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 [^{125}I]ECGF were performed to determine the cross-reactivity of ECGF with bovine acidic pI 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 [^{125}I] ECGF for receptor occupancy. Furthermore, the biological activity of ECGF, acidic FGF, and EDGF-II is potentiated by the glycosylaminoglycan, 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 (pI)[1] fibroblast growth factor (FGF) (4, 10), chondrosarcoma-de-

rived growth factor (23), and basic pI hypothalamus-derived growth factor-I (HDGF), as well as polypeptide mitogens with anionic isoelectric points. These acidic growth factors include endothelial cell growth factor (ECGF) (18, 19), acidic pI brain-derived FGF (9, 24), eye-derived growth factor-II (EDGF-II) (1–3), retina-derived growth factor (RDGF) (6), α-heparin-binding growth factor (α-HGF) (18), and acidic pI HDGF (15). Radioreceptor and immunological assays have recently been developed for the acidic polypeptide, ECGF (20, 22). The availability of purified growth factors and these characterization methods prompted us to determine whether these endothelial cell mitogens share common immunological and cell receptor recognition features. We now report that both acidic FGF and EDGF-II compete with ECGF for receptor occupancy and cross-react with monoclonal antibodies prepared against ECGF. Furthermore, the mitogenic activity of the acidic polypeptide growth factors acidic FGF and EDGF-II on endothelial cells is potentiated by the glycosaminoglycan, heparin, a biological attribute unique to ECGF (22).

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[1] Abbreviations used in this paper: BAEC, bovine aortic endothelial cells; DME–BSA, Dulbecco's modified Eagle's medium, pH 7.4, containing 0.1% bovine serum albumin and 50 mM HEPES; ECGF, endothelial cell growth factor; EDGF-II, eye-derived growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; HDGF, hypothalamus-derived growth factor; HUVEC, human umbilical vein endothelial cell; HGF, heparin-binding growth factor; pI, isoelectric point; RDGF, retina-derived growth factor.

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 to homogeneity from bovine pituitary glands (4) was a generous gift from Dr. Denis Gospodarowicz and ECGF ($M_r \cong 20,000$) and acidic FGF ($M_r \cong 17,000$) was purified from bovine brain by procedures previously described (20, 24) (Burgess, W. H., T. Mehlman, R. Friesel, W. Johnson and T. Maciag, *J. Biol. Chem.*, In press). Purified preparations of EDGF-II were obtained from bovine retina by extraction and Affi-Blue chromatography as described (3), followed by elution of EDGF-II from heparin-Sepharose between 0.9 and 1.1 M NaCl (20). Murine monoclonal antibodies and rabbit polyclonal antisera against ECGF were prepared as previously described (20, 22). The monoclonal antibodies H9, an IgM(k), and H15 an IgG₂(k) are neutralizing antibodies for ECGF (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 μg/cm²) (Armour Pharmaceutical Co.) cell culture dishes in Dulbecco's modified Eagle's medium (DME) containing 10% fetal bovine serum (HyClone Laboratories, Logan, UT) and 2 ng/ml ECGF containing 20 μg heparin/ml. BAEC were propagated in DMEM containing 10% fetal bovine serum. The endothelial cell character of the BAEC and HUVEC populations 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 μg/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]methylthymidine (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, ECGF, was radiolabeled with Na¹²⁵I using Enzymobeads (immobilized glucose-oxidaselactoperoxidase, Bio-Rad Laboratories, Richmond, CA) as previously described (22). The radiolabeled ligand, [¹²⁵I]ECGF, possessed a specific activity of 2 × 10⁴ 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]ECGF 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 ECGF and did not exceed 20% of the total binding.

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

RESULTS

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

As reported previously, [¹²⁵I]ECGF binds to HUVEC and BAEC in a reversible and saturable fashion (22). The apparent K_D 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 EDGF-II, but neither EGF nor cationic FGF, compete for the specific binding of [¹²⁵I]ECGF to both cell types in a concentration-dependent manner. The experimental results obtained with BAEC are shown in Fig.

1. The polypeptides ECGF, EDGF-II, and acidic FGF compete half-maximally at ~10⁻⁹ M. Molarities were calculated for acidic FGF (M_r 17,000), EDGF-II (M_r 17,000), and ECGF (M_r 20,000) based upon protein determinations provided from amino acid compositions.

Acidic FGF and EDGF-II React with Antibodies Against ECGF

Monoclonal antibodies against ECGF 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 ECGF, an observation consistent with the ability of the antibodies to prevent [¹²⁵I]ECGF receptor occupancy (22). The data in Fig. 2 A demonstrate that the murine monoclonal anti-ECGF antibody, HI5, binds ECGF, acidic FGF, and EDGF-II in a concentration-dependent manner. Furthermore, it was possible to demonstrate that the polypeptides are recognized by two distinct anti-ECGF monoclonal antibodies, HI5 and H9, in a manner dependent upon the concentration of the antibody. These data suggest that ECGF, acidic FGF, and EDGF-II share common immunological epitopes which are recognized by two different monoclonal antibodies both of which inhibit ECGF-induced endothelial cell proliferation (20, 22). Denaturation of the polypeptide preparations may explain the relatively small differences in cross-reactivity between EDGF-II, ECGF, and acidic FGF.

