The EMBO Journal vol.8 no.12 pp.3801 - 3806, 1989

Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT-20 cells

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Communicated by D.Stehelin

An endothelial cell growth factor with unique specificity for vascular endothelial cells has been purified from the conditioned medium of the AtT-20 pituitary cell line. This growth factor, which has been characterized as a homodimer composed of two subunits with mol. wts of 23 kd is a potent mitogen for vascular endothelial cells *in vitro* with activity detectable at 50 pg/ml and saturation at 1 ng/ml. It was also angiogenic *in vivo*. In contrast with other endothelial mitogens of the fibroblast growth factor family, it has a unique target cell specificity. It did not stimulate the growth of other cell types of the vascular system such as vascular smooth muscle cells or that of mesoderm and neuroectoderm derived cells. Micro-sequencing revealed an amino-terminal sequence with no homology to any known protein. The release of this novel endothelial cell growth factor by pituitary derived cells and its unique target cell specificity suggest that it could play an important role in the angiogenic process.

Key words: amino acid sequence/angiogenesis/basic FGF/growth factor

Introduction

Several factors that induce angiogenesis have recently been isolated and characterized (Folkman and Klagsbrun, 1987). Of these, only the basic and acidic forms of fibroblast growth factor (FGF) have been shown to control directly all steps of angiogenesis including vascular endothelial cell proliferation, migration and increased expression of plasminogen activator and collagenase activity (Montesano *et al.*, 1986; Gospodarowicz *et al.*, 1987; Saksela *et al.*, 1987).

Despite the evidence that FGF is angiogenic, two puzzling questions point to the existence of other angiogenic factors that could complement the action of FGF. First, FGF lacks the hydrophobic signal sequences that govern secretion (Abraham *et al.*, 1986; Jaye *et al.*, 1986), yet for acceptance as an angiogenic factor any putative mediator should be shown to be a diffusible substance which induces new capillary formation from a microcirculatory bed. Second, FGF is produced by endothelial cells themselves (Moscatelli *et al.*, 1986; Schweigerer *et al.*, 1987; Vlodavsky *et al.*, 1987). If FGF is present in and around endothelial cells and yet the cells are quiescent, other factors must come into play to trigger angiogenesis.

In the course of our investigation on the expression of basic FGF (bFGF) and possible secretory pathways, the pituitary cell line AtT-20 (Gumbiner and Kelly, 1981) was transfected with a chimeric bFGF gene composed of the coding sequence for the growth hormone secretion signal peptide fused with the coding sequence for the bFGF gene (Blam *et al.*, 1988). This cell line was chosen because it has retained normal secretory functions and can be used to study the molecular events involved in packaging and secretion of protein (Gumbiner and Kelly, 1981). Our hope was that the bFGF expressed would have been secreted by these cells either through a constitutive secretory pathway or through a secretory pathway involving secretory granules. When medium conditioned by transfected AtT-20 cells was examined for angiogenic activity a considerable amount of activity was present that could not be immunoneutralized by antibodies against bFGF and which did not cross-react in a radioimmunoassay (RIA) specific for bFGF. Likewise, media conditioned by parental cells that did not express the bFGF gene also contained a considerable amount of bioactivity, suggesting that a factor unrelated to bFGF was responsible for it.

Here we report the isolation and characterization of a new endothelial cell mitogen which is produced and secreted by AtT-20 cells. This factor has a unique target cell specificity, since it stimulates only vascular endothelial cells to proliferate and does not affect other cell types sensitive to FGF. It also has angiogenic activity *in vivo*.

Results

Growth factor isolation and detection

$(NH_4)_2SO_4$ precipitation provided a convenient way of reducing the volume of the collected conditioned medium from the AtT-20 cells to a level suitable for subsequent chromatography. Heparin–Sepharose affinity chromatography (HSAC), which has been used by others for the purification of other growth factors (Klagsbrun and Shing, 1985; Gospodarowicz, 1987) provided an efficient purification step. Material not retained by the column was inactive and accounted for 50% of the total protein loaded (Figure 1). It is likely that the transferrin and insulin components of the cell media were present in the unretained fraction and contributed to the major portion of the proteins. Elution with 0.3 M NaCl yielded a peak of protein with little bioactivity, while elution with 0.8 M yielded a major peak of protein with 163% of the bioactivity applied to the column. Overall, the HSAC resulted in a 42-fold purification, estimated by the protein recovered. Since the growth-promoting activity in the starting material was variable, possibly due to the presence of inhibitor(s), the yield at this step was >100% (Table I).

