A Unique Family of Endothelial Cell Polypeptide Mitogens: The Antigenic and Receptor Cross-Reactivity of Bovine Endothelial Cell Growth Factor, Brain-derived Acidic Fibroblast Growth Factor, and Eye-derived Growth Factor-II

ALAIN B. SCHREIBER,* JOHN KENNEY,* JOSEPH KOWALSKI,* KENNETH A. THOMAS,* GUILLERMO GIMENEZ-GALLEGO,* MARI RIOS-CANDELORE,* JERRY DI SALVO,* DENIS BARRITAULT,* JOSE COURTY,* YVES COURTOIS,* MICHEL MOENNER,* CAMILLE LORET,* WILSON H. BURGESS,† TEVIE MEHLMAN,† ROBERT FRIESEL,‡ WARREN JOHNSON,§ and THOMAS MACIAG

*Institute of Biological Sciences, Syntex Research Institute, Palo Alto, California 94304; *Department of Biochemistry, Merck Institute for Therapeutic Research, Merck, Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065; ‡Unite de Recherches Gerontologiques, Institut National de la Santé et de la Recherche Médicale, 75016 Paris, France; and §Department of Cell Biology, Revlon Biotechnology Research Center, Rockville, Maryland 20850. Address correspondence to T. Maciag, Department of Cell Biology, Revlon Biotechnology Research Center, 2 Research Court, Rockville, MD 20850.

ABSTRACT Bovine brain, hypothalamus, pituitary, and retina contain potent anionic polypeptide mitogens for endothelial cells. Immunological assays using murine monoclonal antibodies against bovine endothelial cell growth factor (ECGF) and radioreceptor assays using [125I]ECGF were performed to determine the cross-reactivity of ECGF with bovine acidic pl brain-derived fibroblast growth factor (acidic FGF) and bovine eye-derived growth factor-II (EDGF-II). We observed that acidic FGF and EDGF-II are recognized by anti-ECGF monoclonal antibodies and compete with [125I]ECGF for receptor occupancy. Furthermore, the biological activity of ECGF, acidic FGF, and EDGF-II is potentiated by the glycosaminoglycan, heparin. These results argue that ECGF, acidic FGF, and EDGF-II belong to a common family of polypeptide growth factors.

Several growth factors of peptidic nature have been isolated and purified from bovine neural tissue (1, 4–6, 9, 10, 14–19, 23, 24). These polypeptides share common biological properties since they are all potent mitogens for murine BALB/c 3T3 cells in vitro. In addition, several of these polypeptide mitogens stimulate endothelial cell proliferation in vitro (1, 5, 6, 11, 13, 16, 18, 19, 23, 24) and may play an important role in homeostatic and pathophysiological processes involving the vascular tree (21). These growth factors include polypeptides of cationic nature such as basic isoelectric point (pl)1 fibroblast growth factor (FGF) (4, 10), chondrosarcoma-de-
MATERIALS AND METHODS

Reagents: Epidermal growth factor (EGF) was purified from male mouse submaxillary glands by reverse-phase high pressure liquid chromatography as previously described (22). Basic FGF, purified homogeneity from bovine pituitary glands (4) was a generous gift from Dr. Denis Gospodarowicz and EGF (Mr, 20,000) and acidic FGF (Mr, 17,000) was purified from bovine brain by procedures previously described (20, 24) (Burgess, W. H., T. Maciag, and W. Johnson and T. Maciag, J. Biol. Chem., In press). Purified preparations of EGF-II were obtained from bovine brain by extraction and Affi-Blue chromatography as described (3), followed by elution of EGF-II from heparin-Sepharose between 0.9 and 1.1 M NaCl (20). Marine monoclonal antibodies and rabbit polyclonal antisera against EGF were prepared as previously described (20, 22). The monoclonal antibodies H9, an IgM(k), and H15 an IgG2(k) are neutralizing antibodies for EGF (22). Heparin and bovine serum albumin (BSA) were obtained from Armour Pharmaceutical Co. (Tarrytown, NY) and heparin-Sepharose 6B was purchased from Pharmacia Fine Chemicals (Piscataway, NJ).

