Proc. Natl. Acad. Sci. USA
Vol. 76, No. 11, pp. 5674–5678, November 1979
Cell Biology

An endothelial cell growth factor from bovine hypothalamus: Identification and partial characterization

(fibroblast growth factor/thrombin)

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Communicated by Arthur B. Pardee, July 12, 1979

ABSTRACT **Extracts of bovine hypothalamus were found to contain a significant level of mitogenic activity when tested in a Swiss 3T3 cell [^{3}H]dThd incorporation assay and in a human umbilical vein endothelial cell growth assay. The mitogenic activity responsible for 3T3 cell activity was purified and characterized as a fibroblast growth factor (FGF)-like mitogen. Neither the biologically active FGF-like mitogen purified from the hypothalamus extracts nor FGF purified from bovine pituitary glands was mitogenic when added to human endothelial cells *in vitro*, suggesting the presence of more than one mitogen in the hypothalamic extracts. The 3T3 and endothelial cell biological activities of hypothalamic extracts were both found to be inactivated by trypsin, subtilisin, and heat treatment, but were stable to dialysis. The endothelial cell growth factor activity could be efficiently separated from the FGF activity by gel exclusion chromatography. The endothelial cell mitogen possessed a molecular weight of approximately 75,000, whereas that of FGF was approximately 15,000. The endothelial cell growth factor activity was found to be inactivated with reducing agents whereas the 3T3 cell mitogenic activity was stable after incubation with 2-mercaptoethanol. Significant levels of endothelial cell mitogenic activity were also found in extracts of bovine brain and pituitary glands.**

The vascular endothelium exists as a functional monolayer interface between blood and tissue (1, 2) that has been previously characterized *in vivo* as a population of cells with a low mitotic index (3). Factors that influence the growth and survival of vascular endothelial cells *in vitro* have important implications not only in the elucidation of normal and pathological states such as thrombus formation (4), the generation of granulation tissue, wound regeneration (5), and tumor growth (6), but also in providing the necessary *in vitro* systems for the study of these processes (7).

Previous investigations have shown that human and bovine vascular endothelial cells could be grown and maintained in culture if the cell culture medium was supplemented with high concentrations of bovine brain or pituitary fibroblast growth factor (FGF) (5). These studies eventually led to the establishment of a bovine vascular endothelial cell line that has a strict requirement for FGF (7). Additional experiments revealed that the mitogenic effect of FGF in vascular endothelial cell culture can be potentiated by the addition of purified human thrombin (5). It was suggested that the enhancement of FGF action by thrombin in the vascular endothelial cell system does not proceed through a mechanism involving the proteolytic digestion or modification of FGF, but as a result of an interaction between the vascular endothelial cells and thrombin (5).

Our studies indicate that human umbilical vein vascular endothelial cells do not respond at low seed density to supplementation with high concentrations of biologically active FGF or human thrombin or both. However, an excellent human endothelial cell mitogenic response was obtained with crude preparations of bovine pituitary, brain, and hypothalamus.

METHODS AND MATERIALS

Cell Culture. Swiss Albino mouse 3T3 embryo fibroblasts were obtained from the American Type Culture Collection. Human umbilical vein endothelial cells (second passage) were obtained from A. Johnson (University of Texas, Dallas, TX). The Swiss albino 3T3 cells were maintained and grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum (FCS), 10 units of penicillin per ml, and 0.7 μg of streptomycin per ml. The human umbilical endothelial cells were grown and maintained in medium 199 (GIBCO) supplemented with 20% FCS (GIBCO), 300 μg of bovine hypothalamic extract, 10 units of penicillin per ml, and 0.7 μg of streptomycin per ml. All cultures were grown at 37°C in an atmosphere of 95% air/5% CO_2. After the completion of the experiments cited in this paper, immunofluorescent antibody staining and enzyme marker assays revealed that the human umbilical vein endothelial cells (passage 14) contained the human Factor VIII antigen and the angiotensin I converting enzyme, kininase II (98–136.5 nmol/hr per mg).

