Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells

(growth factor/microsequencing/proliferation/angiogenesis)

D. GOSPODAROWICZ*, J. A. ABRAHAM†, AND J. SCHILLING†

*Cancer Research Institute, M-1282, University of California Medical Center, San Francisco, CA 94143; and †California Biotechnology Inc., 2450 Bayshore Parkway, Mountain View, CA 94043

Communicated by Robert W. Holley, June 19, 1989

ABSTRACT A growth factor with specificity for vascular endothelial cells has been identified in conditioned medium of pituitary-derived folliculo stellate cells. This factor, named folliculo stellate-derived growth factor (FSdGF), was purified to homogeneity by a combination of heparin-Sepharose affinity chromatography, Bio-Gel P-60 exclusion chromatography, Mono S ion-exchange chromatography, and hydrophobic chromatography on a C₄ reverse-phase HPLC column. FSdGF was characterized as a homodimer composed of two subunits with a molecular mass of 23 kDa. FSdGF was a potent mitogen for vascular endothelial cells with activity detectable at 25 pg/ml and saturation at 500 pg/ml. It did not stimulate the proliferation of other cell types such as bovine vascular smooth muscle cells, corneal endothelial cells, adrenal cortex cells, granulosa cells, BALB/MK cells, or BHK-21 cells. Microsequencing revealed an N-terminal sequence having no significant homology to any known protein. The release of FSdGF by pituitary cells and its target cell specificity raise the possibility that FSdGF may play a role in angiogenesis.

Angiogenesis is a multi-step phenomenon that involves capillary endothelial cell proliferation, migration, and tissue infiltration (1). It plays a central role in a variety of physiological and pathological processes such as embryonic development, wound healing, atherosclerosis, and tumor growth (1, 2). Several factors that induce angiogenesis have recently been isolated and characterized. Among these are the acidic and basic form of fibroblast growth factor (FGF), both of which are capable of stimulating capillary endothelial cell growth *in vitro* as well as being chemotactic for that cell type (2). In addition, both acidic FGF (aFGF) and basic FGF (bFGF) stimulate collagenase activity and plasminogen activator production while decreasing the production of plasminogen inhibitor (3, 4). These enzymes are involved in the breakdown of the capillary basement membrane, an event required in order for angiogenesis to take place (1).

Despite the evidence that FGF is angiogenic, two puzzling questions point to the existence of other angiogenic factors, which could complement the action of FGF. First, FGF lacks the hydrophobic signal sequence that governs secretion (5, 6), yet for acceptance as an angiogenic factor any putative mediator should be shown to be a diffusible substance that induces new capillary formation from a microcirculatory bed. Second, FGF is produced by endothelial cells themselves (7–9). If FGF is present in and around endothelial cells yet the cells are quiescent, other factors must come into play to trigger angiogenesis.

In the course of our study on the localization of bFGF in various tissues, it was observed that, in the pituitary gland, folliculo stellate (FS) cells are the main producers of bFGF (10). Although the medium conditioned by those cells was

found to be strongly mitogenic for capillary endothelial cells, little if any bFGF is present in it, consistent with the lack of a signal sequence on bFGF (10). This observation suggested that FS cells, in addition to synthesizing bFGF, are also capable of producing an endothelial cell mitogen that is released in soluble form. In this report, we describe the purification as well as the biological and biochemical characterizations of this factor provisionally named folliculo stellate-derived growth factor (FSdGF), and which appears to be specific for vascular endothelial cells.

MATERIALS AND METHODS

Cell Culture. Pituitary-derived FS cell cultures were prepared and characterized as previously described (10). Cultures of human umbilical endothelial cells (11), bovine brain and adrenal cortex-derived capillary endothelial (ACE) cells (12), vascular smooth muscle cells (13), bovine granulosa cells (14), adrenal cortex cells (15), corneal endothelial cells (16), baby hamster kidney cells clone 21 (BHK-21) (17), and BALB/MK mouse epidermal keratinocytes (18) (a gift from S. Aaronson, National Cancer Institute) were maintained as previously described (11–18).

