Bovine Brain and Pituitary Fibroblast Growth Factors: Comparison of Their Abilities to Support the Proliferation of Human and Bovine Vascular Endothelial Cells

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ABSTRACT   The mitogenic effects of brain and pituitary fibroblast growth factors (FGF) on vascular endothelial cells derived from either human umbilical vein or bovine aortic arch have been compared. Both brain and pituitary FGF are mitogenic for low density human umbilical endothelial (HUE) cell cultures maintained on either fibronectin- or laminin-coated dishes or on biomatrices produced by cultured cells such as bovine corneal endothelial cells or the teratocarcinoma cell line PF-HR-9. Pituitary FGF triggered the proliferation of HUE cells at concentrations as low as 0.25 ng/ml, with a half-maximal response at 0.55 ng/ml and optimal effect at 2.5 to 5 ng/ml. It was 50,000-fold more potent than commercial preparations of endothelial cell growth factor and 40 times more potent than commercial preparations of pituitary FGF. Similar results were observed when the effect of pituitary FGF was tested on low density cultures of adult bovine aortic endothelial cells. When the activity of brain and pituitary FGF on low density HUE cell cultures was compared, both mitogens were active. To confirm the presence in brain extract of both acidic and neutral, as well as of basic mitogen, for HUE cells, brain tissues were extracted at acidic (4.5), neutral (7.2), and basic (8.5) pH. The three types of extracts were equally potent in supporting the proliferation of either HUE or adult bovine aortic endothelial cells. When the various extracts were absorbed at pH 6.0 on a carboxymethyl Sephadex C-50 column, the neutral and basic extracts had an activity after adsorption similar to that of unadsorbed extracts. In contrast, extracts prepared at pH 4.5 lost 90–95% of their activity which was recovered in the adsorbed fraction containing FGF.

In 1974 we described the presence in extracts of bovine brain and pituitary of a potent mitogen for mouse 3T3 fibroblasts (1). This activity was named fibroblast growth factor (FGF).[1] Both pituitary and brain FGF were later characterized as basic molecules with a pI of 9.6 (2, 3). Both of these factors had common biological activity for a variety of cultured cells derived from the mesoderm (reviewed in references 4–6). In 1975, we reported that brain and pituitary tissues contain, in addition to those basic mitogens, neutral or acidic growth factors which were given the generic name of myoblast growth factor(s), since it was with that cell type that these activities were first detected (7). While the FGF mitogenic activity is extracted preferentially at pH 4.5 (1–3, 7), the neutral or acid activity responsible for increased myoblast proliferation was best extracted at either neutral or basic pH (7).

Both pituitary and brain FGF stimulate the proliferation of vascular endothelial cells derived from either bovine aorta (ABAE cells) or human umbilical vein (HUE cells) (8–10). Their effects on these two cell types differ somewhat. ABAE cells maintained on plastic respond optimally to pituitary or brain FGF when exposed to serum-supplemented medium (8, 9). In contrast, HUE cells maintained on plastic and exposed to serum-supplemented medium require the presence of thrombin to respond to low concentration of FGF (10).

The effect of FGF on the proliferation of human vascular endothelial cells, as well as the nature of the factor involved, have been questioned (11–13). Maciag et al. have reported that pituitary FGF at high concentration, and regardless of whether or not thrombin is present, has no effect on the proliferation of HUE cells (11, 12). Thomas et al. (13) have reported that brain FGF extracted at acidic pH contains an acidic contaminant that contains all of the activity, whereas the basic component has none (13). In contradiction to those earlier studies, the same group (Lemmon et al., 14) has reported recently that highly purified preparation of brain FGF (FGF-A) does indeed consist of basic mitogen, whereas partially purified preparations (FGF-B) contain, in addition to the basic component, an acidic factor. It was reported that while highly purified brain FGF is not very active in stimulating the proliferation of HUE cells, the partially purified preparation is more active, and pituitary FGF, which contains only basic element(s), is inactive (14).

In view of the discrepancy between our early report on the activity of brain and pituitary FGF in supporting HUE cell proliferation (10) and recent reports (11–14), we have re-evaluated the ability of brain and pituitary FGF to stimulate the proliferation of HUE cells. We have compared their activity with that of endothelial cell growth factor (ECGF), a mitogen reported to support the proliferation of HUE cells (11, 12, 14) and commercial preparations of pituitary FGF (Collaborative Research, Inc., Lexington, MA; CR-FGF), which has been reported by others not to support the proliferation of HUE cells (11, 12).

MATERIALS AND METHODS

Materials: Laminin was purified from EHS sarcoma, as described by Timpl et al. (15). Fibronectin was purified from bovine plasma as described by Engvall et al. (16). When analyzed by slab gel polyacrylamide gel electrophoresis under reduced conditions, the purified bovine plasma fibronectin ran as a doublet with a molecular weight in the range of 220,000, while laminin gave two bands with molecular weights of ~200 and 400,000, respectively. Carboxymethyl Sephadex C50 was from Pharmacia, Inc. (Piscataway, NJ). Albumin was obtained from Schwarz-Mann (Orangeburg, NJ). Gelatin and dextran T-40 were obtained from Sigma Chemical Co. (St. Louis, MO). Collagenase (CLS grade, 141 U/mg) was from Worthington Biochemical Corp. (Freehold, NJ). Dulbecco's modified Eagle's medium (DME) and medium 199 (Hanks' salt formulation) were obtained from GIBCO Laboratories (Grand Island, NY). Calf serum and fetal calf serum were obtained from Hy Clone (Sterile Systems, Inc., Logan, UT). Tissue culture dishes were from Falcon Plastics, Gentamicin from Schering Co. (Kenilworth, NJ), and Fungizone from E. R. Squibb & Sons (Princeton, NJ).

Mitogen Preparation: CR-ECGF (lot 82-356) was purchased from Collaborative Research, Inc. Pituitary FGF was purified as previously described (2, 3, 17), with the following modifications. Ion exchange chromatography was done using carboxymethyl cellulose, Whatman CM52 (Whatman Laboratory Products, Inc., Clifton, NJ), as described for brain FGF (3, 17). After lyophilization of the fraction eluting at 0.35 M ammonium formate, the proteins were further purified by isoelectric focusing as described (17). When analyzed by polyacrylamide gel electrophoresis, pituitary FGF gave two closely apposed bands (Fig. 1). Brain FGF was prepared by isoelectric focusing, as already described (12). FGF-2 (Fig. 6B in reference 18) was used in the present study. When the homogeneity of that fraction was analyzed by slab gel electrophoresis, a single band was observed (Fig. 7 in reference 18). CR-Pituitary FGF (lots 82-300 and 83-225) was obtained from Collaborative Research, Inc., and its activity was compared with that of a batch of CR pituitary FGF (lot 748-3) provided by Dr. T. Maciag (Dept. of Pathology, Harvard Medical School Boston, MA) in 1978. This lyophilized sample has been stored in the dark at −20°C with dessicant for the past 5 yr.