Swiss albino 3T3 cells were seeded in microtiter wells (Microtest II tissue culture plates, Falcon Bioquest, Cockeysville, MD) at a cell density of 4×10^3 cells per microtiter well in modified Eagle's medium supplemented with 10% FCS. After confluency was achieved, the medium was aspirated and replaced with 180 μl of depleted modified Eagle's medium/10% FCS (diluted 1:5 with regular modified Eagle's medium) obtained from 6- to 7-day-old Swiss albino 3T3 cell cultures. The depleted modified Eagle's medium/2% FCS microtiter well was supplemented with 20 μl of the growth factor sample (in triplicate), whereas the control microtiter wells were supplemented with 20 μl of FCS (10% FCS control) and 20 μl of modified Eagle's medium (2% depleted FCS control). At time zero, 2.5 μCi of [^{3}H]dThd (New England Nuclear; 1 Ci = 3.7×10^{10} becquerels) was added to each microtiter well without any medium change. After 19 hr of incubation, visual observations were recorded, the cells were fixed, and DNA synthesis was measured by scintillation counting. The results of [^{3}H]dThd incorporation are reported as the percent of the 10% FCS control.

Human umbilical vein endothelial cells were seeded in 35-mm tissue culture dishes (Corning) at a density of 1.6×10^4 cells per dish in medium 199 supplemented with 20% FCS. Samples containing the endothelial cell growth factor were then added to the cell culture dish and the cultures were incubated at 37°C for at least 7 days. The endothelial cell assay cultures

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Abbreviations: FGF, fibroblast growth factor; FCS, fetal calf serum.

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were fed every 2–3 days with the appropriate supplements. Cultures containing 20% FCS in the absence of the endothelial cell growth factor supplement served as the control. Duplicate hemacytometer cell counts were obtained from each cell culture dish after trypsinization with a trypsin/EDTA solution in Hanks' balanced salt solution (GIBCO). Results are reported directly as a number of endothelial cells per cell culture dish.

Preparation of Hypothalamic, Pituitary, and Brain Extracts. The hypothalamic extracts were prepared by homogenization of approximately 370 g of bovine hypothalamus (Pel-Freez) in 500 ml of 0.1 M NaCl at 4°C in a Waring blender for 3–4 min (Collaborative Research, Waltham, MA; CR-ECGS). The homogenate was allowed to stir at 4°C for 2 hr, after which the sample was centrifuged at 13,800 × *g* for 40 min and the supernatant was recovered. The extracts of bovine brain and pituitary glands (Snyder Brothers, Sutton, MA) were prepared in an identical fashion.

The hypothalamic extract was further fractionated with streptomycin sulfate (GIBCO) in order to remove soluble lipid. The supernatant was extracted with 0.5% streptomycin sulfate at pH 7.0 for at least 1 hr, after which the extract was centrifuged at 13,800 × *g* for 40 min and the supernatant was recovered.

Isolation and Separation of Endothelial Cell Growth Factor From FGF. Bovine pituitary FGF and hypothalamic FGF were both purified by the methods previously described by Gospodarowicz and coworkers (8, 9). The hypothalamic endothelial cell growth factor activity was separated from the hypothalamic FGF (3T3 cell) activity by chromatography of the hypothalamic extracts on Sephadex G-100. A Sephadex (Pharmacia) G-100 column (5 × 90 cm) was equilibrated in 50 mM Tris·HCl, pH 7.5/0.5 mM EDTA. The sample of hypothalamic extract was applied in a volume of 10 ml and eluted with the equilibration buffer. Fractions (19 ml) were collected and assayed for absorbance at 280 nm. After completion of the chromatography, the protein profile was separated into seven distinct pools, lyophilized, and resuspended in 10 ml of distilled, deionized water. The Sephadex G-100 column was calibrated with proteins of known molecular weight: bovine serum albumin, M_r 67,000 (Sigma); mouse 7S nerve growth factor, M_r 140,000 (10); and 2.5S nerve growth factor, M_r 26,000 (11).