Preparation of Conditioned Medium. Early passage FS cells were plated onto 600-cm² Nunc plates and grown to confluence over 4–5 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% calf serum, 5% fetal calf serum, and antibiotics (10). Confluent monolayers were washed twice with medium alone prior to the addition of 150 ml per plate of DMEM supplemented with gentamicin (50 µg/ml), Fungizone (2.5 µg/ml), leupeptin (10 µg/ml), insulin (5 µg/ml), and transferrin (10 µg/ml). After 48 or 72 hr, culture fluids were collected and replaced with the same amount of fresh serum-free medium. Collections could be made for a month without visible deterioration of the monolayer.

Isolation Procedure. Conditioned medium collected from the confluent monolayers was centrifuged (10,000 × *g*, 15 min) to remove floating cells and cell debris. The pH of the supernatant was then adjusted to 5.6 with 6 M HCl. (NH₄)₂SO₄ (520 g/liter) was added, and the suspension was kept at 4°C for 6 hr; the precipitate was then collected by centrifugation (10,000 × *g*, 30 min), redissolved in phosphate-buffered saline (PBS), and dialyzed overnight at 4°C against 10 mM Tris-HCl, pH 7.3/50 mM NaCl. The insoluble material was removed by centrifugation (10,000 × *g*, 30 min), and the supernatant was loaded onto heparin-Sepharose resin (20 ml) that had been equilibrated in 10 mM Tris-HCl, pH 7.3/50 mM

Abbreviations: FGF, fibroblast growth factor; aFGF, acidic FGF; bFGF, basic FGF; FS cells, folliculo stellate cells; FSdGF, folliculo stellate-derived growth factor; HSAC, heparin-Sepharose affinity chromatography; RP-HPLC, reverse-phase HPLC; ACE cells, adrenal cortex-derived capillary endothelial cells; TGF- α and - β , transforming growth factors α and β .

NaCl. The resin was washed extensively with the equilibration buffer until the absorbance had returned to baseline and was then eluted stepwise with increasing NaCl concentrations (0.15 M, 0.45 M, 1 M, and 3 M). 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 Bio-Gel P-60 column (100–200 mesh, 1×95 cm) 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 3-fold with 20 mM Hepes (pH 8.3). The sample was then applied onto a Mono S column linked to a fast HPLC system (Pharmacia). Elution was achieved with a multilinear gradient (20 mM Hepes at pH 8.3 to 20 mM Hepes at pH 8.3/1 M NaCl). After fraction aliquots were tested for bioactivity, the active fractions were pooled and loaded onto a Vydac C₄ HPLC column that had been equilibrated in 0.1% (vol/vol) trifluoroacetic acid/20% (vol/vol) 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. Bioactivity was not affected by long-term storage at –70°C or by repeated cycles of thawing and freezing.

Cell Proliferation Assays. The mitogenic activity of the column fractions and purified samples was determined by using as target cells ACE cells (12). For mitogenic assay, cells were seeded in 12-well cluster plates at a density of 5×10^3 cells per well in 1 ml of DMEM supplemented with 10% (vol/vol) calf serum and antibiotics, as described previously (13). Six hours later, a set of triplicate wells was trypsinized, and cells were counted to determine the recovery efficiency. Ten-microliter samples of the column fractions diluted in 0.2% gelatin in PBS were then added to triplicate wells on days 0 and 2. After 4 days in culture, the plates were trypsinized, and cell densities were determined with a Coulter counter.

NaDodSO₄/PAGE. Polyacrylamide gels (15%) were prepared with NaDodSO₄ by the procedure of Laemmli (19). Samples were boiled for 3 min in the presence or absence of 0.1 M dithiothreitol. The gels were fixed and silver-stained (20).

Protein Microsequencing. For protein sequencing, approximately 5 µg (200 pmol) of protein from the active fractions of the C₄ column were redissolved in 50% trifluoroacetic acid and loaded onto an Applied Biosystems 477A gas-phase protein sequencer. Twelve rounds of Edman degradation were carried out by using standard software and chemicals supplied by Applied Biosystems, and identifications of phenylthiohydantoin amino acids were made with an automated on-line HPLC column (model 120A; Applied Biosystems).