HSAC was followed by gel exclusion chromatography using Sephadex G100 (Figure 1B). The bioactivity eluted

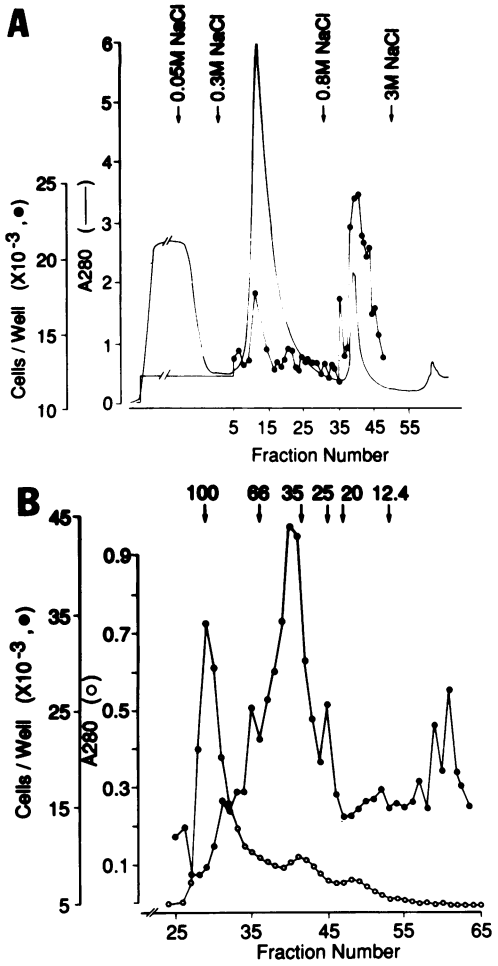

Fig. 1. Purification of AtT-20 endothelial cell-derived growth factor by HSAC and gel exclusion chromatography. (**A**) Approximately 490 ml of the $(NH_4)_2SO_4$ precipitate fractions derived from 30 l of AtT-20 cell-conditioned medium were loaded onto a heparin–Sepharose column (1.5 cm × 12 cm, 25 ml bed volume) at a flow rate of 150 ml/h. The column was then washed with 150 ml of the equilibration buffer (20 mM Tris–HCl, pH 7.3, 50 mM NaCl), and the retained proteins (50% of the total protein applied on the column) were eluted with a stepwise application of increasing NaCl concentrations (0.3, 0.8 and 3 M NaCl). Fraction size was 3 ml, and the flow rate was 60 ml/h. Chromatography was performed at 4°C and absorbancy was monitored at 280 nm. The histogram and closed circles show the relative ability of the different pooled or individual fractions to stimulate the proliferation of low density ACE cell cultures (5×10^3 cells/well, 12 well cluster plates). Conditions for testing were the same as those described in Gospodarowicz *et al.* (1986). The majority of the biological activity was present in the 0.8 M NaCl eluate. In the case of the pooled unabsorbed and washed fraction, an aliquot was diluted 100-fold in 0.2% gelatin in PBS and a 10 μl aliquot was bioassayed (histogram). In the case of the individual 0.3, 0.8 and 3 M NaCl fractions, aliquots were diluted 100-fold in 0.2% gelatin in PBS, and 10 μl aliquots were bioassayed. The majority of the biological activity was present in the 0.8 M NaCl eluate. (**B**) After concentrating the 0.8 M NaCl HSAC bioactive fractions to 1 ml in an Amicon YM10 concentrator, the ultrafiltration retentate was applied on a Sephadex G 100 column (1 × 95 cm), equilibrated and run at 4°C in PBS. The flow rate for development of the column was 6 ml/h, and 3 ml fractions were collected. Absorbancy was monitored at 280 nm (○). The elution positions of molecular mass markers (in kd) were as indicated by the arrows. Aliquots of each fraction from the column were diluted 1 to 100 in 0.2% gelatin in PBS, and 10 μl aliquots were bioassayed in ACE cells in 12 well cluster plates, as described above (●). Most of the bioactivity eluted as a single peak with an apparent mol. wt of 40–45 kd.

Table I. Summary of purification of the AtT-20-derived endothelial cell growth factor

Purification steps	Protein[a] (μg)	ED_{50}[b] (ng/ml)	Total activity	Yield (%)	Purification (fold)
$(NH_4)_2SO_4$	480 000	1500	320 000	100	1
HSAC	180 000	36	522 000	163	42
G100	1500	7	210 000	65	214
Mono S	72	0.4	180 000	56	3750
RP-HPLC C_4	5.1[c]	0.16	31 875	10	9375

[a]Protein was estimated by using the Bradford reagent from BioRad with BSA as a standard.
[b]The ED_{50} was determined as the protein concentration that gave a half stimulation of cell proliferation in the ACE cell assay. It corresponds to one unit of activity.
[c]Protein content was determined by amino acid analysis.

as a major peak with an apparent mol. wt of 40–45 kd. This step resulted in a further 5-fold purification with a recovery of 65% (Table I).

The bioactive fractions from the Sephadex G100 column were pooled and applied on a Mono S column (Figure 2A). The bioactive profile of the eluted fractions consisted of a major bioactive peak eluting at 0.28 M NaCl. The Mono S step gave a further 18-fold increase in specific biological activity over the Sephadex G100 step; recovery was 56% (Table I).

Final purification of the endothelial cell mitogenic activity was achieved by reverse-phase HPLC (RP-HPLC) with a C_4 Vydac column (Figure 2B), a preparative method suitable for amino acid sequence analysis. Although losses in biological activity were encountered, presumably because of the acid conditions and solvent used, these were not serious enough to prevent the detection of bioactive fractions. All of the bioactivity detected was present in two closely apposed sharp peaks of protein which, when iodinated and analyzed by SDS–PAGE under non-reducing conditions, gave the same single band with an apparent mol. wt of 46 kd (Figure 3). In one of the fractions, a small contaminant with a mol. wt of 27 kd whose migration was not affected by reduction was also present. This contaminant did not amount to >5% of the total. When the major peak of activity was rerun under identical condition of a C_4 column, a single peak of protein with a small shoulder was obtained (insert Figure 2B). The RP-HPLC step resulted in a 2.7-fold increase in specific biological activity with a recovery of 10% (Table I).