Cells: Human umbilical vein endothelial cells (HUVEC) and bovine aortic endothelial cells (BAEC) were established from primary cultures. HUVEC were grown on human fibronectin-coated (5 #g/cm²) (Armour Pharmaceutical Co.) cell culture dishes in Dulbecco's modified Eagle's medium (DME) containing 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT) and 2 mg/ml EGF containing 20 #g heparin/ml. BAEC were propagated in DMEM containing 10% fetal bovine serum. The endothelial cell character of the BAEC containing 10% fetal bovine serum (HyClone Laboratories, Logan, UT) and 2 mg/ml EGF was confirmed by the presence of human Factor VIII:Ag and membrane angiotensin-converting enzyme (monoclonal antibody against angiotensin-converting enzyme kindly provided by Dr. R. Auerbach) as previously described (20).

Cell Proliferation Assay: The low seed density human endothelial cell proliferation assay was performed with HUVEC as previously described (21, 25). Briefly, HUVEC were seeded (10³ cells/cm²) on human fibronectin (5 #g/cm²) in 96-well cluster dishes (Costar, Cambridge, MA) in DME supplemented with 10% FBS. Mitogens were added for 3 d at 37°C prior to protein determination as described in Results. The incorporation of [3H]thymidine (New England Nuclear, Boston, MA) into DNA in quiescent BAEC was performed as previously described for murine lung capillary endothelial cells (20, 25).

Radioreceptor Binding Assay: The ligand, EGF, was radiolabeled with Na²¹ using Enzymobeads (immobilized glucose-oxidase-lactoperoxidase, Bio-Rad Laboratories, Richmond, CA) as previously described (22). The radiolabeled ligand, [¹²⁵I]EGF, possessed a specific activity of 2 × 10⁶ cpm/ng protein and was biologically active at 500 pg/ml. Confluent populations of BAEC in 24-well cluster dishes (Costar) were washed twice with DME, pH 7.4, containing 0.1% BSA and 50 mM HEPES (DME–BSA). The cells were incubated at 4°C for 60 min with [¹²⁵I]EGF in the presence or absence of inhibitor polypeptides in DME–BSA, washed three times with DME–BSA, lysed with 0.1 N NaOH, and the cell-associated radioactivity was determined. Nonspecific binding was assessed in the presence of 100-fold molar excess of cold EGF and did not exceed 20% of the total binding.

Double-antibody Immunoprecipitation: The immunoprecipitation was performed as previously described (22). Briefly, polyvinylchloride 96-well plates (Costar) were coated with 50 ng/ml monoclonal rabbit anti-EGF (IgG), coated with 10% normal rabbit serum, incubated with increasing concentrations of polypeptide ligand, washed with DME–BSA, and incubated with monoclonal anti-EGF antibodies (20, 22). The reaction was quantitated spectrophotometrically after incubation with peroxidase-conjugated rabbit antirabbit IgG or IgM (Zymed Laboratories, San Francisco, CA) and orthophenylenediamine substrate conversion.

RESULTS

Acidic FGF and EGF-II Compete for [¹²⁵I]EGF Binding to Endothelial Cell Receptors

As reported previously, [¹²⁵I]EGF binds to HUVEC and BAEC in a reversible and saturable fashion (22). The apparent Kd for receptor occupancy was estimated to be, respectively, 2 and 6 × 10⁻¹⁰ M with 4 and 2 × 10⁴ binding sites per cell (22). Both acidic FGF and EGF share the same receptor binding sites, but neither EGF nor cationic FGF, compete for the specific binding of [¹²⁵I]EGF to both cell types in a concentration-dependent manner. The experimental results obtained with BAEC are shown in Fig. 1. The polypeptides EGF, EGF-II, and acidic FGF compete half-maximally at ~10⁻⁹ M. Molarities were calculated for acidic FGF (M, 17,000), EGF-II (M, 17,000), and EGF (M, 20,000) based upon protein determinations provided from amino acid compositions.

