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THE ISOLATION AND PURIFICATION OF TWO ANIONIC ENDOTHELIAL CELL GROWTH FACTORS FROM HUMAN BRAIN

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Two anionic polypeptides which stimulate the proliferation of human umbilical vein endothelial cells have been isolated and purified to homogeneity from human brain using heparin affinity chromatography. The molecular weights of the polypeptides are 18,500 and 19,300; the isoelectric point for both polypeptides is at pH 5.2. The purified polypeptides differed in their ability to stimulate human endothelial cell growth. The half maximal activities observed for the 18.5 and 19.3 kilodalton polypeptides were at concentrations of 10.0 ng/ml and 0.9 ng/ml respectively.

We have previously identified and partially purified an anionic growth factor from bovine brain which permits the serial propagation of human umbilical vein endothelial cells (1). The growth factor possesses a similar isoelectric point and sensitivity to acid, heat and protease as the partially purified endothelial cell growth factor described by Maciag et al. (2) and Lemmon et al. (3). Thornton et al. (4) have demonstrated that heparin increases the rate of umbilical vein endothelial cell proliferation and decreases the required amount of anionic endothelial cell growth factor, but does not affect the cumulative population doubling levels. Recently, Shing et al. (5) have isolated and purified by heparin-Sepharose affinity chromatography a cationic growth factor from chondrosarcoma extracellular matrix that stimulates capillary endothelial cell growth. Klagsbrun and Shing (6) have also observed that endothelial cell growth supplement (ECGS, Collab. Res.) and endothelial mitogen (EM, Biomed. Tech., Inc.) contain components which bind tightly to heparin. We report the isolation and purification to homogeneity of two acidic polypeptides from human brain which stimulate cell division of human endothelial cells.

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Materials and Methods

Materials: Porcine heparin and type I gelatin were obtained from Sigma Chemical. Falcon tissue culture plates were used. The glutamine, fetal bovine serum and trypsin used were from Grand Island Biological Company. The heparin affinity resin was produced using Sepharose 4B from Pharmacia Fine Chemicals (7). Reagents for electrophoresis and protein assays were obtained from Bio-Rad Laboratories.

Isolation and Purification of Growth Factors: Four human brains and one human hypothalamus (approx. 4000 grams) which were obtained 24-48 h post-mortem were homogenized, extracted and the extract fractionated by ammonium sulfate precipitation as described by Gordon et al. (1). The protein which was precipitated from the extract with saturated ammonium sulfate 30-95% was dissolved in 0.15 M sodium phosphate buffer, pH 6.0, and applied to a Sephadex CM-50 column (5 x 20 cm). Proteins were eluted with a step gradient of 0, 0.15 M and 1.0 M NaCl in 0.15 M sodium phosphate buffer, pH 6.0. Active fractions from the column were dialyzed, lyophilized and 100 mg of the active protein was dissolved in 4 ml of 0.15 M Hepes buffer, pH 7.5. The solubilized protein was applied to a heparin-Sepharose column (1 x 8 cm). The column was eluted using a step gradient of increasing NaCl concentrations up to 3.0 M and 0.5 ml fractions were collected. The active fractions were stored at 4°C and retained significant activity for at least four weeks.

Endothelial Cell Growth Assay: Human umbilical endothelial cells were isolated and maintained in culture by the method of Gordon et al. (1). Cell growth assays were performed by plating 5000 cells per cm^2 on gelatin coated 8 cm^2 tissue culture dishes containing growth media supplemented with 90 ug/ml porcine heparin (4). Samples to be assayed for mitogenic activity were added to the dishes and the dishes were incubated for 72 h. The cells were detached with 0.1% trypsin and quantitated with a hemocytometer. Control plates received an amount of elution buffer equal to the volume of the sample assayed. The cell number per plate recorded for each concentration of growth factor was the average of four replicate plates. One unit of mitogenic activity was defined as the amount of protein which stimulates half maximal cell growth.

Gel Electrophoresis: SDS-PAGE was performed in 16% gels by the method of Laemmli (8). Gels were silver stained according to the method of Merrill (9).