Physical and biological characterization of the growth factor

The purified factor, when run under unreduced conditions, had an estimated mol. wt of 46 kd (Figure 3). This value is in good agreement with its elution position on the sizing Sephadex column run in solvents expected to maintain the native conformation. When run under reduced condition, the apparent mol. wt was 23 kd (Figure 3). From these data, it appears that the mitogen consists of two polypeptide chains with a mol. wt of 23 kd. Given that a single N-terminal sequence was obtained, the dimeric molecule is probably composed of two homologous chains.

Microsequencing revealed a unique N-terminal amino acid sequence and a single amino acid was identified in each of

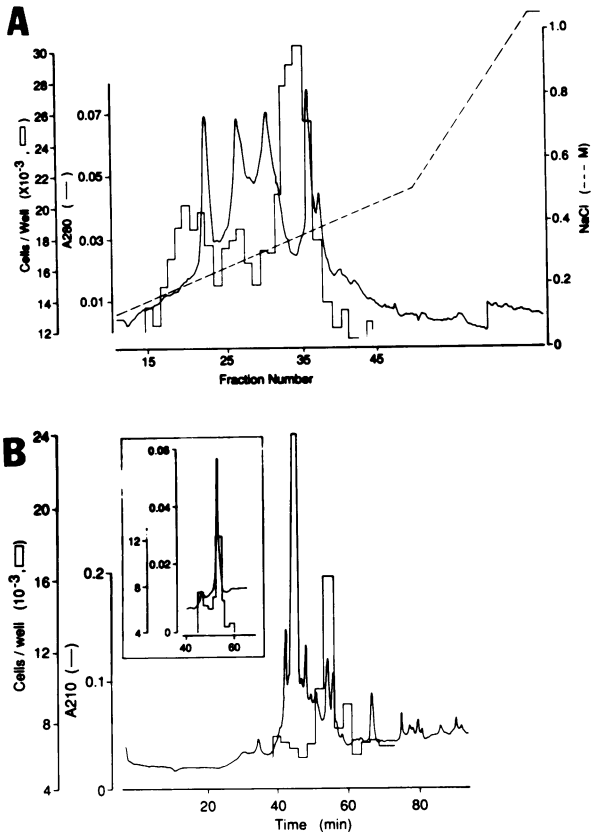

Fig. 2. Mono S ion-exchange chromatography and reverse-phase HPLC of the Sephadex G100-purified and active fractions. (**A**) The bioactive fractions eluted from the Sephadex G100 column were pooled and diluted 3-fold with 20 mM HEPES, pH 8.3. Using a 50 ml Super loop, the sample was then applied on a Mono S HR 5/5 column equilibrated in 20 mM HEPES, pH 8.3, at room temperature. The column was eluted with a multilinear gradient of NaCl (0–1 M) as follows: 0 M NaCl for 5 min, 0 M NaCl to 0.45 M NaCl in 45 min, 0.45 M NaCl to 1 M NaCl in 15 min, 1 M NaCl for 5 min. Absorbancy was monitored at 280 nm. Flow rate was 1 ml/min and 1 ml fractions were collected. Aliquots of each fraction were diluted 1 to 100 in 0.2% gelatin in PBS, and 10 μl aliquots were bioassayed on ACE cells in 12 well cluster plates as described above. The histogram shows the distribution of the biological activity with most of the biological activity eluting in fractions 33–35 (0.28 M NaCl). (**B**) The active Mono S fractions (fractions 33–35) were loaded onto a Vydac C_4 column (25 × 0.46 cm, 5 μm particle size, 300 Å pore size) equilibrated in 0.1% (v/v) TFA, 20% acetonitrile. Protein was eluted with a 115 min linear gradient of 20–45% acetonitrile in 0.1% TFA at a flow rate of 0.6 ml/min, at room temperature. Fractions of 1.5 ml were collected. Aliquots of each fraction were diluted 1 to 10 with 0.2% gelatin in PBS and bioassayed as described above. The histogram shows the distribution of the biological activity. The peak fractions, eluting at 55 and 57.5 min respectively, were used individually for structural studies and further analysis of their biological activity. The major peak of activity was rerun on the same columns as shown in insert, the peak fractions were taken for amino and terminal sequence analysis.

the first 24 cycles, consistent with a homogeneous protein. The yield of the amino terminal residue was 30 pmol. Unambiguous assignments made for cycles 1–10 were as follows: Ala-Pro-Thr-Thr-Glu-Gly-Glu-Gln-Lys-Ala. A search of the NBRF database using the FASTP program of Lipman and Pearson (1985) found no significant homology between this sequence and any known protein.