RESULTS

Presence of Mitogenic Activity in Extracts of Bovine Hypothalamus. To ascertain whether the hypothalamus contained mitogenic components, we prepared water-soluble extracts of the hypothalamus and assayed them for biological activity in two cell culture systems. Incorporation of [^{3}H]thymidine into Swiss albino 3T3 cells in the presence of hypothalamic extract (Fig. 1*A*) revealed that the extract contained a significant amount of biological activity. In addition, supplementation of human umbilical vein endothelial cells with hypothalamic extract at various dilutions also revealed significant *in vitro* mitogenic activity (Fig. 1*B*). The biological activity of the extract in the 3T3 cell culture assay was present in the range of 15–150 μg of protein per ml, whereas the human endothelial cells were active in the range of 150–300 μg of protein per ml. Extracts prepared from bovine brain and pituitary glands also contained significant levels of human endothelial cell and 3T3 cell mitogenic activity (Fig. 2).

Characterization of 3T3 and Endothelial Cell Mitogenic Activity. Because FGF, previously purified from brain and pituitary glands (8, 9), has been characterized as a heat-sensitive and trypsin-labile 3T3 and endothelial cell mitogen (8), studies were performed to determine whether the mitogenic activity

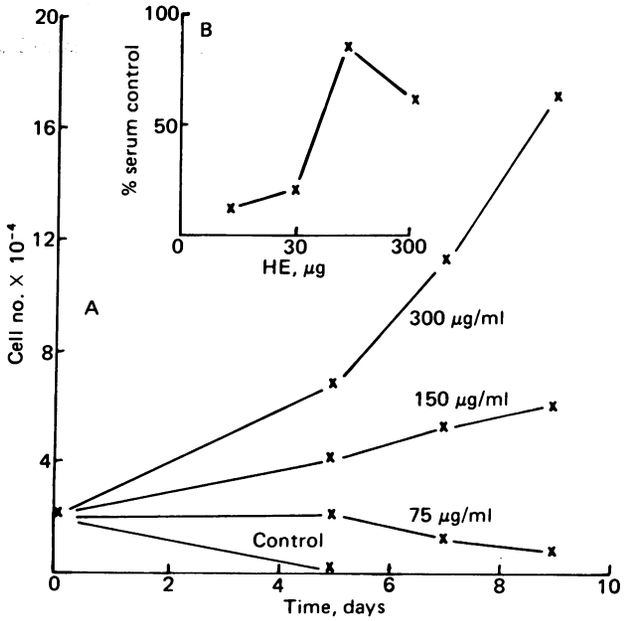

FIG. 1. Stimulation of human umbilical vein endothelial cell division and DNA synthesis in Swiss albino 3T3 cells by hypothalamic extract. (*A*) Human umbilical vein endothelial cells were seeded into multiple cell culture dishes containing medium 199 supplemented with 20% FCS and varying amounts of hypothalamic extract. Cultures containing 20% FCS in the absence of hypothalamic extract served as controls. Endothelial cell number of duplicate plates was determined after 5, 7, and 9 days in culture. (*B*) Hypothalamic extract (HE) was added to confluent Swiss albino 3T3 cell microtiter cultures in 2% depleted FCS. DNA synthesis was measured 19 hr after the addition of [^{3}H]dThd. Microtiter wells containing 2% depleted FCS and 10% FCS served as controls. Data are reported as the percent of [^{3}H]dThd uptake in the 10% FCS control. A sample of 150 μg of hypothalamic extract yielded an incorporation of 215,000 cpm.

of hypothalamic extract responsible for 3T3 and endothelial cell growth was FGF, FGF-like, or indeed a unique mitogen. The data shown in Fig. 3*A* revealed that treatment with trypsin at 25°C for 5 hr or incubation at 60°C for 20 min significantly