Isoelectric Focusing: Electrofocusing was performed in 1 mm slab gels mounted on Gelbond supports (FMC Corporation, Rockland, Maine). Gels were 5% acrylamide, 4% crosslinker and contained 2% Servalyt ampholytes (pH 3-10 or 4-6) and 5% urea as solubilizer. Samples were prepared by dialyzing protein eluted from the heparin affinity column against 0.1 M ammonium acetate pH 6.5, lyophilizing the sample and dissolving the protein in 5% urea, 2% ampholytes. In this manner approximately 1 ug of protein could be applied to the gel in 5-10 ul. Completion of isoelectric focusing was determined by monitoring the rate of decrease in the current flow through the gel. Protein standards were used to determine the pH gradient and isoelectric points of the unknowns.

Results and Discussion

Our protocol for isolation and purification of the endothelial cell growth factors from human brain consists of three steps: ammonium sulfate fractionation of a human brain extract; Sephadex CM-50 ion-exchange chromatography; and heparin affinity chromatography. Virtually all of the

Table 1 Purification of Anionic Endothelial Cell Growth Factors

	Protein Recovery mg	1/2 Maximal Activity	Total Activity units	Specific Activity units/mg	Fold Purification
$(NH_4)_2SO_4$ Fractionation	3.20×10^4	63.0 µg/ml	5.1×10^5	15.9	1.0
Sephadex CM-50	1.95×10^3	4.0 µg/ml	4.9×10^5	2.5×10^2	15.7
Heparin Affinity (I)	0.212	10.0 ng/ml	2.1×10^4	1.0×10^5	6.3×10^3
Heparin Affinity (II)	0.125	0.9 ng/ml	1.4×10^5	1.2×10^6	7.6×10^4

endothelial cell growth promoting activity from the human brain extract was precipitated with ammonium sulfate at 30-95% saturation. The yield of protein from 4 kg of human brain was 32 g, approximately twice the amount obtained from 4 kg of bovine brain (1). The total activity recovered from the human brain extract was similar to that obtainable from an equivalent bovine preparation (Table 1). The elution profile of human brain extract following Sephadex CM-50 chromatography is shown in Figure 1. Approximately 6% of the applied protein was recovered in the active fractions. The interaction which

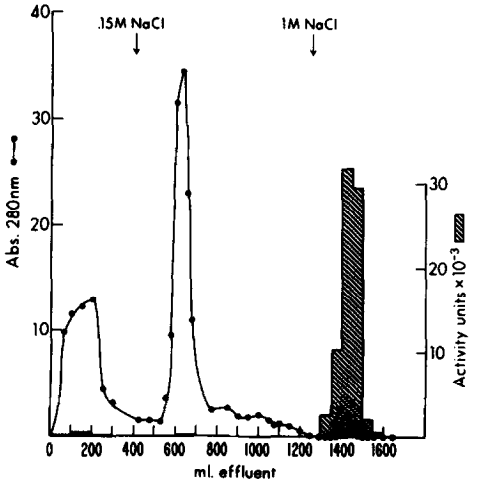

Figure 1. Sephadex CM-50 chromatography of human brain extract precipitated with ammonium sulfate at 30-95% saturation. The soluble portion of the precipitate (5 grams) was applied to the column. Fractions eluted were measured for absorbance at 280 nm (•—•) and for activity in the endothelial cell growth assay (▨).