Brain Crude Extract Preparations: Crude extracts of bovine brain performed at pH 4.5, 7.2, or 8.5 were prepared as previously described (7). 1 kg of brain was homogenized in 2 liters of a solution of 0.15 M ammonium sulfate. The pH of the extract was then adjusted to pH 4.5 with 6 N HCl or to pH 7.2 or 8.5 with 6 N NaOH. The homogenates were stirred at 4°C for 90
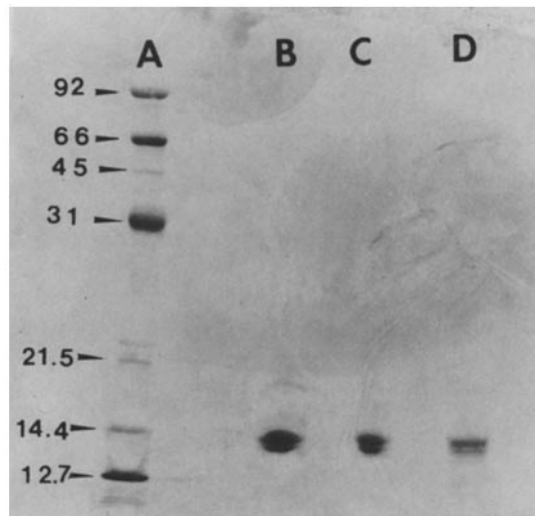

FIGURE 1   SDS PAGE of pituitary FGF purified by isoelectric focusing. Samples containing 10 (B), 5 (C), and 2.5 μg pituitary FGF (D), respectively were added to a sample buffer composed of 15% glycerol, 0.1 M dithiothreitol, 2% SDS, 75 mM Tris-HCl (pH 6.8), 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, 1 mM N-ethylmaleimide, and 1 mM iodoacetic acid. Samples were boiled for 3 min and then applied to an exponential gradient (10-18%) polyacrylamide slab gel with a 4% stacking gel (17, 18). Electrophoresis was performed for 4 h at 20 mA. After electrophoresis, the slab gels were fixed and stained with silver nitrate as described. The migration of the samples was compared with that of protein standards (lane A), which included phosphorylase (M_r = 92,500), BSA (M_r = 66,000), carbonic anhydrase (M_r = 31,000), soybean trypsin inhibitor (M_r = 21,500), lysozyme (M_r = 14,400), and cytochrome c (M_r = 12,700). (A) A similar migration pattern was observed regardless of whether or not the samples were run under reducing conditions.

min and centrifuged at 20,000 g for 45 min. The supernatants were then checked for their pH and dialyzed at 4°C overnight against distilled water. To eliminate insoluble material, the dialyzates were centrifuged and then lyophilized.

Crude extracts of brains prepared at various pH were redissolved in 0.1 M Na phosphate (pH 6.0) and then applied onto a small column (3 ml total volume) of carboxymethyl Sephadex C-50 equilibrate with the same buffer. The columns were washed with 20 ml of 0.1 M Na phosphate (pH 6.0), and the adsorbed proteins were eluted from the columns with 1 M NaCl in 0.1 M Na phosphate (pH 6.0). The optical density of the collected fractions was determined in a Beckman spectrophotometer at 280 nm (Beckman Instruments, Inc., Cedar Grove, NJ).

Cell Culture Conditions: Cultures of bovine corneal endothelial (BCE) cells were established from steer eyes as already described (19, 20). Stock cultures were maintained on tissue culture dishes in DME supplemented with 10% fetal calf serum, 5% calf serum, 50 μg/ml Gentamicin, and 0.25 μg/ml Fungizone. Prior to being used, all media were passed through a Millipore filter (0.2 μm; Millipore Corp., Bedford, MA). Brain FGF (50 ng/ml) was added every other day until the cells were nearly confluent. Cultures of ABAE cells derived from the aortic arch were maintained on tissue culture dishes in DME supplemented with 10% calf serum, 50 μg/ml Gentamicin, and 0.25 μg/ml Fungizone, as previously described (8, 9). During their growth phase, cultures were exposed or not to pituitary FGF (20 ng/ml added every other day) or ECGF (250 μg/ml, added every other day). This resulted in three cell populations, one of which had not been submitted to selective pressure by the use of growth factors, while the other two could have become either FGF- or ECGF-dependent. Stock plates of the unselected population were passaged weekly at a split ratio of 1:4, while the FGF- or ECGF-selected populations were passaged weekly at a split ratio of 1:64. The mouse teratocarcinoma cell line PF-HR-9 was generously provided by Dr. R. Kramer (University of California, San Francisco, CA). PF-HR-9 cells were maintained on tissue culture dishes in DME supplemented with 10% fetal calf serum and passaged upon reaching confluence.

HUE cells were obtained from human umbilical vein by collagenase dissociation, as previously described (10, 21, 22). The cells were seeded onto 10- or

6-cm tissue culture dishes coated with gelatin and fibronectin. Cells were grown in the presence of medium 199 (Hanks salt formulation) buffered with 25 mM HEPES and supplemented with 10^{-8} M selenium (acid form), 10% fetal calf serum, 50 µg/ml Gentamicin, and 0.25 µg/ml Fungizone. During their growth phase cultures were exposed or not to pituitary FGF (20 ng/ml added every other day) or ECGF (250 µg/ml added every other day). This resulted in three cell populations, one of which had not been submitted to selective pressure by the use of growth factors, while the two other cell populations could have become either FGF- or ECGF-dependent. Stock plates of the unselected population were passaged weekly on fibronectin-coated dishes at a split ratio of 1:2, while the FGF- or ECGF-selected populations were passaged weekly at a split ratio of 1:12.

That cultured HUE cells were truly vascular endothelial cells was confirmed by three independent criteria: (a) the presence of Factor VIII antigen, as seen by immunofluorescence; (b) the presence of Weibel Palade bodies; and (c) the morphology of the cells at confluence, which organized themselves into a monolayer composed of small, nonoverlapping cells (Fig. 2).

Coating of Plates with Gelatin, Fibronectin, or Laminin:
To coat the plates with gelatin, a 2-ml solution of 0.2% gelatin in PBS was added to 35-mm tissue culture plates. Plates were allowed to sit overnight at 4°C. The next day, the solution was removed and plates were washed with PBS. To coat the plates with fibronectin or laminin, a 1-ml solution of 100 µg/ml fibronectin or 450 µg/ml of laminin was added to 35-mm tissue culture dishes (23, 24). Plates were left at room temperature for 2 h. The fibronectin or laminin solution was then removed and plates washed with PBS.