RESULTS

Growth Factor Isolation and Detection. Preliminary experiments indicated that medium conditioned by FS cells contained considerable amounts of mitogenic activity for capillary endothelial cells that could not be neutralized by specific aFGF or bFGF neutralizing polyclonal antibodies. Furthermore, when applied to a heparin-Sepharose affinity column in 0.6 M NaCl, the majority of the activity was not retained. In contrast, aFGF and bFGF are both retained under similar conditions (20).

(NH₄)₂SO₄ precipitation provided a convenient way of reducing the volume of the collected conditioned medium from the FS cells to a level suitable for subsequent heparin-Sepharose affinity chromatography (HSAC). Material not retained by the column was inactive and accounted for 50% of the total protein loaded (Fig. 1A). Elution with 0.15 M NaCl

yielded a small peak of protein with no bioactivity, whereas elution with 0.45 M NaCl yielded a major peak of protein with 10% of the bioactivity applied to the column. Elution with 1 M NaCl gave a protein peak in which 90% of the bioactivity applied on the column was recovered (Fig. 1A). Overall, the HSAC resulted in a 30-fold purification and was followed by gel-exclusion chromatography with Bio-Gel P-60 (Fig. 1B). The bioactivity eluted as a major peak with an apparent molecular mass of 40–45 kDa. This step resulted in a further 10-fold purification with a recovery of 108% (Table 1). The pooled Bio-Gel P-60 bioactive fractions were applied on a Mono S column (Fig. 1C). The bioactive profile consisted of two minor peaks of bioactivity eluting, respectively, at 0.23 M NaCl and 0.28 M NaCl, with a major bioactive peak eluting at 0.33 M NaCl. Analysis of the bioactivity present in the various peaks indicated that the 0.33 M NaCl fractions contained 5-fold more activity than either the 0.23 M or 0.28 M NaCl peak (data not shown). The Mono S step gave a further 3-fold increase in specific biological activity over the Bio-Gel P-60 step; recovery was 54% (Table 1). The two other bioactive peaks accounted for the remainder of the bioactivity.

Final purification of the endothelial cell mitogenic activity was achieved by reverse-phase HPLC (RP-HPLC) with a C₄ Vydac column (Fig. 2A), 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 that, when analyzed by NaDodSO₄/PAGE, gave the same single band on the silver-stained gel (Fig. 3). The RP-HPLC step resulted in at least a 8-fold increase in specific biological activity with an overall recovery of 25% (Table 1). Fig. 2B illustrates the relative potency of the various fractions at different stages of purification.

When the bioactive 0.23 M and 0.28 M NaCl pooled Mono S fractions were chromatographed under similar conditions on the C₄ column, a biological profile identical to that observed for the 0.33 M pooled Mono S fractions was obtained (data not shown). The major portion (90%) of the bioactivity coincided with two closely apposed peaks of proteins, which eluted in the same position as those observed with the 0.33 M NaCl Mono S fraction. When analyzed by NaDodSO₄/PAGE, these peaks gave, under reduced conditions, a common band at 23 kDa, migrating in the same position as that observed in Fig. 3. When analyzed by microsequencing, these bands had the same N-terminal sequence as the 0.33 M Mono S fraction. Therefore, the bioactive peptides present in the 0.23 M, 0.28 M, and 0.33 M NaCl Mono S fractions represent microheterogeneous forms of the same growth factor.

Physical and Biological Characterization. Microsequencing of the C₄-purified material (200 pmol) revealed an N-terminal amino acid sequence that had no significant homology to other proteins, and a single amino acid was identified in each of the first 12 cycles, consistent with a homogeneous protein. The yield of the N-terminal residue was 150 picomol. Unambiguous assignments made for cycles 1–12 were as follows: Ala-Pro-Met-Ala-Glu-Gly-Gly-Gln-Lys-Pro-His-Glu. A search of the National Biomedical Research Foundation data base using the FASTP program of Lipman and Pearson (21) found no significant homology between this sequence and any known protein.