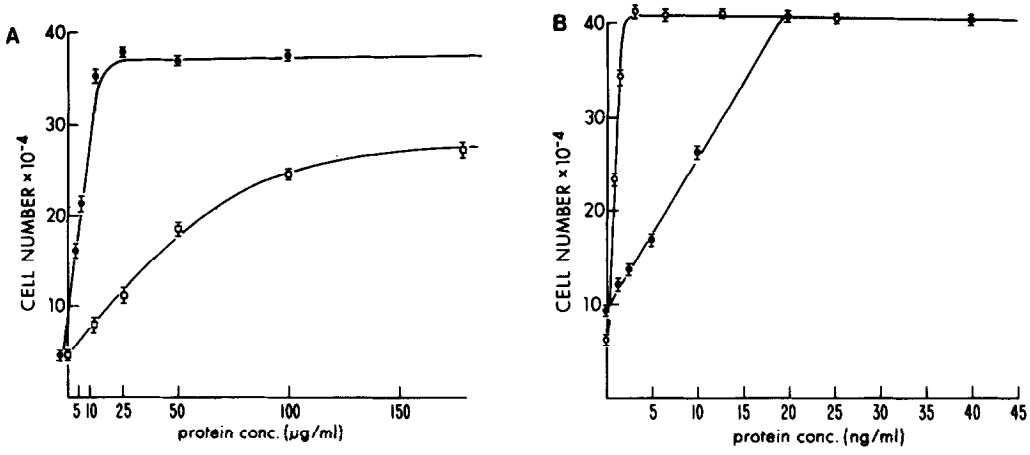

Figure 2. Mitogenic response of human umbilical vein endothelial cells to growth factors isolated from the human brain.
A. Plot of protein concentration vs. cell number for the ammonium sulfate precipitate of human brain extract (□) and the active fraction from Sephadex CM-50 chromatography (●).
B. Plot of protein vs. cell number for the two active fractions I (●) and II (○) isolated by heparin affinity chromatography.

permits the anionic polypeptides to bind to Sephadex CM-50 at pH 6.0 is unknown.

The endothelial cell growth response curves generated with the active samples after ammonium sulfate fractionation and Sephadex CM-50 chromatography are shown in Figure 2A. The active fractions after ammonium sulfate precipitation reached a plateau in the growth curve at approximately 2.7×10^5 cells/plate while the active fractions following Sephadex CM-50 chromatography reached a maximum of approximately 4.0×10^5 cells/plate. We have not determined if addition of protein from the ammonium sulfate precipitate to the growth assays of the CM-50 fractions would lower the maximal growth attainable.

The pattern of elution of protein and mitogenic activity from the heparin-Sepharose affinity column is presented in Figure 3. Two heparin binding growth factors were recovered following heparin affinity chromatography. Both growth factors were homogeneous as determined by SDS-PAGE analysis (Fig. 3). An 18.5 kilodalton polypeptide eluted from the affinity column at 1.2 M NaCl and exhibited half maximal activity at 10.0 ng/ml (Fig.