The dose–response curve for the growth factor depicted in Figure 4 illustrates that as little as 50 pg/ml stimulates

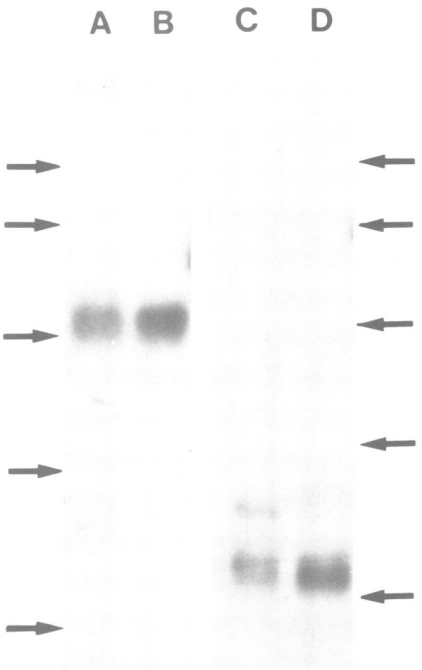

Fig. 3. SDS–PAGE of the bioactive fractions purified by RP C_4 HPLC ^{125}I-labelled protein samples of the fractions eluting at 55 min and 57.5 min were analyzed individually under unreduced (**lanes A** and **B**) or reduced conditions (**lanes C** and **D**). After electrophoresis the gels were stained with Coomassie Blue, destained, dried and subjected to autoradiography. Migration of the samples was compared to that of unreduced (**left**) or reduced (**right**) protein standards: 97, 66, 43, 30, 21 kd.

proliferation of adrenal cortex-derived capillary endothelial (ACE) cells. Saturation was observed at 1 ng/ml with an ED_{50} of 160 pg/ml (Figure 4B). These values compared favorably with the range of concentrations where bFGF promotes the proliferation of ACE cells (minimal effect at 10 pg/ml saturation at 200 pg/ml and ED_{50} at 50 pg/ml) (Gospodarowicz *et al.*, 1986). However, the final density of the culture grown in the presence of the AtT-20-derived endothelial cell growth factor was half that of cultures exposed to optimal concentrations of bFGF. Nevertheless, if one considers that the mol. wt of the AtT-20-derived endothelial cell growth factor is 2.5 times that of bFGF, this new factor has essentially the same potency on a molar basis as bFGF. In addition to its ability to stimulate the proliferation of ACE cells, the AtT-20-derived endothelial cell growth factor did stimulate the growth of bovine brain-derived capillary endothelial cells (not shown) as well as that of human umbilical endothelial (HUE) cells (Figure 4A). These results indicate that the mitogenic effect of the factor is not limited by species variation nor by the origin of the vascular endothelial cells. However, and in contrast with bFGF, the factor is not mitogenic for BHK-21 (baby hamster kidney-derived fibroblast clone 21) cells (Figure 4C), nor is it mitogenic for adrenal cortex cells, corneal endothelial cells, granulosa cells, BALB/MK cells, or vascular smooth cells (Figure 4D). Therefore, and in contrast with FGF, it seems to have a unique specificity for vascular endothelial cells. In agreement with its ability to stimulate the proliferation of vascular endothelial cells *in vitro*, the

