actual tumor volumes and glucose concentrations in nondiabetic mice. Thus, the parameters Ω and V_E must be calculated for volumes V'. The value of V' ranges from 38 ± 14 mm^3 ($\equiv V_0$) to 4864 ± 3254 mm^3 at day 30 (the last day of the experiment). (The estimated errors of V' are 2 standard deviations obtained by propagating the error of the parameters of the model.) For realistic values of V' in our system, self-incitement contributes up to 10 percent (Fig. 4).

Our model does not indicate mechanistic details of self-incitement; here SICRI's and glucose provide only phenomenological correlates of the tumor-proliferating activity. However, some mechanisms can be envisaged. For example, an increase in the amount of circulating growth hormone parallels tumor growth, elevations in SICRI concentration, and decreases in glucose concentration (*3*, *10*). In tumor extracts no growth hormone was found, showing that this substance, unlike SICRI's, is not of tumor origin. Moreover, growth hormone concentrations correlate well with those of blood glucose ($r = -0.59$) (*10*), and depression of blood sugar amount is known to cause the release of growth hormone into the circulation (*17*). This hormone could close the feedback loop by inciting tumor proliferation; tumor-promoting effects of growth hormone on some (mainly lymphoproliferative) tumors are well documented (*18*). Our ongoing experiments do not exclude autocrine, paracrine, or endocrine activity of SICRI's, in analogy with what has been suggested for the action of various growth factors (*19*). Fluctuations in glucose concentrations related to SICRI's have been reported in patients bearing different types of tumors (*1–5*). Furthermore, changes in the concentration of growth hormone in response to such fluctuations have been shown to occur in Hodgkin's disease (*3*).

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Heparin Binds Endothelial Cell Growth Factor, the Principal Endothelial Cell Mitogen in Bovine Brain

Abstract. *Endothelial cell growth factor (ECGF), an anionic polypeptide mitogen, binds to immobilized heparin. The interaction between the acidic polypeptide and the anionic carbohydrate suggests a mechanism that is independent of ion exchange. Monoclonal antibodies to purified bovine ECGF inhibited the biological activity of ECGF in crude preparations of bovine brain. These data indicate that ECGF is the principal mitogen for endothelial cells from bovine brain, that heparin affinity chromatography may be used to purify and concentrate ECGF, and that the affinity of ECGF for heparin may have structural and perhaps biological significance.*

Endothelial cells in vivo are responsible for the formation of a nonthrombogenic interface between blood and tissue and have a prominent mechanistic role in the pathophysiology of angiogenesis, wound repair, thrombosis, and atherogenesis (*1*). Thus the discovery of factors that regulate endothelial cell proliferation and differentiation can contribute to our knowledge of the developmental biology and physiology of the vascular system (*2*).

Endothelial cell growth factor (ECGF) is an acidic polypeptide mitogen—purified and characterized from bovine neural tissue (*3*)—which supports the proliferation and serial propagation of human endothelial cells in vitro (*4*). The addition of heparin to a crude preparation of ECGF increases the specific activity of the ECGF preparation as an endothelial cell mitogen, reduces the endothelial cell population doubling time, and permits the establishment of stable human endothelial cell clones (*5*). Although the mechanism of heparin action is unclear, the synergistic mitogenic activity between the anionic carbohydrate and acidic polypeptide mitogen (*3*) suggests the possibility of a structural interaction between these biological response modifiers. We report that (i) the anionic polypeptide ECGF is the principal endothelial cell mitogen in extracts of bovine neural tissue and (ii) heparin-Sepharose chromatography can be used to purify ECGF. We further suggest that the biological activity of heparin as a potentiator of ECGF activity may involve a structural interaction between the carbohydrate and the polypeptide.

Crude preparations of ECGF from bovine brain, at neutral *p*H and low ionic strength, were subjected to streptomycin sulfate precipitation (*6*). Fractions containing the 20K, low molecular weight ECGF were obtained from gel exclusion chromatography of the bovine brain extract (*3*). These fractions were used as the starting material for heparin-Sepharose studies; half-maximum stimulation of human endothelial cell growth occurred at approximately 5 μg/ml.