Preparation of Extracellular Matrix-coated Dishes:
For production of extracellular matrix (ECM), BCE cells were seeded at an initial density of 4×10^4 cells per 35-mm dish and grown in the presence of DME supplemented with 10% calf serum, 5% dextran T-40, and brain FGF (20 ng/ml added every other day) (25–27). HR-9 cells were seeded at high density (2 $\times 10^5$ cells per 35-mm dish) on fibronectin-coated dishes. This reinforced the adhesion of the HR-9 ECM to the plastic substratum. HR-9 cells were grown in the presence of DME supplemented with 10% calf serum. Once cultures had been confluent for 5 (HR-9 cells) or 7 d (BCE cells), the media were removed and the confluent cultures were washed once with PBS. The cells were then exposed to 0.02 M NH$_4$OH in distilled water for 5 min, followed by washing with PBS (28, 29). This results in the denudation of the extracellular matrices produced by both types of cells. While cellular elements are absent from the ECM produced by BCE cells (30), little cellular debris remains closely associated with the HR-9 matrix (28).

Cell Seeding:
Cell monolayers from ABAE or HUE stock plates were dissociated by exposure (2–3 min, 24°C) to a solution containing 0.9% NaCl, 0.01 M sodium phosphates (pH 7.4), 0.05% trypsin, and 0.02% EDTA (STV solution, Difco Laboratories, Inc., Detroit, MI). When cells rounded up, they were suspended in DME supplemented with 10% calf serum for ABAE cells or HEPES buffered 199 medium supplemented with 10^{-8} M selenium and 20% fetal calf serum for HUE cells. An aliquot of the cell suspension was then counted in a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) and cells were distributed at an initial cell density of 2 or 4×10^4 cells per 35-mm dish.

Cell Growth Measurement and Culture Lifetime Determination:
For cell growth measurements, cells were seeded, as described above, at an initial density of 2 or 4×10^4 cells per 35-mm dish. Various mitogens or brain extracts were dissolved in DME containing 0.5% BSA, and aliquots (10 µl per dish) containing the various concentrations of factors indicated in the figure legends were added to the plates. For growth-rate determinations, triplicate plates were trypsinized every day or every other day, and the cells counted in a Coulter counter. For determination of potency of the various preparations, increasing concentrations (ranging from 0.1 ng to 100 µg/ml) of the factors were added to triplicate plates, and plates were trypsinized and counted when those exposed to the highest concentration became confluent (ordinarily within 5 to 6 d after seeding).

RESULTS

Comparison of the Ability of Various Substrates to Support the Proliferation of HUE Cells

The proliferative response of HUE cells to various mitogens such as pituitary or brain FGF and ECGF has been reported to depend on the substrate upon which cells are maintained (10, 12, 27). Although HUE cells, maintained on plastic, proliferate poorly in response to serum and pituitary or brain FGF or ECGF (10, 12), they show a definite improvement in their ability to respond to these various mitogens when maintained on fibronectin- or BCE-ECM-coated dishes (12, 27). We have therefore first analyzed the influence of various substrates on the proliferative response of HUE cells to serum factors and pituitary FGF. As shown in Fig. 2A, low density HUE cell cultures (4×10^4 cells per 35-mm dish) in their first
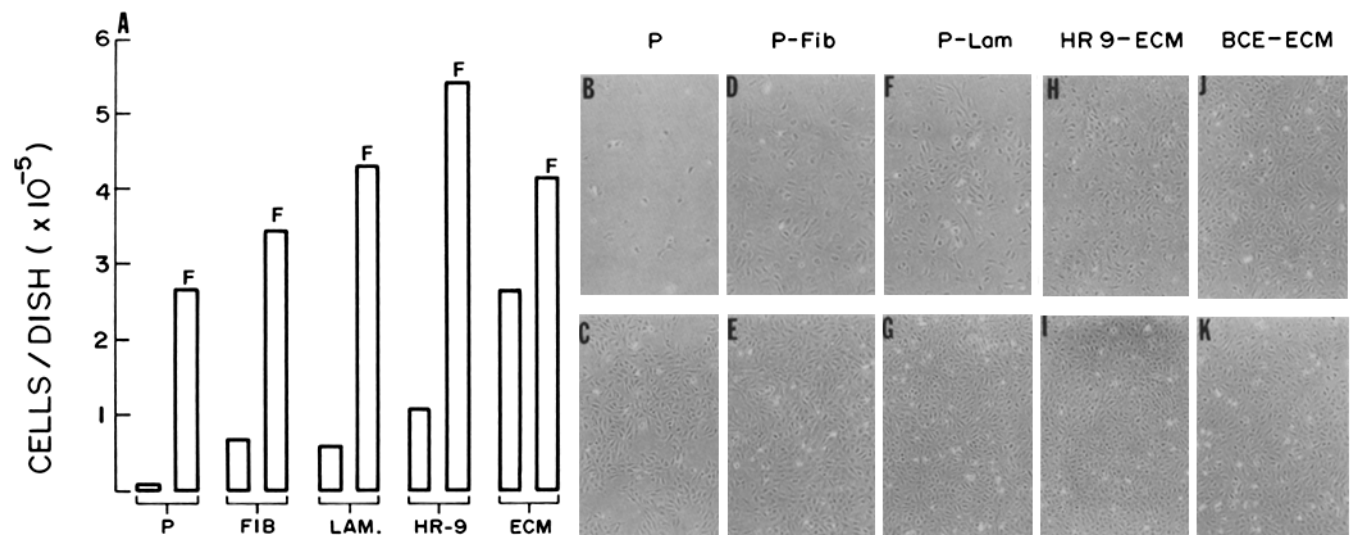

FIGURE 2 Comparison of the effect of various substrates on the proliferative response of low density HUE cell cultures to pituitary FGF. (A) 4×10^4 HUE cells were seeded on either 35-mm plastic tissue culture dishes (P) and coated or not with fibronectin (Fib), laminin (Lam), HR-9-ECM (HR-9), and BCE-ECM (ECM). Cells were exposed to HEPES (25 mM) buffered medium 199 supplemented with 10^{-8} M selenium, 20% fetal calf serum, and without (left bar) or with pituitary FGF (right bar F, 10 ng/ml) added every other day. After 6 d, cultures were trypsinized and cells were counted in a Coulter counter. Standard deviation was <10%. (B–K) HUE cell cultures were seeded and maintained as described above. Cells were maintained on plastic- (P, B, and C), fibronectin- (P-Fib, D and E), laminin- (P-Lam, F and G), HR-9-ECM- (H and I), or BCE-ECM- (J and K) coated dishes. Cultures were exposed to HEPES buffered medium 199 supplemented with 10^{-8} M selenium, 20% fetal calf serum, and without (B, D, F, H, J) or with pituitary FGF (C, E, G, I, and K, 10 ng/ml added every other day). After 6 d in culture, photomicrographs were taken under phase-contrast microscopy. × 100.