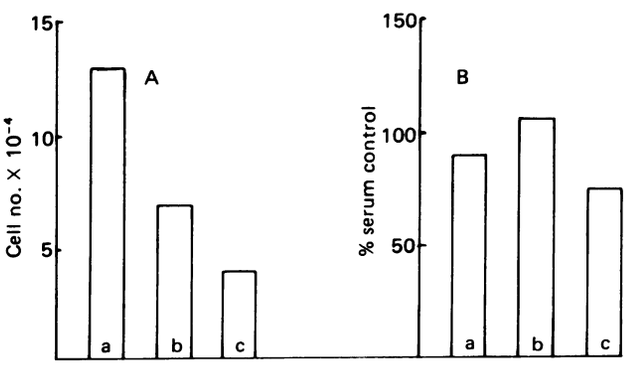

FIG. 2. Stimulation of human umbilical vein endothelial cell division and DNA synthesis in Swiss albino 3T3 cells in the presence of bovine brain, hypothalamus, and pituitary extracts. (*A*) Human umbilical vein endothelial cell cultures were supplemented with 20% FCS in medium 199 containing bovine hypothalamic extract (180 μg/ml) (*a*), pituitary extract (270 μg/ml) (*b*), and brain extract (115 μg/ml) (*c*). Cultures were fed every second day, and the cell number was obtained on the seventh day. (*B*) Incorporation of [^{3}H]dThd in confluent Swiss Albino 3T3 cells was measured in response to hypothalamic extract (180 μg/ml) (*a*), pituitary extract (270 μg/ml) (*b*), and brain extract (115 μg/ml) (*c*) in the presence of 2% depleted FCS. DNA synthesis was measured 19 hr after the addition of [^{3}H]dThd. Results are reported as the percent of the 10% FCS control.

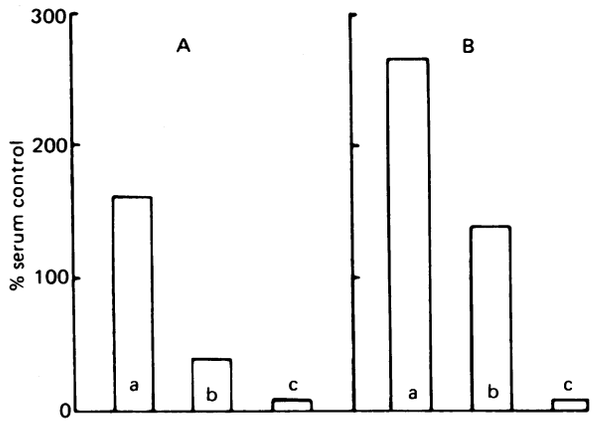

FIG. 3. Effect of heat and trypsinization on the Swiss albino 3T3 biological activity of the FGF from hypothalamic extract and purified bovine pituitary. (*A*) Samples of hypothalamic extract (1 mg/ml) were incubated for 3 hr at 37°C with 5 μg of trypsin (6000 units/mg) in 0.1 M Tris·HCl (pH 9.5). Soybean trypsin inhibitor (10 μg/ml) was added to the reaction vessel and the trypsin-treated extract (150 μg/ml) was assayed for [^{3}H]dThd incorporation in the Swiss albino 3T3 microtiter assay. Samples of hypothalamic extract (10 mg/ml) were also heat treated (60°C, 15 min) and assayed (300 μg/ml) for biological activity. Data are reported as the percent of the 10% FCS control for hypothalamic extract control (300 μg/ml) (*a*), trypsin-treated extract (*b*), and heat-treated extract (*c*). (*B*) Trypsinization and heat-treatment experiments were repeated with purified bovine FGF (10 μg/ml). Data are reported as the percent of the 10% FCS control with 100 ng of FGF control (*a*), trypsin-treated FGF (*b*), and heat-treated FGF (*c*) per ml.