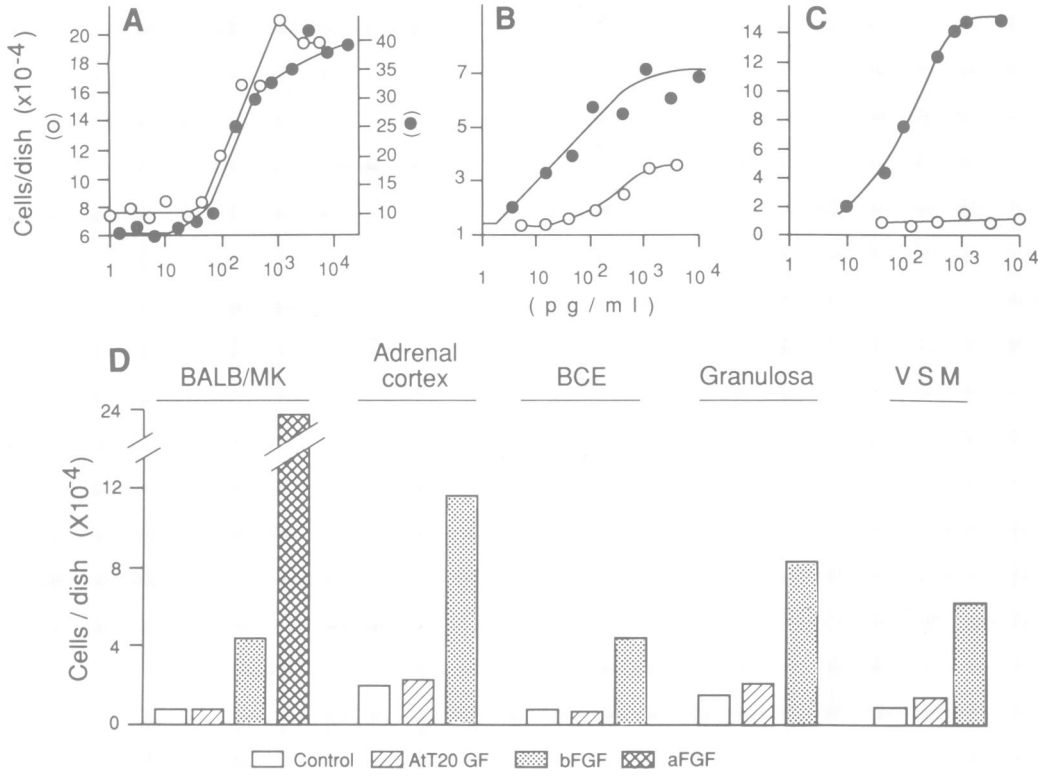

Fig. 4. Comparison of the ability of pituitary-derived bFGF versus the AtT-20 derived endothelial cell growth factor to stimulate the growth of vascular endothelial cells and other unrelated cell types. (**A**) Low-density cultures of HUE cells (13) (5×10^3 cells per 22 mm diameter gelatinized well) were exposed to HEPES (25 mM)-buffered medium 199 supplemented with 100 μg heparin per ml, 10^{-8} M selenium, 20% FCS) and increasing concentrations of either pituitary derived bFGF (○) or AtT-20-derived endothelial cell growth factor (●). Heparin was added only once at the time of seeding while both bFGF and AtT-20-derived endothelial cell growth factor was added every other day. After 6 days in culture, triplicate wells were trypsinized and cell counted. The final density of cultures exposed to 20% FCS alone was 7.4×10^4 cells/well. Standard deviation was $<10\%$ of the mean. (**B**) Low-density cultures of ACE cells (5×10^3 cells per 22 mm diameter well) were exposed to DMEM supplemented with 10% CS and increasing concentrations of either pituitary-derived bFGF (●) or the AtT-20-derived endothelial cell growth factor (○) added every other day. After 5 days in culture, triplicate wells were trysinized and cell counted. The final density of culture exposed to 10% CS alone was 1.3×10^4 cells/well. Standard deviation was $<10\%$ of the mean. (**C**) Low-density cultures of BHK-21 cells (5×10^3 cells per 22 mm diameter gelatinized tissue culture dishes) were exposed to 2 ml of DMEM-F12 (11 v/v) supplemented with 2.5 μg/ml Fungizone, 50 μg/ml gentamicin, 10 μg/ml transferrin, 5 μg/ml insulin and increasing concentrations of either pituitary-derived bFGF (●) or the AtT-20-derived endothelial cell growth factor (○). Insulin and transferrin were added only once, bFGF and AtT-20-derived endothelial cell growth factor were added every other day. After 4 days in culture triplicate dishes were trypsinized and cell counted. The final density of culture exposed to transferrin and insulin alone was 1.05×10^4 cells/well. Standard deviation was $<10\%$ of the mean. (**D**) BALB/MK cells were seeded at a density of 5×10^3 cells per 22 mm diameter wells in low Ca-modified Eagle's medium supplemented with 10% FCS (Carpenter and Zendequi, 1985). 5×10^3 BCE cells, granulosa cells, adrenal cortex cells or VSM cells per 22 mm wells were seeded in their respective media (DMEM supplemented with 10% FCS, 5% CS or 10% CS for BCE and adrenal cortex cells, F-12 medium supplemented with 2.5% CS for granulosa cells and DMEM supplemented with 10% platelet-poor plasma serum for VSM cells). bFGF (2 ng/ml), aFGF (10 ng/ml in the case of BALB/MK cells and 100 ng/ml in the case of VSM cells), or AtT-20-derived endothelial cell growth factor (1.5 ng/ml) were added every other day. After 6 days in cultures, cells were trypsinized and counted in a Coulter counter. Standard deviation was $<10\%$ of the mean.

AtT-20-derived endothelial cell growth factor was also angiogenic *in vivo* when tested on the developing vascular system of the chick chorioallantoic membrane (Figure 5).

Discussion

In the present study, an angiogenic factor that seems to have a distinct specificity for vascular endothelial cells has been identified. This growth factor released by AtT-20 cells was purified using a combination of HSAC, exclusion gel chromatography, cation exchange chromatography and RP-HPLC. The purification resulted in an apparent increase in specific activity of 10 000-fold with a 13% recovery of growth-promoting activity (Table I).

The AtT-20-derived endothelial cell mitogen has a unique target cell specificity, stimulating only the proliferation of vascular endothelial without affecting that of vascular smooth muscle cells. In addition, its ability to stimulate the proliferation of bovine brain-derived capillary endothelial cells as well as that of HUE cells indicates that its mitogenic effect is not limited by species variation nor by the origin of the vascular endothelial cells. Surprisingly, it did not stimulate the proliferation of even BHK-21 cells, a cell line known to respond to a wide variety of mitogens including transforming growth factor (TGF) α and β, epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and acidic FGF (aFGF) or bFGF (Neufeld *et al.*, 1986), nor was it mitogenic for other mesoderm- or neuroectoderm-derived cells. The present data established that AtT-20 cells which have retained many important chemical and physiological properties of pituitary corticotroph, in particular the ability to synthesize and release as major