passage after primary and maintained on plastic in the presence of HEPES-buffered medium 199 supplemented with 20% fetal calf serum and antibiotics died over a period of 6 d, as indicated by the final cell density of the cultures, which was fourfold lower (1.1 × 10⁴ cells per dish) than the seeding density. In contrast, when pituitary FGF was added to the culture, cells proliferated actively, so that after a period of 6 d their final cell density was 2.7 × 10⁵ cells per dish. Coating dishes with either fibronectin or laminin prevented cell death but had little effect in supporting cell proliferation. Addition of pituitary FGF to the cultures triggered active cell proliferation, so that after 6 d in culture the final cell densities were 3.4 and 4.3 × 10⁵ cells, respectively, for cultures maintained on fibronectin- versus laminin-coated dishes. Cultures maintained on fibronectin-coated dishes were more disorganized and were composed of cells with a larger surface area (Fig. 2, D and E) than cells maintained on laminin-coated dishes (Fig. 2, E and G). When the abilities of HR-9-ECM- and of BCE-ECM-coated dishes to support HUE cell proliferation were compared, cells not exposed to FGF proliferated minimally when maintained on HR-9-ECM but, and as previously reported (27), proliferated actively on BCE-ECM. In both cases, addition of FGF to the medium induced rapid cell proliferation, and the final cell density reached by the cultures was higher on HR-9-ECM than on BCE-ECM. The morphological appearance of cells maintained on HR-9 matrix (Fig. 2, H and I) was that of small and closely apposed cells forming a uniform monolayer. In contrast, cells grown on BCE-ECM-coated dishes were larger in size (Fig. 2, J and K).

Comparison of the Effect of Pituitary FGF, CR-FGF, and ECGF on the Proliferation of Low Density Cultures of HUE and ABAE Cells

HUE cells were seeded at low density (4 × 10⁴ cells/35-mm dish) on fibronectin-coated dishes and exposed to medium supplemented with 20% fetal calf serum and increasing concentration of pituitary FGF (ranging from 0.1 ng/ml to 25 ng/ml), CR-FGF (lot 83-225, ranging from 1 ng to 250 ng/ml), or CR-ECGF (lot 82-356, ranging from 1 μg to 250 μg/ml). After 5 d in culture, the final cell density of the cultures was determined. As shown in Fig. 3A, pituitary FGF was active at concentrations as low as 0.25 ng/ml, at 0.55 ng/ml half-maximal response was observed, and at 2.5 ng/ml it was saturating. CR-FGF was also active, although to a lesser extent than our preparation. Stimulation of cell proliferation could be observed readily at 5 ng/ml with a half-maximal effect at 25 ng/ml and saturation at 250 ng/ml. CR-ECGF was also active. Minimal effects were seen at 5 μg/ml, and saturation was observed at 100 to 250 μg/ml. That concentration is 40,000-fold higher than that required to observe a maximal effect of pituitary FGF.

To insure that the above results were not unique to the HUE cells, we repeated a similar test using ABAE cells seeded at low density (2 × 10⁴ cells per 35-mm dish) on plastic dishes and exposed to medium supplemented with 10% calf serum. As shown in Fig. 3B, the results were identical to those observed with HUE cells; the half-maximal response was at 0.4 and 7.5 ng/ml, respectively, for pituitary FGF and CR-FGF.

Since the activity of CR-FGF could have been limited to the particular batch we used, we decided to compare the activity of pituitary FGF to older batches of CR-FGF (lots
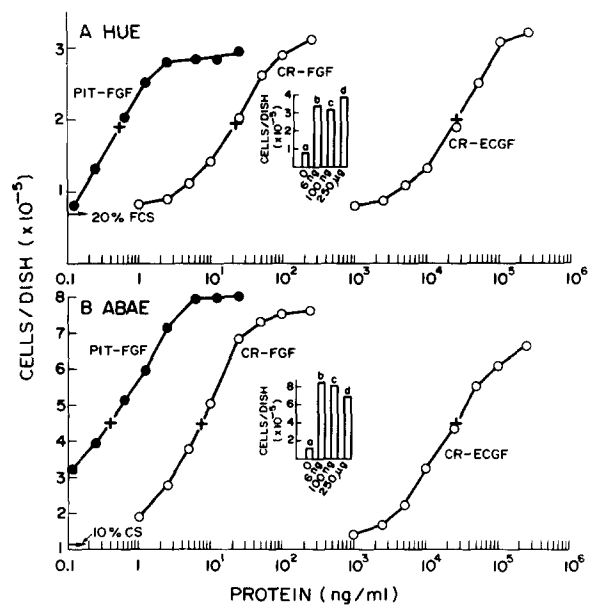
FIGURE 3   Effect of increasing concentrations of pituitary FGF, CR-FGF, and CR-ECGF on the proliferation of low density HUE and ABAE cell cultures. (A) Low density HUE cells (4 × 10⁴ cells per 35-mm dish) in their second passage were seeded on fibronectin-coated dishes and exposed to HEPES (25 mM) buffered medium 199 supplemented with 10⁻⁸ M selenium, 20% fetal calf serum and increasing concentrations of pituitary FGF (PIT-FGF, ●), CR-FGF (lot 83-225, ○), or CR-ECGF (lot 82-356, ○). PIT-FGF or CR-FGF were added every other day in 10-μl aliquots of medium. Due to the high concentration tested, CR-ECGF was added in 25-μl aliquots. After 5 d in culture, triplicate dishes were trypsinized and counted in a Coulter counter. The crosses indicate the concentrations at which a half-maximal response was observed for each mitogen. The final cell density of cultures exposed to 20% fetal calf serum was 7.2 × 10⁴ cells (arrow, 20% FCS). The inset shows the final density of HUE cell cultures originally seeded at 4 × 10⁴ cells per 35-mm dish, as described above, and exposed to medium 199 supplemented with 20% fetal calf serum alone (a) or supplemented with 10 ng/ml pituitary FGF (b), 100 ng CR-FGF (lot 748-3) (c), or 250 μg CR-ECGF (lot 82-300) (d). (B) Low density ABAE cells (2 × 10⁴ cells per 35-mm dish) were seeded on plastic dishes and exposed to DME supplemented with 10% calf serum and increasing concentrations of pituitary FGF (PIT-FGF, ●), CR-FGF (lot 83-225, ○), or CR-ECGF (lot 82-356, ○) added every other day, as described above. After 6 d in culture, triplicate dishes were trypsinized and cells were counted in a Coulter counter. The crosses indicate the concentrations at which a half-maximal response was observed for each mitogen. The final cell density of cultures exposed to 10% calf serum alone was 1.1 × 10⁵ cells (arrow, 10% CS). The inset shows the final cell density of ABAE cells seeded at 2 × 10⁴ cells per 35-mm dish, as described above, and exposed to DME supplemented with 10% calf serum alone (a) or with 10 ng/ml pituitary FGF (b), 100 ng/ml CR-FGF (lot 748-3) (c), or 250 μg/ml CR-ECGF (lot 82-300) (d).