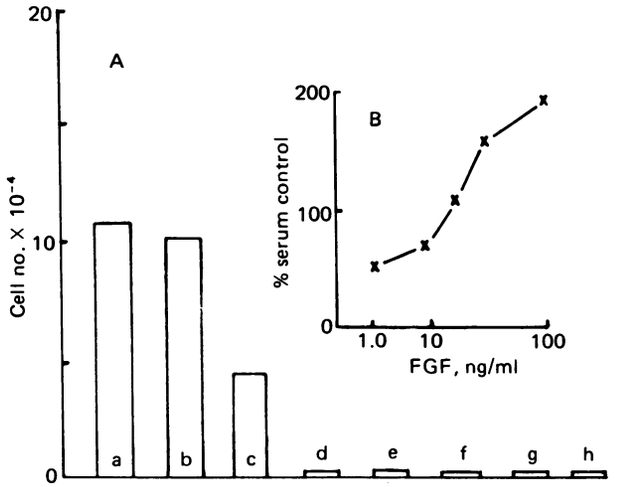

FIG. 4. Characterization of hypothalamic extract and the response of human endothelial cell growth and DNA synthesis in Swiss albino 3T3 cells to purified pituitary and hypothalamic FGF. (*A*) Effect of hypothalamic extract (300 μg/ml) on human endothelial cell growth in culture after supplementation (6 days) with 20% FCS and hypothalamic extract control (*a*), dialysis against 0.15 M NaCl (4°C, 18 hr) (*b*), digestion with subtilisin (Sigma, 7 units/mg) for 24 hr in 20 mM sodium phosphate, pH 7.0/5 mM $CaCl_2$ (*c*), and denaturation with heat (60°C, 15 min) (*d*) and after supplementation with 1 μg of purified pituitary FGF (*e*), pituitary FGF and human thrombin (2.07 × 10^3 NIH units/mg) (*f*), purified hypothalamic FGF (*g*), and hypothalamic FGF and human thrombin (*h*). (*B*) Incorporation of [^{3}H]dThd in confluent Swiss albino 3T3 cells as a function of the concentration (ng/ml) of purified bovine pituitary FGF.

reduced the 3T3 cell culture activity of the extract. Similar results were also obtained with purified bovine pituitary gland FGF (Fig. 3*B*), suggesting that the 3T3 cell biological activity of hypothalamic extract possessed FGF or FGF-like properties. Data obtained from studies using human endothelial cells revealed that the endothelial cell activity of hypothalamic extract was also labile to heat, sensitive to incubation with subtilisin, and resistant to dialysis (Fig. 4*A*), suggesting that the mitogenic component of the extract responsible for 3T3 and endothelial cell growth was either FGF or an FGF-like mitogen. The response of human endothelial cell growth in the presence of purified bovine pituitary gland FGF was also studied. We did not observe any significant increase in human endothelial cell growth in the presence of purified FGF at concentrations as high as 1 μg/ml (Fig. 4*A*) even though the FGF used in these studies was biologically active in the 3T3 cell microtiter assay (Fig. 4*B*). Supplementation of the FGF with human thrombin (1 μg/ml) also failed to produce an endothelial cell mitogenic response (Fig. 4*A*).

Purification of Bovine Hypothalamus FGF. Because it remained a possibility that the human endothelial cell growth-promoting activity of hypothalamic extract may have been due to the presence of a unique form of FGF present in the extract preparations, the hypothalamic FGF was purified by procedures previously described for bovine pituitary FGF (8). Gel exclusion chromatography of the biological activity of hypothalamic FGF eluted with a molecular weight of approximately 15,000 (Fig. 5*A*). Biological assay of the hypothalamic FGF Sephadex G-75 fraction (peak 4) demonstrated that the purified mitogen was active in the low nanogram range (Fig. 5*B*). The hypothalamic FGF fraction was also tested for biological activity in the human endothelial cell culture assay. We observed no increase in endothelial cell number in cultures supplemented with either purified hypothalamic FGF or hypothalamic FGF and purified human thrombin (Fig. 4*A*).