The purified factor when run under nonreducing conditions had an estimated molecular mass of 46 kDa (Fig. 3). This value is in good agreement with its elution position on the sizing Bio-Gel P-60 column eluted with solvents expected to maintain the native conformation. When run under reducing conditions, the apparent molecular mass was 23 kDa (Fig. 3). From these data, it appears that the mitogen consists of two

FIG. 1. Purification of FSdGF by HSAC, gel-exclusion chromatography, and Mono S ion-exchange chromatography. (A) Approximately 350 ml of the $(\text{NH}_4)_2\text{SO}_4$ precipitate fractions derived from 21 liters of FS cell-conditioned medium and dialyzed against 10 mM Tris-HCl, pH 7.3/50 mM NaCl was loaded onto a heparin-Sepharose column (1.5 cm $\times$ 12 cm, 25-ml bed volume) at a flow rate of 150 ml/hr. 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 were eluted with a stepwise application of increasing NaCl concentrations (0.15 M, 0.45 M, 1 M, and 3 M). The fraction size was 2 ml, and the flow rate was 60 ml/hr. The histogram and open 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 per well). In the case of the pooled fractions (eluate, wash, and 0.15 M NaCl), aliquots were diluted 10-fold in 0.2% gelatin in PBS, and 10- μ l aliquots were bioassayed. In the case of the individual 0.45 M and 1 M NaCl fractions, aliquots were diluted 100-fold, and 10- μ l aliquots were bioassayed. (B) After concentrating the 1 M NaCl HSAC bioactive fractions 126–133 to 1 ml in an Amicon YM 10 concentrator, the ultrafiltration retentate was applied on a Bio-Gel P-60 column (100–200 mesh, 1 $\times$ 95 cm) equilibrated and run at 4°C in PBS. The flow rate for development of the column was 6 ml/hr, and 1.45-ml fractions were collected. The elution positions of molecular mass markers (in kDa) are indicated by the arrows. Aliquots of each fraction were diluted 100-fold, and 10- μ l aliquots were bioassayed in ACE cells in 12-well cluster plates. (C) The bioactive fractions 26–29 eluted from the Bio-Gel P-60 column were pooled and diluted 3-fold with 20 mM Hepes (pH 8.3). 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). The flow rate was 1 ml/min, and 1-ml fractions were collected. Aliquots of each fraction were diluted 100-fold, and 10- μ l aliquots were bioassayed on ACE cells in 12-well cluster plates. Fractions indicated by the asterisks were pooled and further examined by RP-HPLC with a C_4 column.

polypeptide chains with a molecular mass of 23 kDa. Given that a single N-terminal sequence was obtained, the dimeric molecule is probably composed of two identical or at least very homologous chains.

The dose-response curves for FSdGF, depicted in Figs. 2B and 4, illustrate that as little as 25 pg/ml stimulates ACE cell proliferation. Saturation was observed at 500 pg/ml with an ED_{50} of 65 pg/ml (Fig. 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; ref. 12 and Fig. 4B). However, the final density of the culture grown in presence of FSdGF was half that of cultures exposed to optimal concentrations of bFGF. Nevertheless, if one considers that the molecular mass of FSdGF is 2.5 times that of bFGF, FSdGF has essentially the same potency on a molar

Table 1. Summary of purification of FSdGF

Purification step	Protein, μ g	Maximal stimulation, ng/ml	ED_{50} ,* ng/ml	Total activity, units $\times 10^{-5}$	Fold purification	Yield, %
$(\text{NH}_4)_2\text{SO}_4$ precipitate	405,000	750 [†]	140 [†]	29 [†]	1	— [†]
HSAC (1 M NaCl pool)	13,464 [‡]	100	15	9	30 [§]	100
Bio-Gel P-60	1,477 [‡]	10	1.5	9.8	300	108
Mono S fractions 39–42	244 [‡]	8	0.5	4.9	900	54
C_4 HPLC	15 [¶]	0.5	0.05	2.3	6923	25

*The ED_{50} was determined as the protein concentration that gave a half stimulation of cell proliferation in the ACE cell assay. It corresponded to one unit of activity.