82-356 and 734-3), one of which we received from Dr. Maciag in 1978 (lot 734-3). This particular batch of FGF was just as active in stimulating HUE cell proliferation as the newly acquired batch of CR-FGF (Fig. 3A, inset), and it had a similar effect when tested for its ability to support the proliferation of low density ABAE cell cultures maintained on plastic and exposed to 10% calf serum (Fig. 3B, inset). CR-FGF lot 82-356 was 80% as active as lot 83-225 when tested on both cell types (unpublished results).

Comparison of the Ability of FGF and ECGF to Stimulate the Proliferation of Vascular Endothelial Cell Cultures Previously Maintained in Their Presence or Absence

Endothelial cell cultures used in the above studies were exposed from the beginning of their in vitro life span to either brain or pituitary FGF. The possibility therefore exists that their ability to respond to FGF reflects the selection of a subset of endothelial cells particularly responsive to that factor.

To analyze this possibility, primary cultures of HUE and ABAE cells were grown either in the total absence of growth factors or in the presence of either pituitary FGF or ECGF. The response of the three cell populations in their second passage after primary to either pituitary FGF or ECGF was then analyzed. As shown in Fig. 4, low density cultures of HUE or ABAE cells that have never been exposed to growth factors in the initial stage of their in vitro life span did respond to either FGF or ECGF (Fig. 4, A and D). The proliferative response as a function of growth factor concentration was similar to that observed with cell populations previously grown in the presence of either ECGF (Fig. 4, B and E) or FGF (Fig. 4, C and F). Since cells grown in the absence of growth factors responded equally well to FGF or ECGF, it is unlikely that exposing endothelial cells to either mitogen would select for a subset population that would be responsive to either FGF or ECGF during primary or early-passage culture.

Comparison of the Ability of Brain and Pituitary FGF to Stimulate HUE Cell Proliferation

It has been proposed that the mitogenic activity present in brain FGF that stimulates HUE cell proliferation is caused by an acidic contaminant (13). We have recently reported the purification by isoelectric focusing of brain FGF (18). All of the activity focused in the basic range (pI 9.4 to 9.6), excluding the possibility of an acidic contaminant in those preparations that have been shown to be active in promoting the growth of ABAE cells (18). To see if brain FGF is also active on HUE cells, we compared its ability to stimulate HUE cell proliferation with that of pituitary FGF. As shown in Fig. 5A, a mitogenic effect of brain FGF was readily detectable at 1 ng/ml, with half-maximal response at 5 ng/ml and saturation at 20 ng/ml. When its activity was compared with that of pituitary FGF, it was sixfold less active. The effects of brain and pituitary FGF on the growth rate of low density, first-passaged HUE cells (2 × 10⁴ cells) maintained on plastic and exposed to medium supplemented with 20% fetal calf serum alone was also analyzed, and is shown in Fig. 5B. Low density HUE cell cultures (first passage) maintained on plastic and exposed to serum-supplemented medium alone had a progressive decline (90%, over a period of 12 d) in their initial cell density. In contrast, cultures exposed to either brain or pituitary FGF divided actively (averaging doubling-time of 48 h) and by day 10 had a 10-fold increase in cell density. Due to cell death in cultures maintained in absence of FGF, the density of cultures exposed to pituitary or brain FGF were 100-fold higher after 12 d in culture than in their absence (Fig. 5B).

Early-passage (P_2) HUE cells seeded on fibronectin-, BCE-ECM-, or HR-9-ECM-coated dishes also responded to the
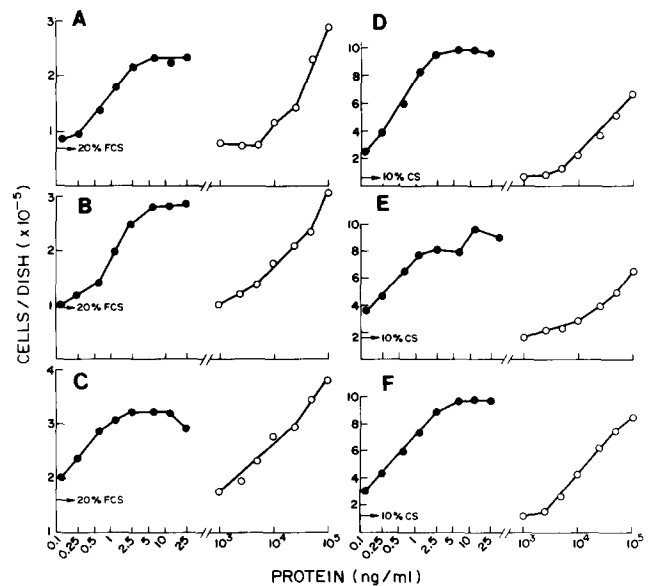

FIGURE 4   Comparison of the effects of pituitary FGF and ECGF on the proliferative response of HUE and ABAE cells previously cultured in the presence of serum alone or serum supplemented with either FGF or ECGF. First-passage HUE cells previously grown in presence of medium supplemented with serum (A) or with serum and either ECGF (B) or FGF (C) were seeded at low density (4 × 10⁴ cells) on 35-mm fibronectin-coated dishes. (A–C) Cells were exposed to HEPES (25 mM) buffered medium 199 supplemented with 10⁻⁸ M selenium, 20% fetal calf serum, and increasing concentrations of pituitary FGF (●; ranging from 0.1 to 25 ng/ml) or ECGF (○; lot 82-356, ranging from 1 to 100 μg/ml). Mitogens were added every other day. After 5 d in culture triplicate dishes were trypsinized and counted in a Coulter counter. The final cell density of cultures exposed to 20% fetal calf serum was 6.4 × 10⁴ cells for cultures not previously exposed to mitogens (A) and 9.4 (B) or 1.6 × 10⁵ cells (C), respectively, for cultures previously exposed to either ECGF or FGF. First-passage ABAE cells previously grown in presence of medium supplemented with serum (D) or with serum and either ECGF (E) or FGF (F) were seeded at low density (2 × 10⁴ cells) on 35-mm plastic tissue culture dishes. (D–F) Cells were exposed to DME supplemented with 10% calf serum and increasing concentrations of pituitary FGF (●; ranging from 0.1 to 25 ng/ml) or ECGF (○; lot 82-356, ranging from 1 to 200 μg/ml). Mitogens were added every other day, and after 5 d in culture triplicate dishes were trypsinized and counted in a Coulter counter. The final cell density of cultures exposed to 10% calf serum was 6 × 10⁴ cells for cultures not previously exposed to mitogens (D) and 1.6 (E) or 1.2 × 10⁵ cells (F), respectively, for cultures previously exposed to either ECGF or FGF.