We were also able to determine that the endothelial cell growth factor activity present in extracts of the hypothalamus is chemically distinct from FGF. Incubation of the hypothalamic extract with 2-mercaptoethanol resulted in the complete loss of endothelial cell mitogenic activity with no effect on the 3T3 cell activity of the hypothalamic preparation (Fig. 6). In

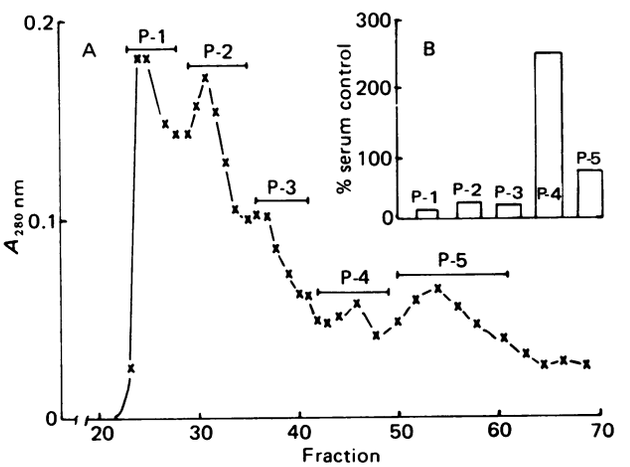

FIG. 5. Biological activity of hypothalamic FGF in Swiss albino 3T3 cells after purification on Sephadex G-75. (*A*) Hypothalamic FGF sample was obtained as a result of 1 M NaCl elution from a carboxymethyl-Sephadex C-50 column equilibrated in 0.1 M Na_2HPO_4 (pH 5.9) as described (8). The sample was dialyzed, lyophilized, and reconstituted in 50 mM sodium phosphate, pH 6.7/0.15 M NaCl and applied to a Sephadex G-75 column (1.5 × 95 cm) equilibrated in the reconstitution buffer. The column was monitored by measuring $A_{280\ nm}$. Fractions (2 ml) were combined into five separate pools and lyophilized. (*B*) Ability of the individual pools after Sephadex G-75 chromatography to stimulate [^{3}H]dThd incorporation was measured in the Swiss albino 3T3 cell microtiter assay. Each fraction was assayed at a concentration of 100 ng/ml; the level of DNA synthesis was reported as the percent of the 10% FCS control.

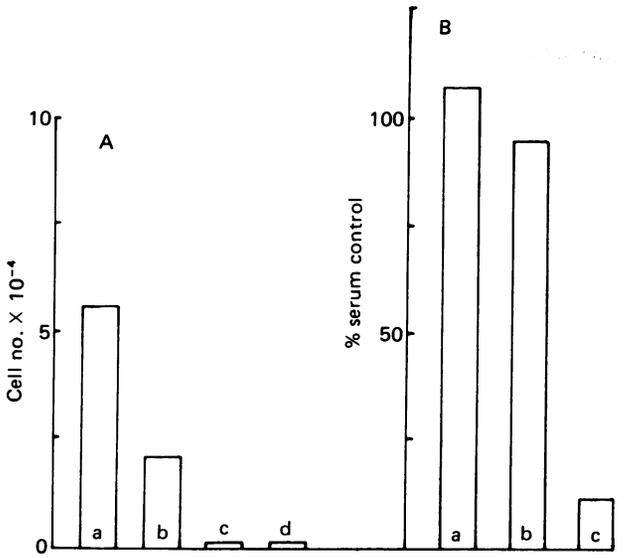

FIG. 6. Biological activity of hypothalamic extract after incubation with acid and 2-mercaptoethanol. The extract (10 mg/ml) was incubated with either 0.1 M acetic acid or 0.1% 2-mercaptoethanol for 30 min at 37°C. The samples were dialyzed against 50 mM Tris·HCl, pH 7.6/0.5 mM EDTA and assayed in the endothelial cell and 3T3 cell assays. (*A*) Effect of hypothalamic extract (200 mg/ml) on human endothelial cell growth (7 days) after supplementation with 20% FCS and hypothalamic extract control (*a*), acetic acid-treated extract (*b*), 2-mercaptoethanol-treated extract (*c*), and 10 μg of purified bovine pituitary FGF per ml (*d*). (*B*) BALB/c 3T3 microtiter cultures in 2% depleted FCS were supplemented with 100 μg of hypothalamic extract control (*a*), 2-mercaptoethanol-treated extract (*b*), and acetic acid-treated extract (*c*) per ml. The level of [^{3}H]dThd incorporation is reported as percent of the 10% FCS control.

contrast, both the endothelial cell and 3T3 cell biological activities present in the hypothalamic extract appeared to be susceptible to treatment with 0.1 M acetic acid (Fig. 6).