[†]The growth-promoting activity in the $(\text{NH}_4)_2\text{SO}_4$ fraction was variable, maybe due to the presence of growth inhibitor. Maximal stimulation at saturation was also much lower than that observed for other partially purified fractions (see Fig. 2B). Therefore, the yield was based on the total activity present in the first partially purified fraction (HSAC, 1 M NaCl).

[‡]Protein was estimated by using the Bradford reagent from Bio-Rad with bovine serum albumin as standard.

[§]The purification in the HSAC step was estimated at 30-fold based on the amount of protein recovered.

[¶]Protein was estimated by amino acid analysis.

FIG. 2. RP-HPLC of the bioactive Mono S-purified fractions and comparison of the ability of FS cell-conditioned medium at various stages of purification to stimulate the proliferation of low-density ACE cell cultures. (A) The active Mono S fractions (fractions 38–42; Fig. 1C) were loaded onto a Vydac C₄ column (25 $\times$ 0.46 cm, 5- μ m particle size, 300-Å pore size) equilibrated in 0.1% (vol/vol) trifluoroacetic acid/20% acetonitrile. The arrows show the times of injection. Protein was eluted with a 115-min linear gradient of 20–45% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.6 ml/min at room temperature. Fractions of 1.5 ml were collected, except in the region where the bioactivity was expected to elute; in this region, fraction volumes were manually limited to the size of the individual peak fractions. Aliquots of each fraction were diluted 10-fold and bioassayed. The histogram shows the distribution of the biological activity. The peak fractions indicated by the asterisks were used individually for structural studies and further analysis of their biological activity. (B) Low-density ACE cell cultures (5×10^3 cells per well) were seeded, and their proliferation was measured. Samples tested were $(NH_4)_2SO_4$ precipitate (Δ), pool of the HSAC nonretained material ($\blacktriangle$), pool of the HSAC 0.45 M NaCl fractions ($\circ$), pool of the HSAC 1 M NaCl fractions ($\bullet$), pool of the bioactive Bio-Gel P-60 fractions ($\square$), pool of the bioactive Mono S fractions (∇), bioactive C₄ fractions ($\diamond$). Individual points are the means of triplicate determinations.

basis as bFGF. In addition to its ability to stimulate the proliferation of ACE cells, FSdGF stimulated the growth of bovine brain-derived capillary endothelial cells (data not shown) as well as that of human umbilical endothelial cells (Fig. 4A). These results indicate that the mitogenic effect of the factor is not limited by species variation or by the origin of the vascular endothelial cells. However, and in contrast with bFGF, the factor is not mitogenic for BHK-21 cells (Fig. 4C), and it is not mitogenic for adrenal cortex cells, corneal endothelial cells, granulosa cells, BALB/MK cells, or vascular smooth muscle cells (Fig. 4D). Therefore, and in contrast with FGF, this factor seems to have a specificity for vascular endothelial cells.

DISCUSSION

In the present study, we have identified an endothelial cell mitogen factor that seems to have a distinct specificity for vascular endothelial cells. This growth factor (FSdGF), released by FS cells, was purified with a combination of HSAC,

FIG. 3. NaDodSO₄/PAGE of the bioactive fractions purified by C₄ RP-HPLC. Protein samples (10 μ l) of the fractions indicated by the asterisks in Fig. 2A were analyzed individually under nonreducing (lanes A and B) or reducing (lanes C and D) conditions. After electrophoresis, the gels were silver-stained. The position of each molecular mass marker (in kDa) is indicated by an arrow.

exclusion-gel chromatography, cation-exchange chromatography, and RP-HPLC. The purification resulted in an apparent increase in specific activity of 7000-fold, with a 25% recovery of growth-promoting activity (Table 1 and Fig. 2B).