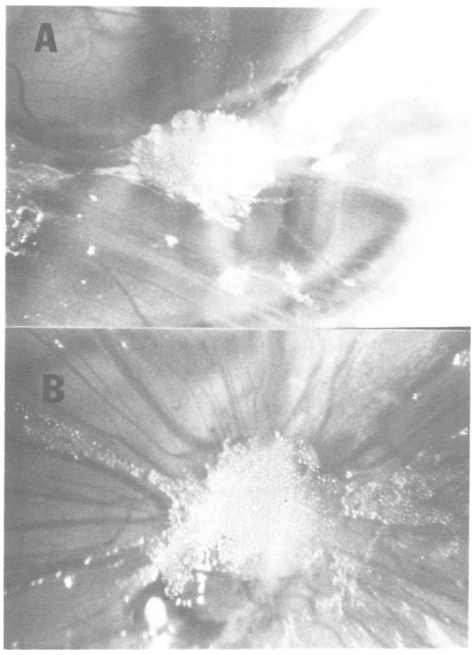

Fig. 5. Induction of angiogenesis in the chick chorioallantoic membrane by the AtT-20-derived endothelial cell growth factor. Test substances were incorporated into Sephadex G200 and transplanted onto 9 day chorioallantoic membranes as described in Materials and methods. (**A**) The result of control implant (10 μl of Sephadex G200 swell in PBS, 88%, n = 26). (**B**) The results of an implant containing 200 ng of AtT-20-derived endothelial cell growth factor (83% positive results, n = 30). The zone of implantation was photographed after 4 days (×40).

secretory products ACTH, S lipotropin and β-endorphin (Gumbiner and Kelly, 1981), do also produce an angiogenic factor.

The physical properties of the AtT-20-derived endothelial cell growth factor (mol. wt 46 kd, basic p*I*, affinity for HS, N-terminal sequence) and biological properties (mitogenic for vascular endothelial cells) indicate that it is distinct from other known growth factors such as EGF, TGFα, PDGF, TGFβ or the recently reported keratinocyte growth factor (Rubin *et al.*, 1989). Its lack of recognition by neutralizing polyclonal antibodies directed against aFGF or bFGF as well as its lack of cross-reactivity in RIA specific for aFGF or bFGF (unpublished results) indicates that it is distinct from FGF. It seems also to be distinct from the recently reported platelet-derived endothelial cell growth factor (PDECGF) (Miyazono *et al.*, 1987; Ishikawa *et al.*, 1989). Although PDECGF and AtT-20-derived endothelial cell growth factor have the same apparent target cell specificity and similar molecular mass, they differ by 20-fold in potency and by their secondary structure, PDECGF being a single chain polypeptide while the AtT-20-derived endothelial cell growth factor has a dimeric structure.

The unique target cell specificity and N-terminal sequence lead us to conclude that the AtT-20-derived endothelial cell growth factor represents a previously unreported growth factor. Although the present study clearly established that this novel growth factor is mitogenic for capillary endothelial cells, it is not yet known whether it can stimulate other events linked to angiogenesis. These include chemotaxis of capillary endothelial cells and activation of the synthesis of cellular enzymes such as collagenase and plasminogen activator which are involved in the breakdown of capillary basement membrane (Saksela *et al.*, 1987). In view of its preferential activity on vascular endothelial cells as compared to other mesoderm derived cells, we propose, provisionally, the name of vasculotropin for this growth factor.

Available structural data should allow studies on the cloning, structure, topology, expression and regulation of the growth factor gene in both physiological and pathological conditions. These studies may provide clues as to its physiological functions including angiogenesis.

Materials and methods

Reagents

The protein assay kit and low mol. wt standards for SDS−PAGE were from BioRad. Heparin−Sepharose and Mono S column HR5/5 were obtained from Pharmacia. The Vydac C_4 reverse-phase column was purchased from The Separation Group. Media and serum were from the Cell Culture Center, (University of California, San Francisco). Twelve-well cluster plates (22 mm diameter well) were from Corning, large-scale Nunc culture plates (600 cm^2) were from Applied Scientific. Gentamicin was obtained from Schering Co., and Fungizone was purchased from E.R.Squibb and Sons. Leupeptin, gelatin, transferrin and insulin were from Sigma. Pituitary-derived bFGF and neutralizing rabbit polyclonal antibodies directed against bFGF were prepared as previously described (Gospodarowicz *et al.*, 1984, 1988).

Cell culture

The AtT-20 cell line was obtained from the Cell Culture Center (University of California, San Francisco). Confluent cultures were dissociated by brief exposure to STV. The cells collected were then seeded at a split ratio of 1:10 into large-scale culture plates and grown in the presence of DMEM-H21 supplemented with 10% FCS, 50 μg/ml gentamicin, and 0.25 μg/ml Fungizone. Upon reaching confluency, cultures were further passaged or exposed to serum-free medium (see below). Cultures of human umbilical endothelial cells (Gospodarowicz *et al.*, 1983), bovine brain and adrenal cortex-derived capillary endothelial cells (Gospodarowicz *et al.*, 1986), bovine granulosa cells (Gospodarowicz *et al.*, 1977a), bovine adrenal cortex cells (Gospodarowicz *et al.*, 1977c), bovine corneal endothelial cells (Gospodarowicz *et al.*, 1977b), bovine vascular smooth muscle cells (Gospodarowicz *et al.*, 1988), baby hamster kidney cells, clone 21 (BHK-21) (Neufeld *et al.*, 1986) and BALB/MK mouse epidermal keratinocytes (Weissman and Aaronson, 1983) (a gift from Dr S.Aaronson, NIH NCI, Bethesda, MD) were maintained as previously described.