Separation of Endothelial Cell Growth-Promoting Activity from 3T3 Cell Mitogenic Activity in Extracts of Hypothalamus. Attempts were made to separate the endothelial cell growth-promoting activity from the 3T3 cell mitogenic activity present in the hypothalamic extract preparations. Separation was achieved by Sephadex G-100 chromatography of the extract preparation. We observed that the extract eluted from the Sephadex G-100 exclusion column as three distinct protein peaks which were subdivided into a total of seven individual fractions (Fig. 7*A*). The biological activity of each fraction was determined in the human endothelial cell assay and the 3T3 microtiter assay (Fig. 7*B*). The results revealed that the human endothelial cell growth-promoting activity was associated with a high molecular weight fraction (fraction 3), whereas a majority of the 3T3 cell mitogenic activity was associated with a low molecular weight fraction (fraction 7). The apparent molecular weights of the human endothelial cell and 3T3 cell mitogens were approximately 75,000 and 15,000, respectively.

DISCUSSION

FGF is a well-characterized biological mitogen present in bovine pituitary (8) and brain (9) capable of eliciting a mitogenic response in a wide variety of mesoderm-derived cells *in vitro* (5). It has been reported that the addition of purified FGF and human thrombin to cultures of human umbilical endothelial cells can prevent the premature senescence of the endothelial cell cultures and stimulate a high index of mitosis (5). Repeated attempts to confirm these observations with purified bovine pituitary FGF were not successful. We have consistently observed that the *in vitro* addition of purified bovine pituitary FGF (1 μg/ml) to human umbilical vein endothelial cells in the presence and absence of human thrombin (1 μg/ml) does not elicit a mitogenic response or prevent the precocious senescence of the endothelial cell culture. Similar results have also been reported for primary bovine aortic arch endothelial cells (12).