Analysis by NaDodSO₄/PAGE showed that the factor is a dimeric molecule with an apparent molecular mass of 46 kDa. The factor is retained on a Mono S column when applied at pH 8.3 and elutes at high salt concentration (0.33 M NaCl), indicating that the factor has a basic isoelectric point. The biological properties of FSdGF seem to be unique to this growth factor, since of all the cells tested it stimulated the growth of only vascular endothelial cells without affecting that of vascular smooth muscle 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 α (TGF- α), epidermal growth factor, platelet-derived growth factor, TGF- β , and aFGF or bFGF (reviewed in ref. 17).

The physical properties of the growth factor described in this paper (molecular mass of 46 kDa, basic isoelectric point, affinity for heparin-Sepharose, and its N-terminal sequence) and biological properties (mitogenic for vascular endothelial cells) indicate that it is distinct from other known growth factors such as epidermal growth factor, TGF- α , platelet-derived growth factor, TGF- β , or the recently reported keratinocyte growth factor (22). Its lack of recognition by neutralizing polyclonal antibodies directed against aFGF or bFGF as well as its lack of crossreactivity 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 (PDEC GF; ref. 23). Although PDEC GF and FSdGF have the same apparent target cell specificity and similar molecular mass, they differ by 20-fold in potency and by their secondary structure: PDEC GF is a single-chain polypeptide, whereas FSdGF has a dimeric structure.

The target cell specificity and N-terminal sequence of FSdGF lead us to conclude that it represents a previously unreported growth factor. Although the present study clearly established that it is mitogenic for capillary endothelial cells, it is not yet known whether FSdGF can stimulate other events linked to angiogenesis. These include chemotaxis of

FIG. 4. Comparison of the ability of pituitary-derived bFGF versus FSdGF to stimulate the growth of vascular endothelial cells and other unrelated cell types. (A) Low-density cultures of human umbilical endothelial cells (5×10^3 cells per 2.2-cm-diameter gelatinized well) were exposed to Hepes (25 mM) buffered medium 199 supplemented with heparin (100 $\mu\text{g/ml}$), 10 nM selenium, 20% FCS, and increasing concentrations of either pituitary-derived bFGF ($\circ$) or FSdGF ($\bullet$). Heparin was added only once at the time of seeding, whereas both bFGF and FSdGF were added every other day. After 6 days in culture, triplicate wells were trypsinized, and cells were counted. (B) Low-density cultures of ACE cells (5×10^3 cells per 2.2-cm-diameter well) were exposed to DMEM supplemented with 10% calf serum and increasing concentrations of either pituitary-derived bFGF ($\circ$) or FSdGF ($\bullet$) added every other day. After 5 days in cultures, triplicate wells were trypsinized and cells were counted. The right ordinate represents the final density of cultures exposed to FSdGF, whereas the left one represents that of cultures exposed to bFGF. (C) Low-density cultures of BHK-21 cells (2×10^4 cells per 35-mm gelatinized tissue culture dishes) were exposed to 2 ml of DMEM/F-12 medium (1:1, vol/vol) supplemented with Fungizone (2.5 $\mu\text{g/ml}$), gentamicin (50 $\mu\text{g/ml}$), transferrin (10 $\mu\text{g/ml}$), insulin (5 $\mu\text{g/ml}$), and increasing concentrations of either pituitary-derived bFGF ($\circ$) or FSdGF ($\bullet$). Insulin and transferrin were added only once; bFGF and FSdGF were added every other day. After 4 days in culture, triplicate dishes were trypsinized, and cells were counted. (D) Bovine corneal endothelial (BCE) cells, granulosa cells, adrenal cortex cells, or vascular smooth muscle (VSM) cells (2×10^4) were seeded in their respective medium in 35-mm dishes (DMEM supplemented with 10% calf serum for bovine corneal endothelial cells and adrenal cortex cells, F-12 medium supplemented with 2.5% calf serum for granulosa cells, and DMEM supplemented with 10% platelet-poor plasma serum for vascular smooth muscle cells). BALB/MK cells were seeded at a density of 3×10^4 cells per 35-mm dishes in low- Ca^{2+} modified Eagle's medium supplemented with 10% fetal calf serum. bFGF (2 ng/ml), aFGF (100 ng/ml in the case of vascular smooth muscle cells or 10 ng/ml in the case of BALB/MK cells), or FSdGF (5 ng/ml) were added every other day. After 6 days in cultures, cells were trypsinized and counted.