addition of the mitogens with an increased growth-rate (24 h average doubling time during the logarithmic growth phase). Control cultures maintained on fibronectin- or BCE-ECM-coated dishes and exposed to 20% fetal calf serum showed a slow increase in cell density (72 h average doubling time) (Fig. 5B). Cultures maintained on HR-9-ECM-coated dishes did not proliferate (Fig. 5B).

Comparison of the Ability of Crude Brain Extracts Prepared at Acidic, Neutral, and Basic pH to Stimulate the Proliferation of Low Density HUE Cell Cultures

We have previously shown that brain crude extract prepared at acidic pH (pH 4.5) contains mostly basic mitogen(s) such

as FGF, while those prepared at neutral (pH 7.2) or basic pH (pH 8.5) contains mostly neutral or acidic mitogens (7). We have therefore compared the activity of those brain extracts on the proliferation of low density HUE cells (4 × 10⁴ cells per 35-mm dish) seeded on fibronectin-coated dishes. As shown in Fig. 6, when those extracts were tested at concentrations ranging from 1 to 250 μg/ml, they were equally active in stimulating the proliferation of HUE cells. This tends to demonstrate that the basic mitogens that predominate in acidic extract are as potent in stimulating HUE cell proliferation as neutral or acidic mitogens predominating in neutral or basic extract. To determine further whether basic mitogens are mostly present in acidic extract whereas neutral or acidic ones are present in neutral and basic extract, all three types of extract were dissolved in 0.1 M Na phosphate, pH 6.0 and then applied on a carboxymethyl Sephadex C50 column. The unadsorbed proteins and the adsorbed fraction eluted with 1 M NaCl in 0.1 M Na phosphate (pH 6.0) were then tested for their ability to stimulate the proliferation of low density ABAE cells (2 × 10⁴ cells/35-mm dish) maintained on plastic. The elution profile of each extract is shown in the insets of Fig. 6. The unadsorbed fraction of the pH 4.5 brain crude extract was 10-fold less potent than the input, while the adsorbed fraction, presumably containing FGF, was 20-fold more potent than the input (saturation at 2.5 μg protein/ml versus 50 μg protein/ml for the input). In contrast with both crude extracts prepared at either pH 7.2 or 8.5, chromatography onto a carboxymethyl Sephadex column did not decrease

their activity. On the contrary, the unadsorbed fractions were as active (pH 7.2) or more active (pH 8.5) than the input. The adsorbed fractions eluting with 1 M NaCl contained so little protein that it could not be tested for activity. It is therefore likely that most of the activity (90–95%) present in the acidic brain extract is due to basic mitogens which would be adsorbed on the carboxymethyl Sephadex column, whereas that present in neutral or basic extract is composed of neutral or acidic protein that would not be adsorbed onto the column.

DISCUSSION

The present results emphasize the role of bovine pituitary and brain FGF in the in vitro control of proliferation of vascular endothelial cells. As reported in 1977, both pituitary and brain FGF are mitogenic for human and bovine vascular endothelial cells (4, 8–10), and in both cases the substrate upon which the cells are maintained plays an important role in the cells' response to the mitogens. Bovine vascular endothelial cells maintained on plastic and exposed to serum-supplemented media respond to FGF with an increased proliferative rate (4, 8, 9). Maintenance of the same cells on the ECM produced by cultured BCE cells will allow the cells to proliferate in the absence of FGF at an optimal rate (average doubling time 18 h) in response to plasma factors (28). These factors are likely to be transferrin and high density lipoproteins, which together support the growth of ABAE cells exposed to serum-free medium (31).
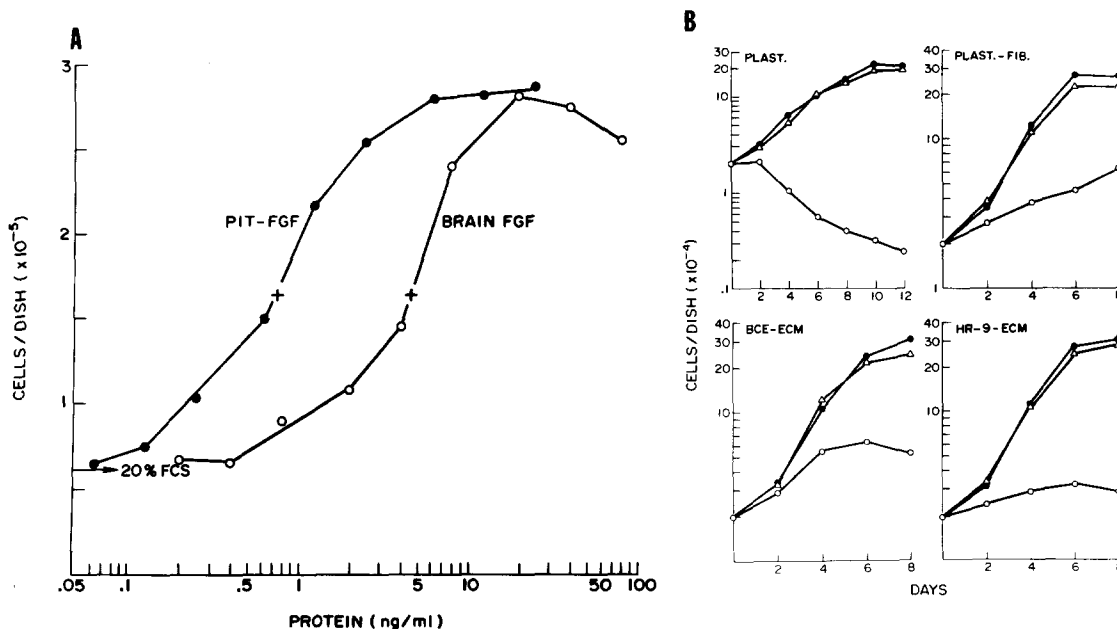