Acidic FGF and EGF-II React with Antibodies Against EGF

Monoclonal antibodies against EGF have proven valuable as reagents to assess the immunological character of the endothelial cell growth-promoting activity present in crude preparations of bovine brain (20). Subsequently, these antibodies have also been used to inhibit the biological activity of purified EGF, an observation consistent with the ability of the antibodies to prevent [¹²⁵I]EGF/EGF receptor occupancy (22). The data in Fig. 2A demonstrate that the murine monoclonal anti-EGF antibody, H15, binds EGF, acidic FGF, and EGF-II in a concentration-dependent manner. Furthermore, it was possible to demonstrate that the polypeptides are recognized by two distinct anti-EGF monoclonal antibodies, H15 and H9, in a manner dependent upon the concentration of the antibody. These data suggest that EGF, acidic FGF, and EGF-II share common immunological epitopes which are recognized by two different monoclonal antibodies both of which inhibit EGF-induced endothelial cell proliferation (20, 22). Denaturation of the polypeptide preparations may explain the relatively small differences in cross-reactivity between EGF-II, EGF, and acidic FGF.

The Mitogenic Activity of EGF, Acidic FGF, and EGF-II Is Potentiated by Heparin

It is established that the mitogenic activity of crude (25) and purified preparations of EGF (20, 22) is potentiated by the glycosaminoglycan, heparin. Since EGF, acidic FGF, and EGF-II share common receptor binding and immunological characteristics, we examined the ability of heparin to potentiate the biological activity of EGF-II and acidic FGF. As demonstrated in Fig. 3, EGF, acidic FGF, and EGF-II
are indeed potent endothelial cell mitogens. In addition, the biological activities of EDGF-II, acidic FGF, and ECGF are potentiated by heparin. The effect of heparin on the potentiation of biological activity is very similar among these polypeptides. The half-maximal mitogenic response for EDGF-II, ECGF, and acidic FGF is between 2 and 4 ng/ml in the presence of heparin.

**DISCUSSION**

A number of laboratories have recently purified anionic polypeptides from a variety of bovine tissues. These polypeptides share a common feature since they are potent biological mitogens for endothelial cells in vitro (1–3, 5, 7, 15, 17, 19, 24). They also share common chemical and physical characteristics including similar isoelectric points (pI's) and molecular weights and the ability to avidly bind immobilized heparin (5, 6, 15, 20). These polypeptides have been designated ECGF (18), acidic FGF (9, 24), EDGF-II (1–3), RDGF (6), α-HGF (17), and acidic HDGF (15). We report that three of these endothelial cell mitogens share considerable biochemical homology which includes (a) heparin affinity, (b) cross-reactivity to polyclonal ECGF antisera and monoclonal ECGF antibodies, (c) competition with EGF for binding to a high affinity endothelial cell–derived receptor (22), and (d) potent biological activity as endothelial cell mitogens which is potentiated by heparin (22). Furthermore, antibodies prepared against ECGF inhibit the mitogenic activity of acidic FGF and EDGF-II (data not presented); this observation is consistent with the ability of anti-ECGF antibodies to prevent receptor occupancy by ECGF (22). These biological attributes, together with biochemical similarities among ECGF, acidic FGF, and EDGF-II (1, 19, 24), strongly argue that these endothelial cell mitogens are either identical or belong to the same family of polypeptide growth factors. Furthermore, our data suggest that this new polypeptide growth factor family, as defined by biological, immunological (20), and radioreceptor (22) criteria, is unique and distinct from other traditional growth factor families such as nerve growth, EGF, bovine basic FGF, insulin-like growth factors I and II, and platelet-derived growth factor (13).