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

It is established that the mitogenic activity of crude (25) and purified preparations of ECGF (20, 22) is potentiated by the glycosaminoglycan, heparin. Since ECGF, acidic FGF, and EDGF-II share common receptor binding and immunological characteristics, we examined the ability of heparin to potentiate the biological activity of EDGF-II and acidic FGF. As demonstrated in Fig. 3, ECGF, acidic FGF, and EDGF-II
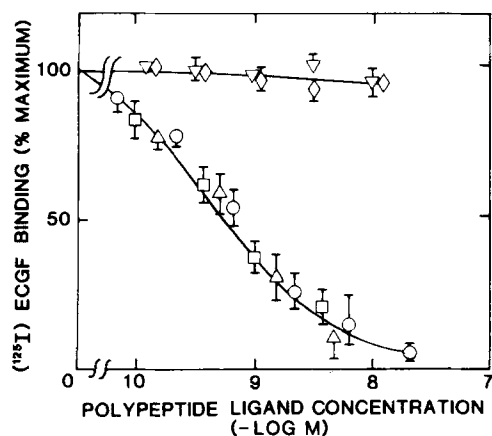

FIGURE 1   Competition of binding of [¹²⁵I]ECGF to BAEC by ECGF, EDGF-II, and acidic FGF. BAEC grown to confluence in DME supplemented with 10% fetal bovine serum. The cells were incubated at 4°C for 1 h with 10⁻⁹ M [¹²⁵I]ECGF in the absence or presence of increasing concentrations of unlabeled ECGF (O), acidic FGF (Δ), EDGF-II (□), cationic FGF (∇), or EGF (◊). Results are reported as the percent [¹²⁵I]ECGF bound as a function of the concentration of unlabeled competing ligand.
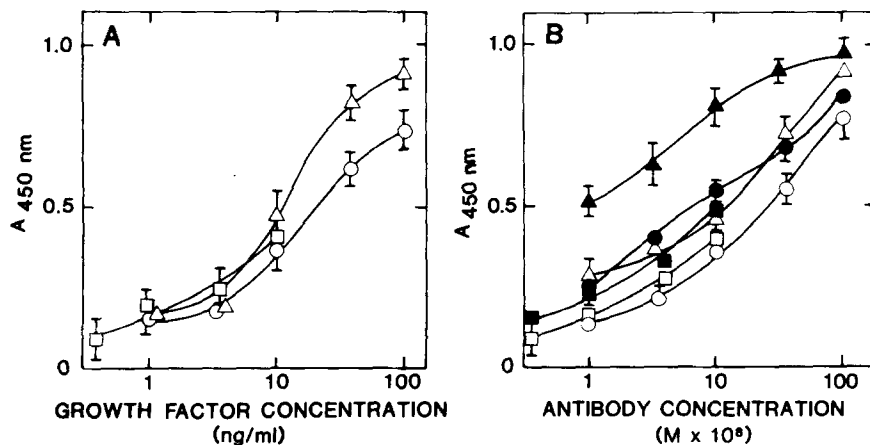
FIGURE 2   Reactivity of anti-ECGF antibodies with ECGF, EDGF-II, and acidic FGF. (A) Rabbit polyclonal anti-ECGF antibodies (50 μg/ml) were adsorbed to solid-phase polyvinylchloride plates. After countercoating, increasing concentrations of either ECGF (O), acidic FGF (△), or EDGF-II (□) were incubated at 37°C for 2 h. The plates were washed, further incubated at 37°C for 2 h with 10⁻⁶ M HI5 murine monoclonal IgG anti-ECGF, washed, and the binding of HI5 IgG was determined spectrophotometrically after incubation with rabbit anti-mouse IgG antiserum coupled to perioxidase. The results are reported as antibodies at 450 nm (orthophenylenediamine conversion.) (B) Same protocol as in A except that the ligand concentration remained constant at 100 ng/ml of either ECGF (O), acidic FGF (△), or EDGF-II (□). Increasing concentrations of either HI5 murine monoclonal anti-ECGF (open symbols) or H9 murine monoclonal anti-ECGF (closed symbols) were added at 37°C for 2 h. Results are reported as orthophenylenediamine conversion (antibodies at 450 nm) as a function of the concentration of the monoclonal anti-ECGF antibody.

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 ECGF 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 radiorecep-
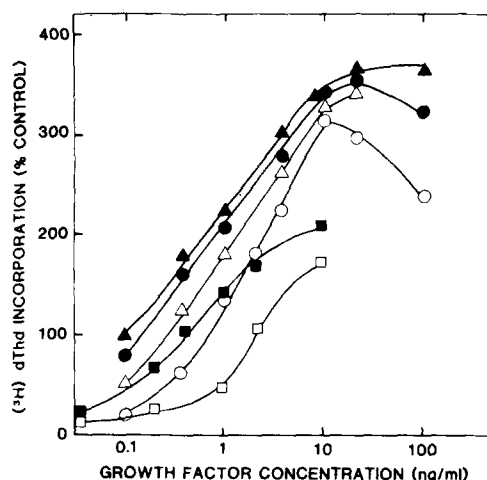

FIGURE 3   Potentiation of the mitogenic activity of ECGF, EDGF-II, and acidic FGF by heparin. The mitogenic response of BAEC to ECGF (O), acidic FGF (△), and EDGF-II (□) was determined in the absence (open symbols) and presence (closed symbols) of heparin (50 μg/ml) as described previously (20, 22). The results are reported as the percent incorporation of [³H]dThd into DNA versus the protein concentration of the various polypeptide ligands.

tor (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.

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