FIGURE 5 Proliferation as a function of concentration of brain and pituitary FGF and as a function of time of early passage HUE cells maintained on plastic, fibronectin-, BCE-ECM-, or HR-9-ECM-coated dishes and exposed or not to pituitary or brain FGF. (A) Low density HUE cells (4 × 10⁴ cells per 35-mm dish) in their first passage after primary and previously grown in the presence of pituitary FGF were seeded on fibronectin-coated dishes and exposed to 25 mM HEPES buffered medium 199 supplemented with 10⁻⁸ M selenium, 20% fetal calf serum, and increasing concentrations of pituitary FGF (PIT-FGF, ●) or brain FGF (○). Both mitogens were added every other day. After 5 d in culture, triplicate dishes were trypsinized and counted in a Coulter counter. The crosses indicate the concentration at which a half-maximal response was observed for each mitogen. The final cell density of HUE cell cultures exposed only to 20% fetal calf serum was 6 × 10⁴ cells per plate (arrows, 20% FCS). (B) Low density HUE cells (2 × 10⁴ cells per 35-mm dish) in their first passage after primary and previously grown in the presence of pituitary FGF were seeded on plastic (PLAST), fibronectin (PLAST-FIB), BCE-ECM-, or HR-9-ECM-coated 35-mm dishes and exposed to 25 mM HEPES buffered medium 199 supplemented with 10⁻⁸ M selenium, 20% fetal calf serum alone (○) or with brain FGF (50 ng/ml, △) or pituitary FGF (10 ng/ml, ●) added every other day. Triplicate dishes were trypsinized every other day and counted in a Coulter counter.
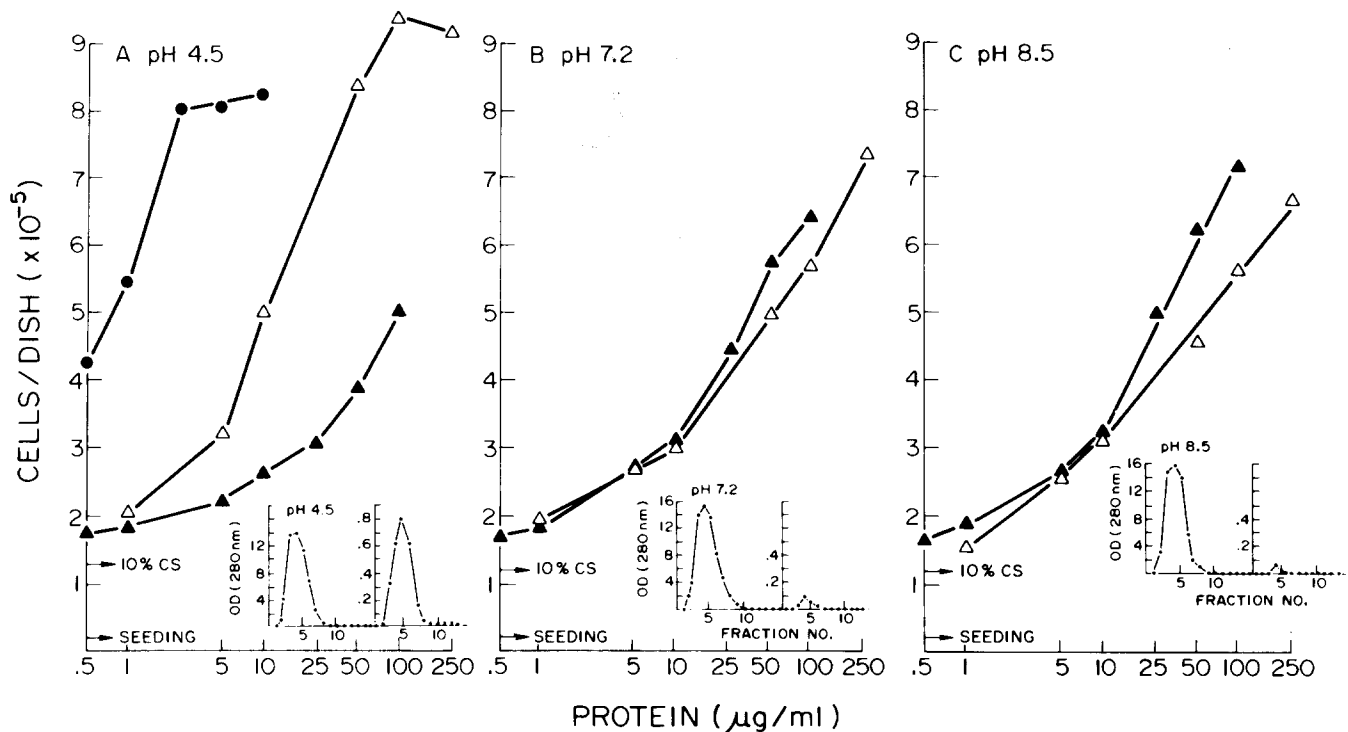
FIGURE 6 Effects of increasing concentrations of crude brain extracts prepared at pH 4.5, 7.2, or 8.5 and chromatographed on carboxymethyl Sephadex C-50 column on the proliferation of low density HUE cells. Brain crude extracts were prepared as described in Materials and Methods and reference 7. 20 mg of lyophilized extract was dissolved in 2 ml of 0.1 M Na phosphates buffer, pH 6.0 and applied on a small (3 ml total volume) column of carboxymethyl Sephadex C-50. The column was washed with 0.1 M Na phosphates pH 6.0, as described (2, 3, 17). 1-ml fractions were collected and their optical density at 280 mm was determined (inset). Protein concentration was determined on the basis of an extinction coefficient of $E^{1\%}_{1cm} = 0.8$. After adequate dilution of the unadsorbed and adsorbed fractions with DME, aliquots (10 μl) containing the various protein concentrations were then added every other day to low density ABAE cells (2 × 10^4 cells per 35-mm dish, arrow SEEDING) maintained on plastic dishes and exposed to DME supplemented with 10% calf serum. After 6 d in culture, triplicate dishes were trypsinized and cells counted in a Coulter counter. The cell density of control cultures exposed to 10% calf serum was 1.2 × 10^5 cells after 6 d (arrow, 10% CS). (A) Crude extract prepared at pH 4.5: △, crude extract; ▲, unadsorbed fraction; ●, adsorbed fraction. Inset shows the chromatographic profile (left) of the unadsorbed fraction, and the elution profile of the adsorbed fraction (right). (B) Crude extract prepared at pH 7.2: △, crude extract; ▲, unadsorbed fraction. (C) Crude extract prepared at pH 8.5: △, crude extract; ▲, unadsorbed fraction.