Preparation of condition medium

AtT-20 cells were plated onto 600 cm^2 plates and grown to confluence over 4−5 days in DMEM-H21 supplemented with 10% FCS and antibiotics as described above. The monolayers were washed twice with 25 ml of phosphate-buffered saline prior to the addition of 150 ml per plate of DMEM supplemented with 50 μg/ml gentamicin, 2.5 μg/ml Fungizone, 10 μg/ml leupeptin, 5 μg/ml insulin and 10 μg/ml transferrin. After 48 h, culture fluids were collected and replaced with the same amount of fresh serum-free medium. Collections could be made for up to 2 weeks without visible cell deterioration.

Isolation procedure

Conditioned medium collected from the confluent monolayers was centrifuged (10 000 *g*, 15 min) in order to remove floating cells and cell debris. The pH of the supernatant was then adjusted to 5.6 with 6 N HCl. $(NH_4)_2SO_4$ (520 g/l) was added, and the suspension was set for 24 h at 4°C, the precipitate was then collected by centrifugation (10 000 *g*, 30 min), redissolved in PBS and stored at −70°C.

For final isolation, the precipitates from six collections (30 l total of conditioned medium, starting material) were thawed, pooled and then dialyzed overnight at 4°C against 10 mM Tris−HCl, pH 7.3, 50 mM NaCl. Following dialysis, the insoluble material was removed by centrifugation (10 000 *g*, 30 min) and the supernatant was loaded onto a heparin−Sepharose resin (25 ml) that had been equilibrated in 10 mM Tris−HCl, pH 7.3, 50 mM NaCl. Flow rate was 150 ml/h. The resin was washed extensively with the equilibration buffer until the absorbance had returned to baseline,

and eluted stepwise with increasing NaCl concentrations (0.3, 0.8 and 3 M NaCl). Aliquots were removed from the fractions for cell proliferation assays, and fractions with the highest bioactivity were pooled and concentrated to 1 ml with an Amicon ultrafiltration cell (Model 12) equipped with a Diaflo YM-10 ultrafiltration membrane.

The concentrated sample was loaded onto a Sephadex G100 column equilibrated at 4°C in PBS and was eluted with PBS. Aliquots of each fraction were taken for cell proliferation assay and the bioactive fractions were pooled, and diluted 2-fold with 20 mM HEPES, pH 8.3. The sample was then applied with a Super loop onto a Mono S column linked to a FPLC system (Pharmacia). Elution was achieved with a multilinear gradient (20 mM HEPES, pH 8.3, to 20 mM HEPES, pH 8.3, 1 M NaCl). After fraction aliquots were tested for bioactivity, the active fractions were pooled and loaded onto a Vydac C_4 HPLC column that had been equilibrated in 0.1% trifluoracetic acid (TFA), 20% acetonitrile. The column was eluted with a linear gradient of 20–45% acetonitrile. Aliquots for the bioassay were then taken, and the column fractions were stored frozen at −70°C.

Cell proliferation assays

The mitogenic activity of the column fractions and purified samples was determined by using as target cells bovine adrenal cortex-derived capillary endothelial cells (ACE cells) (Gospodarowicz *et al.*, 1986). Cells were seeded at 5×10^3 cells per 22 mm diameter wells; 6 h later, a set of triplicate wells was trypsinized, and cells were counted to determine the plating efficiency. Ten microliter aliquots were then added in triplicate to wells on days 0 and 2. After 4 days in culture, the wells were trypsinized, and cell densities were determined with a Coulter counter.

The mitogenic activity of the final purified material was also tested on human umbilical endothelial cells, bovine granulosa cells, bovine adrenal cortex cells, bovine corneal endothelial cells, bovine vascular smooth muscle cells, BHK-21 cells and BALB/MK mouse epidermal keratinocytes. For assaying, cells were seeded at an initial density of 5×10^3 cells/per 22 mm diameter wells. Assays were conducted as described for bovine vascular endothelial cells.

Chorioallantoic membrane (CAM) angiogenesis assay

The CAM of the chick embryo was used as the *in vivo* system to study the vascular response induced by the purified preparation. A 1 cm window was cut into the eggshell of 9 day old eggs, and the CAM was dislocated from the shell membrane by the false air sac technique (Harris-Hooker *et al.*, 1983). The growth factor preparations were diluted at concentrations ranging from 1 to 20 μg/100 μl, and 3 mg Sephadex G-200 beads were added. After an overnight incubation at 4°C, 5 μl of this material were implanted on CAMs and the membranes were examined daily for 4 days.

SDS–PAGE

Protein was iodinated by the chloramine T method (McConahey and Dixon, 1966) and run, after being reduced (0.1 M DTT, 37°C, 60 min) or unreduced on a discontinuous gel system (3% stacking gel, 15% resolving gel) (Laemmli, 1970). After electrophoresis, the gels were stained with 0.1% Coomassie Blue in 50% trichloroacetic acid for 15 min and destained overnight with 7% acetic acid. The gels were then dried and subjected to autoradiography at −70°C using X-Omat Kodak AR film and Du Pont Lightning Plus intensifying screens. Low mol. wt protein markers were used to determine the mol. wt of the samples.