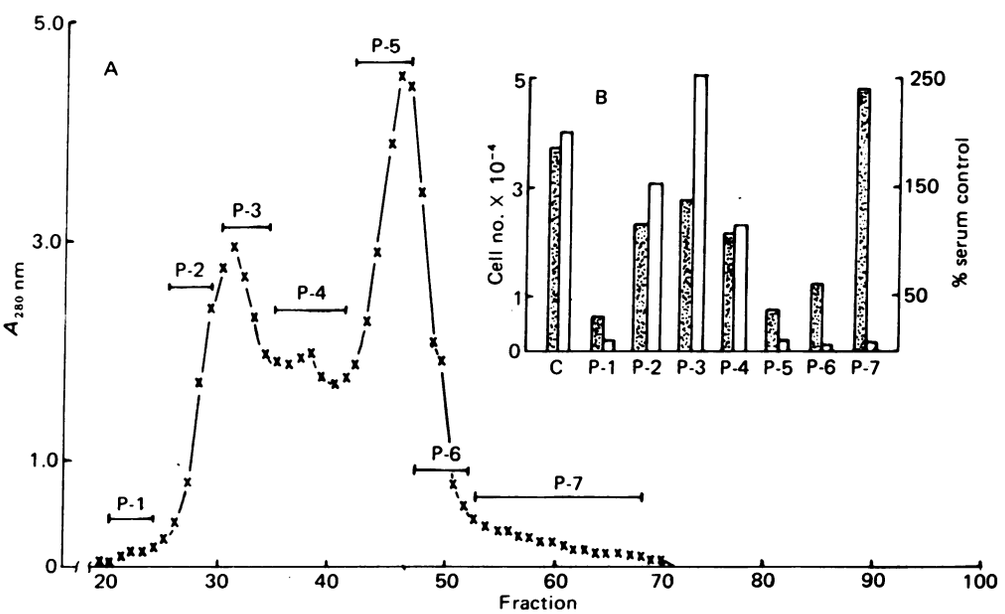

FIG. 7. Separation of human endothelial cell growth factor activity from FGF activity by Sephadex G-100 chromatography of hypothalamic extract. (*A*) Hypothalamic extract (1.3×10^3 mg of protein) was applied to a Sephadex G-100 column equilibrated in 50 mM Tris·HCl, pH 7.5/0.5 mM EDTA. The column was monitored by assaying absorbance of the fractions (19 ml) at 280 nm. The fractions were combined into seven individual pools and lyophilized. (*B*) The human endothelial cell assay (open bar graph) and the Swiss albino 3T3 cell microtiter assay (stippled bar graph) were performed on the pooled fractions after Sephadex G-100 chromatography (1:50 dilution). The hypothalamic extract (300 μg/ml) served as the control (C). Results of the [^{3}H]dThd assay are reported as percent of the 10% FCS control, whereas the results of the human endothelial cell assay are reported as cell number after 6 days in culture.

Significant levels of mitogenic activity were found in extracts prepared from bovine hypothalamus. Preparations of hypothalamic extracts were capable of stimulating DNA synthesis in resting Swiss albino 3T3 cells and the growth of human umbilical vein endothelial cells at very low seed densities *in vitro*. Similar biological activities were also found in extracts prepared from bovine brain and pituitary glands. The 3T3 cell activity of the hypothalamic mitogen was characterized as a protease-sensitive, mercaptoethanol-stable, acid-sensitive, heat-labile, cationic protein with an M_r of approximately 15,000. These data are consistent with the physical and biological properties of both pituitary and brain FGF. The supplementation of human endothelial cells with purified hypothalamic FGF in the presence and absence of human thrombin also failed to elicit a mitogenic response *in vitro*, suggesting that the mitogenic hypothalamic component responsible for human endothelial cell growth was not FGF. We were able to determine that the endothelial cell activity of the hypothalamic extract was completely destroyed as a result of reduction with mercaptoethanol. These results suggest that the endothelial cell growth factor component of the hypothalamic extract contains intramolecular disulfide bonds which are required for the maintenance of biological function. Because the major 3T3 cell growth factor component was not susceptible to treatment with reducing reagents, the data suggest that the endothelial cell growth factor and the 3T3 cell mitogen are chemically distinct. This observation is consistent with the report that the primary structure of brain FGF does not contain cysteine (9).

Additional evidence supporting the uniqueness of the human endothelial cell mitogen was obtained from the discriminatory behavior of the human endothelial cell biological activity from the FGF activity by gel exclusion chromatography. It was possible to separate the endothelial cell biological activity present in hypothalamic extracts from the 3T3 cell mitogen by Sephadex G-100 chromatography. The 3T3 cell biological activity eluted with a molecular weight of approximately 75,000. Although the biologically active 3T3 cell fraction was devoid of human endothelial cell activity, the endothelial cell fraction was biologically active in the 3T3 cell assay. The results suggest that, although the mitogen responsible for the growth of human endothelial cells in culture is physically, chemically, and biologically distinct from FGF, there appears to be some biological 3T3 cell activity associated with the biologically active endothelial cell fraction. Thus, the endothelial cell growth factor activity can be interpreted as representing either a macromolecular binding protein–growth factor complex related to FGF or a new and unique endothelial cell growth factor.

We thank Drs. Eric Jaffe and Alice Johnson for their assistance and performance of the Factor VIII and kininase II assay and Dr. John Fenton for his generous gift of purified human α-thrombin. We also thank Drs. Gordon Sato and M. R. Iyengar for stimulating discussions and Rosely Greenlee for expert secretarial assistance.

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