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 (3).

Available structural data should allow studies on the cloning, structure, topology, expression, and regulation of the FSdGF gene in both physiological and pathological conditions. These studies may provide clues as to the distribution of FSdGF in normal versus malignant tissues as well as to its physiological functions.

We recognize the early contribution of Drs. Plouet and Ferrara, who provided evidence for a growth factor with a molecular mass of 39 kDa. This study was supported by Grants HL20197 and EY02186 from the National Institutes of Health.

1. Folkman, J. (1986) *Cancer Res.* **46**, 467-473.
2. Gospodarowicz, D., Ferrara, N., Schweigerer, L. & Neufeld, G. (1987) *Endocr. Rev.* **8**, 95-114.
3. Saksela, O., Moscatelli, D. & Rifkin, D. B. (1987) *J. Cell Biol.* **107**, 957-962.
4. Montesano, R., Vassalli, J. D., Baird, A., Guillemin, R. & Orsi, L. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 7297-7301.
5. Abraham, J. A., Mergia, A., Whang, J. L., Tumolo, A., Friedman, J., Hjerrild, K. A., Gospodarowicz, D. & Fiddes, J. C. (1986) *Science* **233**, 545-547.
6. Jaye, M., Howk, R., Burgess, W., Ricca, G. A., Chiu, I. M., Ravera, M. W., O'Brien, S. J., Modi, W. E., Maciag, T. & Drohan, W. N. (1986) *Science* **233**, 541-545.
7. Schweigerer, L., Neufeld, G., Friedman, J., Abraham, J. A., Fiddes, J. C. & Gospodarowicz, D. (1987) *Nature (London)* **325**, 257-259.

8. Moscatelli, D., Presta, M., Silverstein, J. & Rifkin, D. B. (1986) *J. Cell. Physiol.* **129**, 273-276.
9. Vlodavsky, I., Folkman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasse, J. & Klagsburn, M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 2292-2296.
10. Ferrara, N., Schweigerer, L., Neufeld, G., Mitchell, R. & Gospodarowicz, D. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 5773-5777.
11. Gospodarowicz, D., Cheng, J. & Lirette, M. (1983) *J. Cell Biol.* **97**, 1677-1685.
12. Gospodarowicz, D., Massoglia, S., Cheng, J. & Fujii, D. K. (1986) *J. Cell. Physiol.* **127**, 121-136.
13. Gospodarowicz, D., Ferrara, N., Haaparanta, T. & Neufeld, G. (1988) *Eur. J. Cell Biol.* **46**, 144-151.
14. Gospodarowicz, D., Ill, C. R. & Birdwell, C. R. (1977) *Endocrinology* **100**, 1108-1120.
15. Gospodarowicz, D., Ill, C. R., Hornsby, P. J. & Gill, G. N. (1977) *Endocrinology* **100**, 1080-1089.
16. Gospodarowicz, D., Mescher, A. L. & Birdwell, C. R. (1977) *Exp. Eye Res.* **25**, 75-89.
17. Neufeld, G., Massoglia, S. & Gospodarowicz, D. (1986) *Regul. Pept.* **13**, 293-305.
18. Weissman, B. E. & Aaronson, S. A. (1983) *Cell* **32**, 599-606.
19. Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685.
20. Gospodarowicz, D., Cheng, J., Lui, G.-M., Baird, A. & Böhlen, P. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 6963-6967.
21. Lipman, D. G. & Pearson, W. R. (1985) *Science* **227**, 1435-1441.
22. Rubin, J. S., Osada, H., Finch, P. W., Taylor, W. G., Rudikoff, S. & Aaronson, S. A. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 803-806.
23. Ishikawa, F., Miyazono, K., Hellman, U., Drexler, H., Wernstedt, C., Hagiwara, K., Usuki, K., Takaku, F., Risau, W. & Heldin, C. H. (1989) *Nature (London)* **338**, 557-561.