Protein microsequencing

For protein sequencing, ~2 μg (= 80 pmol) of protein from the active fractions of C4 column were redissolved in 50% TFA and loaded onto an Applied Biosystems 477A gas-phase protein sequenator. Twenty-four rounds of Edman degradation were carried out using standard software and chemicals supplied by Applied Biosystems, and identifications of PTH amino acids were made with an automated on-line HPLC column (model 120A, Applied Biosystems).

References

Abraham,J.A., Mergia,A., Whang,J.L., Tumolo,A., Friedman,J., Hjerrild,K.A., Gospodarowicz,D. and Fiddes,J.C. (1986) *Science*, **233**, 545–547.

Blam,S., Mitchell,R., Tischer,E., Rubin,J.S., Silva,M., Silver,S., Fiddes,J.C., Abraham,J.A. and Aaronson,S.A. (1988) *Oncogene*, **3**, 129–136.

Carpenter,G. and Zendequi,J. (1985) *Anal. Biochem.*, **153**, 279–282.

Folkman,J. and Klagsbrun,M. (1987) *Science*, **235**, 442–447.

Gospodarowicz,D. (1987) *Methods Enzymol.*, **147**, 106–119.

Gospodarowicz,D., Ill,C.R. and Birdwell,C.R. (1977a) *Endocrinology*, **100**, 1108–1120.

Gospodarowicz,D., Mescher,A.L. and Birdwell,C.R. (1977b) *Exp. Eye Res.*, **25**, 75–89.

Gospodarowicz,D., Ill,C.R., Hornsby,P.J. and Gill,G.N. (1977c) *Endocrinology*, **100**, 1080–1089.

Gospodarowicz,D., Cheng,J. and Lirette,M. (1983) *J. Cell. Biol.*, **7**, 1677–1685.

Gospodarowicz,D., Cheng,J., Lui,G.M., Baird,A. and Böhlen,P. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 6963–6967.

Gospodarowicz,D., Massoglia,S., Cheng,J. and Fujii,D.K. (1986) *J. Cell. Physiol.*, **127**, 121–136.

Gospodarowicz,D., Ferrara,N., Schweigerer,L. and Neufeld,G. (1987) *Endocrine Rev.*, **8**, 95–114.

Gospodarowicz,D., Ferrara,N., Haaparanta,T. and Neufeld,G. (1988) *Eur. J. Cell. Biol.*, **46**, 144–151.

Gumbiner,B. and Kelly,R.B. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 318–332.

Harris-Hooker,S.A., Gajdusek,C., Wight,T.N. and Schwartz,S.M. (1983) *J. Cell. Physiol.*, **114**, 302–312.

Ishikawa,F., Miyazono,K., Hellman,U., Drexler,H., Wernstedt,C., Hagiwara,K., Usuki,K., Takaku,F., Risau,W. and Heldin,C.H. (1989) *Nature*, **338**, 557–561.

Jaye,M., Howk,R., Burgess,W., Ricca,G.A., Chiu,I.M., Ravera,M.W., O'Brien,S.J., Modi,W.E., Maciag,T. and Drohan,W.N. (1986) *Science*, **233**, 541–545.

Klagsbrun,M. and Shing,Y. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 805–809.

Laemmli,U.K. (1970) *Nature*, **227** 680–685.

Lipman,D.G. and Pearson,W.R. (1985) *Science*, **227**, 1435–1441.

McConahey,P. and Dixon,F.J. (1966) *Int. Arch. Allergy*, **29**, 185–189.

Miyazono,K., Okabe,T., Urabe,A., Takaku,F. and Heldin,C.H. (1987) *J. Biol. Chem.*, **262**, 4098–4103.

Montesano,R., Vassali,J.D., Baird,A., Guillemin,R. and Orsi,L. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 7297–7301.

Moscatelli,D., Presta,M., Joseph Silverstein,J. and Rifkin,D.B. (1986) *J. Cell. Physiol.*, **129**, 273–276.

Neufeld,G., Massoglia,S. and Gospodarowicz,D. (1986) *Regulatory Peptides*, **13**, 293–305.

Rubin,J.S., Osada,H., Finch,P.W., Taylor,W.G., Rudikoff,S. and Aaronson,S.A. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 803–806.

Saksela,O., Moscatelli,D. and Rifkin,D.B. (1987) *J. Cell. Biol.*, **107**, 957–962.

Schweigerer,L., Neufeld,G., Friedman,J., Abraham,J.A., Fiddes,J.C. and Gospodarowicz,D. (1987) *Nature*, **325**, 257–259.

Vlodavsky,I., Folkman,J., Sullivan,R., Fridman,R., Ishai-Michaeli,R., Sasse,J. and Klagsbrun,M. (1987) *Proc. Natl. Acad. Sci. USA*, **84**, 2292–2296.

Weissman,B.E. and Aaronson,S.A. (1983) *Cell*, **32**, 599–606.

Received on July 5, 1989; revised on August 11, 1989

Note added in proof

Further microsequencing of the AGT-20-derived growth factor showed that it has significant homology with the follicle stellate cell-derived growth factor [Gospodarowicz *et al.* (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 7311–7315]. It is therefore likely that it represents the mouse form of this newly identified family of vascular endothelial cell mitogens.