; soybean trypsin inhibitor, 21,500; lysozyme, 14,400.

Figure 4

Dose-responsive growth of adrenal-cortex derived capillary endothelial in the presence of purified VEGF. Cells were seeded at the density of 1 x 10^4/well in 12 well plates. The indicated amounts of VEGF were added a few hours after plating in 5 ul/ml aliquots. After five days, cells were counted in a Coulter counter. The results shown represent mean values of three separate experiments conducted in duplicate. Duplicates in each experiment varied less than 10%.

Figure 5

Effects of VEGF on the growth of different cell types. CEC, corneal endothelial cells; BAC, bovine adrenal cortex cells; KTC, keratynocytes; LEC, lens epithelial cells; BHK-21, baby hamster kidney cells, clone 21; ACC, adrenal cortex capillary endothelial cells; BBC, bovine brain capillary endothelial cells; HUVE, human umbilical vein endothelial cells; FBAE, fetal bovine aortic endothelial cells; ABAE, adult bovine aortic endothelial cell. Cells were seeded in their respective growth media, incubated with a maximal concentration of VEGF and counted after 4 or 5 days. Results are expressed as percent of appropriate control.

sieve with a TSK G 3000 SW column (data not shown), suggested a M_r in the range of 40-43,000, the possibility that the factor in native conditions is a dimer was considered. This was strongly suggested by the finding that the purified material had an apparent M_r of ~45,000 in a silver stained SDS-PAGE under non-reducing conditions (Fig. 3).

As illustrated in Fig. 4, the dose response curve for the purified growth factor revealed a half maximal effect on adrenal cortex-derived endothelial cells proliferation at 100-150 pg/ml and a maximal effect at 1-1.2 ng/ml. These values were derived from protein sequencing and were found to be in good agreement with those obtained by comparing the relative intensities of bands with standards in silver stained SDS/PAGE.

Gas phase microsequencing of the purified material demonstrated unambiguously a single N-terminal amino acid sequence. The first five residues are: Ala-Pro-Met-Ala-Glu. A computer search revealed that such sequence does not display significant homology to any previously known protein.

The bioactivity of the growth factor was also tested with different cell types. As shown in Figure 5, appreciable activity was observed only in cell types of vascular endothelial origin, such as fetal and adult bovine aortic endothelial cells, bovine brain capillary endothelial cells and human umbilical vein endothelial cells. In contrast, adrenal cortex cells, lens epithelial cells, corneal endothelial cells, BHK-21 fibroblasts and keratinocytes failed to show any significant mitogenic response.

DISCUSSION

We describe for the first time the identification, purification and biological characterization of a heparin-binding endothelial cell growth factor from culture media conditioned by pituitary FC. Analysis of the purified material by SDS PAGE revealed a M_r of ~45,000 under non reducing conditions. When the material was analyzed in the presence of 2-mercaptoethanol, a single band with a M_r of 23,000 was visualized, indicating that the growth factor is probably a dimer composed of two subunits of identical apparent molecular weight.

The purified growth factor was able to stimulate the proliferation of vascular endothelial cells at concentrations between ~25 pg and 1-1.2 ng/ml. These values, assuming a M_r of 45,000, correspond respectively to 0.55 pM and 22-26 pM. Such values are in the same range as those obtained with bFGF (13, 23). However, the novel growth factor did not induce any appreciable mitogenic effect on corneal endothelial cells, lens epithelial cells, BHK-21 fibroblasts, adrenal cortex cells, or keratynocytes. In contrast, bFGF and aFGF are both potent mitogens for all of these cell types (13, 23). Also, the growth factor appears different from a recently purified (25) and cloned (26) endothelial cell growth factor isolated from human platelets (PD-ECGF). Although PD-ECGF and the factor by us isolated have the same molecular mass, they differ in their N-terminal sequence, secondary structure and in biological potency. Unlike the growth factor secreted by FC, PD-ECGF is constituted by a single polypeptide chain and also appears ~10 fold less potent in promoting endothelial cell growth.

On the basis of its apparent target cell selectivity, we propose to name this novel factor, at least provisionally, vascular endothelial growth factor (VEGF).

The ability of VEGF to bind heparin may have implications as to its in vivo function and regulation. Heparan sulphates are fundamental components of the extracellular matrix and have been proposed to play a crucial role in determining contact between target cells and heparin-binding growth factors (27, 28).

The presence of VEGF in pituitary FC strongly suggests a role for these cells in the development, organization and maintainance of a differentiated stage of the complex microvasculature of the adenohypophysis.

It is presently unknown whether VEGF is expressed in organs other than the pituitary gland. However, considering the fundamental role of vascular endothelial cells growth and angiogenesis in a great variety of normal and pathological proliferations (28), it is tempting to speculate that the distribution of the growth factor is likely to be found more widespread. Within this context, it is of interest that PDGF, EGF, TGF alfa, TGF beta, FGF, NGF, which were initially believed to be restricted to specific cells or tissues, were later found to have a much broader and sometimes ubiquitous distribution (29).

The genes for bFGF and aFGF, the best characterized endothelial cell mitogens, do not code for a conventional signal peptide (5, 6). Accordingly, these growth factors appear to be sequestered inside the cells of origin and apparently do not have direct access to target cells (8,9,13). It has been suggested that bFGF may be incorporated into the basement membrane and be subsequently released in a soluble form only when the matrix is degraded following the action of specific enzymes (27). Such a mechanism of release suggests a role for the growth factor mostly or exclusively in events which involve degradation of the basement membrane or cell lysis, such as organ remodeling, wound healing or neoplasia (28).

In contrast, a soluble endothelial cell growth factor such as VEGF may play a more dynamic role in the physiological regulation of vascular endothelial cells proliferation, either in the cyclical growth of blood vessels which takes place in organs such as the corpus luteum (30) or in the tonic maintenance of the differentiated stage of the endothelium in the vascular tree.

Finally, unlike bFGF or aFGF, which are active on a very broad spectrum of cells (13,23), VEGF appears to be specific for vascular endothelial cells. It is tempting to speculate therefore that it might be of special therapeutic significance for conditions in which a selective action on the vascular endothelial cells, in the absence of excessive connective tissue proliferation, is desirable, such as diabetic ulcers or traumatic vascular injuries.

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