Preparations of low molecular weight ECGF were processed through a column consisting of heparin covalently coupled to Sepharose 4B. Most of the protein passed through the column and contained no human endothelial cell growth–promoting activity (Fig. 1A). Elution of the heparin-Sepharose column with 1*M* NaCl resulted in the recovery of the endothelial cell growth–promoting activity. The titration of the biological activity present in these fractions revealed that the mitogen was capable of promoting half-maximum human endothelial cell growth at concentrations of 10 to 15 ng/ml (Fig. 1B). The significant increase in the specific activity of ECGF could also

be shown by subjecting the fractions eluted from the heparin-Sepharose column to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and silver stain analysis of the gel (Fig. 1C). The biologically active ECGF fraction eluted from the heparin-Sepharose column was heterogeneous, as reflected by the number of bands in the 15- to 30-kD range. The heterogeneity of the ECGF fractions obtained with heparin-Sepharose chromatography is consistent with the low nanogram range of biological potency of the mitogen. We estimate that this preparation of ECGF is approximately 5 percent pure (*7*). Although we have observed, on occasion, less heterogeneous preparations of ECGF with higher specific biological activity, our present data provide a conservative estimate of the purity of the mitogen after heparin-Sepharose chromatography.

Fibroblast growth factor (FGF), a mitogen for many mesenchymal cells, including fibroblasts and endothelial cells, is also present in extracts of bovine brain (*6*). FGF is a cationic polypeptide (*8*), which also may have been adsorbed to heparin-Sepharose through an ionic interaction. To eliminate the possibility that FGF contributed to the biological activity on endothelial cells as a contaminant of ECGF, the ECGF fractions obtained with heparin-Sepharose chromatography were subjected to isoelectric focusing. We observed that the biological activity eluted from the heparin-Sepharose column contained a single species of anionic growth factor having an apparent isoelectric point between *p*H 5 and 6 (Fig. 2). These results are consistent with the reported isoelectric point for ECGF (*3*) and related polypeptides isolated from bovine neural tissue and retina (*9*).

We have also used heparin-Sepharose chromatography to adsorb the biological activity of ECGF from crude preparations of bovine brain. Since neural tissue contains various substances with mammalian cell growth–promoting activity (*10*), it is conceivable that these active substances may also be adsorbed to immobilized heparin and thus contribute to the biological activity of ECGF. This is important since crude preparations of ECGF are routinely used to propagate endothelial cells serially in vitro (*4, 5, 11*). To demonstrate that ECGF is the primary endothelial cell mitogen in extracts prepared from bovine brain at neutral *p*H, we prepared murine monoclonal antibodies against highly purified preparations of ECGF (*7*). The murine antibody H18 is a monoclonal immunoglobulin M (IgM) preparation that recognizes and binds ECGF by a solid-phase enzyme-linked immunosorbent assay (ELISA) procedure, whereas H9 and H22 are murine monoclonal IgM preparations that bind ECGF and also inhibit the ability of purified ECGF to stimulate [^{3}H]thymidine incorporation into murine lung capillary endothelial cells (*12*). The antibody M63 is a murine monoclonal IgM prepared against the human epidermal growth-factor receptor (*13*). The four monoclonal IgM preparations were assessed for the ability to inhibit the biological activity of crude bovine brain extract to stimulate murine endothelial cell proliferation. The data in Fig. 3 demonstrate that the H9 and H22 monoclonal antibodies to ECGF are capable of inhibiting more than 80 percent of the ECGF biological activity present in crude preparations of brain extract. In contrast, the H18 monoclonal antibody preparation does not inhibit the ability of crude brain extract to stimulate murine capillary endothelial cell proliferation—an observation which suggests that the H18 monoclonal IgM preparation binds to an epitope on ECGF that is not involved in the generation of the mitogenic response. Furthermore, the absence of an inhibitory response with the murine IgM monoclonal antibodies prepared against the epidermal growth-factor receptor is consistent with the suggestion that the inhibition of ECGF activity is not mediated through a nonspecific interaction between the constant region of the