Although we have not established a relationship between RDGF (6), α-HGF (17), and acidic HDGF (15) at the immunological and receptor level, comparative biochemical and biological information does exist which suggest that acidic HDGF, α-HGF, and RDGF are indeed homologous with acidic FGF, EDGF-II, and ECGF (6, 15, 17). These homologies are substantiated by common molecular weights, acidic pI's, avid affinity for immobilized heparin (6, 15, 17), and, in the case of α-HGF, similar amino acid compositions (17, 24).
(Burgess W. H., and T. Maciag, unpublished observations). Although these similarities are tentative and will require experimental verification at either the protein or gene level, we propose that α-HGF, acidic HDGF, and RDGF will ultimately belong to the family of endothelial cell polypeptide mitogens which presently includes ECGF, acidic FGF, and EDGF-II.

Historically, interest in polypeptide mitogens for endothelial cells has been stimulated by the ubiquitous nature of the endothelium and the potential role of the endothelial cell in maintenance of homeostasis (7, 8, 12, 21). Clearly, polypeptides capable of inducing endothelial cell proliferation may have a significant impact on our understanding of the biochemical responsibilities of the endothelium during the development of the vascular tree, wound healing, tumor development, and atherogenesis (21). Biochemical interest in the structure of these endothelial cell polypeptide mitogens has been driven by the need to elucidate the contribution of the endothelial cell to normal and pathophysiological neovascularization (7, 8, 12, 21). This study demonstrates that ECGF, EDGF-II, and acidic FGF belong to the same family of endothelial cell polypeptide mitogens.

The authors thank L. Peterson for expert secretarial assistance. G. Gimenez-Gallego is on sabbatical leave from Consejo Superior Investigaciones Cientificas Centro de Investigaciones Biologicas, Madrid, Spain. Dr. A. B. Schreiber's new address is Armour/Meloy Labs, Inc., 6715 Electronic Drive, Springfield, Virginia 22151. Dr. Barritault's present address is Department of Biotechnology, Universite Paris 12, Creteil, France.

This work was supported by grants HL 310765 and AG 04807 from the National Institutes of Health to T. Maciag, as well as ATP CNRS 955341 and a grant from the League de Cancer de Van de Marne to D. Barriault. J. Courty was a resident from the Association pour la Recherche sur le Cancer.