Human vascular endothelial cells, which are far more difficult to culture than bovine cells, have a similar substrate requirement for optimal proliferation in response to serum factors and exogenous growth factors. On plastic, early-passage HUE cells seeded at low density did not proliferate actively. The survival of HUE cells was greatly increased when the plastic surface was coated with various agents such as fibronectin, laminin, or biomatrices produced by either HR-9 or BCE cells. On all these substrata, HUE cells responded to the addition of pituitary or brain FGF by proliferating actively.

Of all substrata tested, cells were best organized at confluence when maintained on laminin- or HR-9-ECM-coated dishes. In both cases, pituitary or brain FGF was required for HUE cells to proliferate actively. In contrast, more disorganized cultures maintained on either fibronectin- or BCE-ECM-coated dishes was observed. In this regard, it is worth noting that the HR-9 matrix, which is composed mostly of collagen type IV, heparin sulfate proteoglycans, laminin, and entactin (32–34) has a composition very close to that seen in vivo in capillary endothelial cell basement membrane (35, 36). In contrast, the BCE-ECM (on which HUE cells are more disorganized) contains, in addition to the components present in the HR-9 matrix, elastin, collagen type I and III, fibronectin, and dermatan sulfate proteoglycans (37–39). These addi-

tional components could be responsible for the disorganization of the confluent cultures. As reported earlier (10), we have been able to passage HUE cell cultures weekly at a split ratio of 1:10 using either BCE or HR-9 matrix for at least 50 doublings. FGF is required to observe an optimal growth rate. Although on BCE-ECM increased cellular pleiomorphism as a function of passage number was observed, this was less obvious in the case of cultures maintained on HR-9 matrix (unpublished results).

In previous studies we reported that HUE cells maintained on plastic respond poorly to FGF and require the presence of thrombin to respond optimally (10). In the present study, we have observed that coating the plastic surface with fibronectin markedly improved the response of the cells to FGF. This raises the possibility that thrombin, which has no intrinsic ability to stimulate HUE cell growth (4, 10), may act in a manner similar to that of fibronectin.

In our hands, CR pituitary-FGF is 1,000- and 20-fold more potent as a mitogen for low density HUE cell cultures than crude (11, 12) or partially purified ECGF preparation (12, 40). The mitogenic activity of CR-FGF, observed with newly acquired preparations, was also shared by older preparations provided by Dr. Maciag. Although it is conceivable that the early negative results obtained with pituitary FGF (11) could have been caused by the plastic substrate on which cells were

maintained, in later reports fibronectin-coated dishes were used (12), and FGF preparations obtained either from Collaborative Research or purified by Lemmon et al. (14) remained inactive. One possible explanation for the lack of activity of FGF on these cells could be that stock cells were grown in the presence of ECGF (12, 14). This factor could have caused a down-regulation of FGF binding sites similar to the down-regulation of EGF binding sites observed when cells are exposed to either platelet-derived growth factor (PDGF) or FGF (41–44), and thereby could have made the cells FGF-resistant. This possibility has been analyzed in the present study by looking at the proliferative response to FGF and ECGF of endothelial cell populations that have never been exposed to growth factors other than those present in serum versus the response of populations previously exposed to either FGF or ECGF. It is clear from our results that possible selection of vascular endothelial cells resulting from growing primary cultures in the presence of FGF does not make them resistant to ECGF. The converse is also true. Growing cells in the presence of serum alone does not seem to select for cell populations that do not respond to either FGF or ECGF. It is possible, however, that unselected cell populations grown in the presence of a given growth factor could develop an addiction for that growth factor when passaged repeatedly at low density. This would not, however, prevent them from responding to other growth factors, since early- or late-passage ABAE cells grown continuously in the presence of FGF respond equally well to ECGF when exposed to it (Figs. 3 and 4). Likewise, fibroblast, granulosa, vascular smooth muscle, and corneal endothelial cells continuously grown in the presence of FGF can respond equally well to EGF (4).

The possibility has been raised that the mitogen active in FGF preparation is an acidic contaminant (13) similar in nature to ECGF (12). Since both pituitary and brain FGF used in the present study have a pI of 9.6 (17, 18), the possibility that an acidic contaminant could be involved in the mitogenic activity of FGF has been eliminated. This does not mean, however, that neutral or acid mitogens cannot trigger the proliferation of HUE cells. We have previously reported that the proliferation of HUE cells could be triggered both by FGF (a basic mitogen) and by EGF (an acidic mitogen). The action of both mitogens could be potentiated by thrombin (10). In 1975, we also reported the presence of mitogen(s) in both brain and pituitary extracts prepared at either neutral or basic pH that were shown by their chromatographic behavior on DEAE and carboxymethyl Sephadex column to be either neutral or acidic (7). While basic mitogens are best extracted at acidic pH (4.5), the neutral and acidic ones are best extracted at neutral (7.2) or basic pH (8.5) (7). This situation is reminiscent of what occurred with pituitary hormones, which have solubilities that vary with the pH of extraction. In 1961, Ellis (45), in what was considered to be a classic paper, described the sequential extraction of bovine pituitary glands with water at pH 5.5, 0.1 M $(NH_4)_2SO_4$ at pH 4.0, 0.25 M $(NH_4)_2SO_4$ at either pH 5.5 or 7.5, and finally with 50% ethanol at pH 9.8 to 10. The extracts were bioassayed for the anterior pituitary hormones and for the proteinases I and II. With certain exceptions, the first extract contained mainly follicle stimulating hormone and proteinase I; the second, luteinizing hormone and thyroid stimulating hormone; the third growth hormone and proteinase II; and the fourth, prolactin. These methods of extraction are commonly used for the purification of the various pituitary hor-

mones (46, 47). It is also worthwhile to observe that FGF (a basic mitogen) is commonly found as a contaminant in preparations of LH (48), a hormone that, according to Ellis (45), is preferentially solubilized at pH 4.0, but not in either growth hormone or prolactin preparations (7), which are extracted at neutral or basic pH. Our previous studies dealing with the solubility of mitogens at different pH (7) have recently been confirmed by others (49), who have reported that acidic or neutral mitogens are insoluble when pituitary tissue is extracted at an acidic pH of 4.5. In the present study, we have confirmed that neutral or acidic mitogens extracted at either neutral or basic pH and first shown to be active on myoblasts are also active on HUE cells. It is likely that they are the same as the recently identified ECGF which is purified from neutral (pH 7.2) extract of hypothalamus (40).

In conclusion, just as in a previous study (18) we were unable to confirm the existence of an acidic contaminant in brain FGF, in the present study we were unable to confirm that pituitary FGF is not mitogenic for human umbilical vein endothelial cells. On the contrary, using unselected cell populations, we have observed that FGF was even more active on HUE cells than on bovine vascular endothelial cells.

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