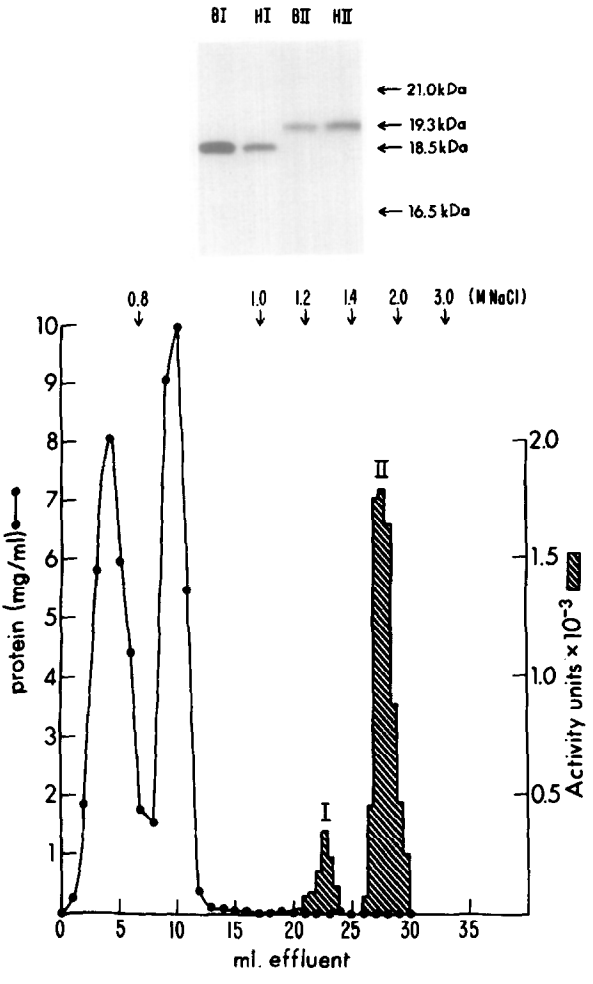

Figure 3. Heparin affinity chromatography of the active fraction (human brain) from Sephadex CM-50 chromatography. Two peaks of mitogenic activity eluted from the column at 1.2 M and 1.4 M NaCl (labeled I and II respectively). The upper part of the figure shows an SDS-PAGE analysis of the two active fractions isolated from human brain (HI and HII) along with corresponding fractions isolated by heparin affinity chromatography from bovine brain (BI and BII). A preparation of rabbit skeletal muscle myosin light chains was used for molecular weight standards.

2B). The 19.3 kilodalton polypeptide eluted at 1.4 M NaCl showed half maximal activity at concentrations of 0.9 ng/ml.

The fold purification for the 18.5 and 19.3 kilodaltons components was 6,300 and 76,000 respectively (Table 1). In analytic isoelectric focusing both polypeptides focused as sharp single bands at pH 5.2. We have recently purified the 19.3 kilodalton polypeptide from human brain using only ammonium sulfate fractionation and heparin affinity chromatography. We have not been

able to purify the 18.5 kilodalton polypeptide in high yield using a two step procedure. The SDS-PAGE analysis of endothelial cell growth factors isolated and purified from bovine brain using similar methodology is also shown in Figure 3. The polypeptides purified from the bovine brain exhibited similar molecular weights and focused at pH 5.2.

In comparing the acidic polypeptides with other purified growth factors, the isoelectric points are similar to that of the acidic fibroblast growth factor purified by Thomas et al. (10) from bovine brain. The acidic fibroblast growth factor, which is a mitogen for BALB/C 3T3 fibroblasts, was purified as two unseparated forms with reported molecular weights of 16,000 and 16,800.

The molecular basis for the differences between the two anionic polypeptides remains to be determined. The relationship of the polypeptides to each other and the acidic fibroblast growth factor will require analysis of their target cells as well as further characterization.

The isolation and purification of two polypeptides with similar functions yet exhibiting differences in heparin binding affinity, molecular weight in SDS-PAGE, and a ten-fold difference in their ability to stimulate endothelial cell growth, will permit an analysis of the structural features which affect both heparin binding and growth promoting activity. A direct correlation between the heparin binding affinity of these polypeptides and their ability to stimulate endothelial cell growth was observed which substantiates Klagsbrun and Shing's proposal (6) of a growth factor:heparin:endothelial cell interaction in the stimulatory process of endothelial cells.

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References

1. Gordon, P.G., Sussman, I., and Hatcher, V.B. (1983) In Vitro 19, 661-671.
2. Maciag, T., Hoover, G.A., Stemerman, M.B., and Weinstein, R. (1981) J. Cell Biol. 91, 420-426.
3. Lemmon, S.K., Riley, M.C., Thomas, K.A., Hoover, G.A., Maciag, T., and Bradshaw, R.A. (1982) J. Cell Biol. 95, 162-169.
4. Thornton, S.C., Mueller, S.N., and Levine, E.M. (1983) Science 623-625.
5. Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J., and Klagsbrun, M. (1984) Science 223, 1296-1298.
6. Klagsbrun, M., and Shing, Y. (1984) Fed. Proc. 43, 3, Abst. 1376.
7. Elgart, E.S., and Rosenberg, M.D. (1975) Biochim. Biophys. Acta 410, 178-192.
8. Laemmli, U.K. (1970) Nature 227, 680.
9. Merril, C.R., Goldman, D., Sedman, S.A., and Ebert, M.H. (1981) Science 211, 1437-1439.
10. Thomas, K.A., Rios-Candelore, M., and Fitzpatrick, S. (1984) Proc. Natl. Acad. Sci. USA, 81, 357-361.