REFERENCES

1. Barritault, D., J. Plouet, J. Courty, and Y. Courtois. 1982. Purification, characterization and biological properties of the eye-derived growth factor from retina: analogies with brain-derived growth factor. J. Neurosci. Res. 8:477-490.
2. Barritault, D., C. Arruti, and Y. Courtois. 1981. Is there an ubiquitous growth factor in the retina? Differentiation. 18:59-64.
3. Courty, J., C. Loret, M. Moenner, B. Chevalier, O. Lagente, Y. Courtois, and D. Barritault. 1985. Bovine retina contains three growth factor activities with different affinity for heparin; eye-derived growth factors. Biochem. Biophys. Res. Commun. 128:262-268.
4. Bohlen, P., A. Baird, F. Each, N. Ling, and D. Gospodarowicz. 1984. Isolation and partial molecular characterization of pituitary fibroblast growth factor. Proc. Natl. Acad. Sci. USA. 81:5304-5308.
5. Conn, G., and V. B. Hatcher. 1984. The isolation and purification of two anionic endothelial cell growth factors from human brain. Biochem. Biophys. Res. Commun. 128:262-268.
6. D'Amore, P. A., and M. Klagsbrun. 1984. Endothelial cell mitogens derived from retina and hypothalamus: biochemical and biological similarities. J. Cell Biol. 99:1543-1549.
7. Folkman, J. 1975. Tumor angiogenesis: a possible control point in tumor growth. Ann. Intern. Med. 82:96-100.
8. Folkman, J. 1984. Angiogenesis: initiation and modulation. Symp. Fundam. Cancer Res. 36:201-208.
9. Gamberini, A. G., and H. A. Armelin. 1982. Purification and partial characterization of an acidic fibroblast growth factor from bovine pituitary. J. Biol. Chem. 257:9692-9697.
10. Gospodarowicz, D., F. M. Liu, and J. Cheng. 1982. Purification in high yield of brain fibroblast growth factor by preparative isoelectric focusing at pH 9.6. J. Biol. Chem. 257:12266-12276.
11. Gospodarowicz, D., J. Cheng, and M. Liorette. 1983. Bovine brain and pituitary fibroblast growth factors: comparison of their abilities to support the proliferation of human and bovine vascular endothelial cells. J. Cell Biol. 97:1677-1685.
12. Greensblatt, M. J. 1970. Tumor angiogenesis. Microvasc. Res. 2:342-349.
13. James, R., and R. A. Bradshaw. 1984. Polypeptide growth factors. Annu. Rev. Biochem. 53:259-292.
14. Kellett, J. G., T. Tanaka, J. M. Rowe, R. P. C. Shiu, and H. G. Friesen. 1981. The characterization of growth factor activity in the brain. J. Biol. Chem. 256:54-58.
15. Klagsbrun, M., and Y. Shing. 1983. Heparin affinity of basic and cationic capillary endothelial cell growth factors: analysis of hypothalamic-derived growth factors and fibroblast growth factors. Proc. Natl. Acad. Sci. USA. 82:805-809.
16. Lemmon, S. K., M. C. Riley, K. A. Thomas, G. A. Hoover, T. Maciag, and R. A. Bradshaw. 1982. Bovine fibroblast growth factor: comparison of brain and pituitary preparations. J. Cell Biol. 95:162-169.
17. Lobb, R. R., and J. W. Fett. 1984. Purification of two distinct growth factors from bovine neural tissue by heparin affinity chromatography. Biochemistry. 23:6295-6309.
18. Maciag, T. 1984. Angiogenesis. Prog. Hemostasis Thromb. 7:167-182.
19. Maciag, T., J. Cerundolo, S. Hiley, P. R. Kelley, and R. Forand. 1979. An endothelial cell growth factor: the principal endothelial cell mitogen in bovine brain. Science (Wash. DC). 205:969-971.
20. Maciag, T., M. A. South, R. Forand, and R. A. Bradshaw. 1982. Bovine fibroblast growth factor: comparison of brain and pituitary preparations. J. Cell Biol. 95:162-169.
21. Maciag, T., G. A. Hoover, and R. Weinstein. 1982. High and low molecular weight forms of endothelial cell growth factor. J. Biol. Chem. 257:5333-5336.
22. Maciag, T., T. Mehlin, R. Fried, and A. B. Schreiber. 1984. Heparin binds endothelial cell growth factor; the principal endothelial cell mitogen in bovine brain. Science (Wash. DC). 225:932-934.
23. Schreiber, A. B., J. Kenney, W. J. Kowalski, R. Fried, T. Mehlin, and T. Maciag. 1985. The interaction of endothelial cell growth factor with heparin: characterization by receptor and antibody recognition. Proc. Natl. Acad. Sci. USA. In press.
24. Shing, Y., J. Folkman, R. Sullivan, C. Butterfield, J. Murray, and M. Klagsbrun. 1984. Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. Science (Wash. DC). 223:1296-1299.
25. Thomas, K. A., M. Rios-Candelore, and S. Fitzpatrick. 1984. Purification and characterization of acidic fibroblast growth factor from bovine brain. Proc. Natl. Acad. Sci. USA. 81:357-361.
26. Thomson, S. C., S. N. Mueller, and E. M. Levine. 1983. Human endothelial cells use heparin in cloning and long-term serial propagation. Science (Wash. DC). 222:623-625.