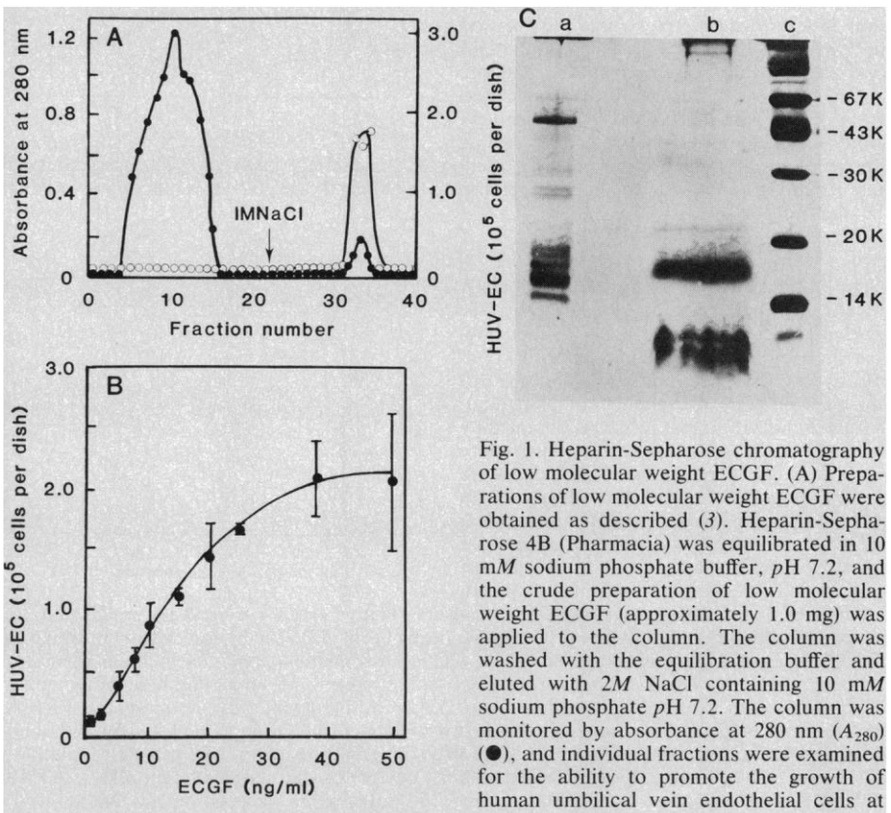

Fig. 1. Heparin-Sepharose chromatography of low molecular weight ECGF. (A) Preparations of low molecular weight ECGF were obtained as described (*3*). Heparin-Sepharose 4B (Pharmacia) was equilibrated in 10 m*M* sodium phosphate buffer, *p*H 7.2, and the crude preparation of low molecular weight ECGF (approximately 1.0 mg) was applied to the column. The column was washed with the equilibration buffer and eluted with 2*M* NaCl containing 10 m*M* sodium phosphate *p*H 7.2. The column was monitored by absorbance at 280 nm (A_{280}) (●), and individual fractions were examined for the ability to promote the growth of human umbilical vein endothelial cells at low cell seed density (○). The biological growth assay was performed as described (*3, 4*). The human umbilical vein endothelial cell seed density was 10^3 cells/cm^2. The A_{280} values are reported as total A_{280} per fraction. (B) Fractions eluted from the heparin-Sepharose column were pooled and dialyzed against 10 m*M* sodium phosphate, *p*H 7.2. The biologically active ECGF pool was titrated to determine the concentration of ECGF which stimulated half-maximum growth of human umbilical vein endothelial cells (HUV-EC). The assay system consisted of human endothelial cells (passage 7) seeded at 10^3 cells per square centimeter on a matrix containing human fibronectin (10 μg/cm^2) in Medium 199 (Gibco) and 10 percent fetal bovine serum (Hyclone). Various concentrations of the heparin-Sepharose preparation of ECGF were added to the culture system. The assay was fed with the appropriate reagents every 2 to 3 days, and human endothelial cells not receiving ECGF supplementation served as controls. The culture dishes were harvested after 10 days and the viable human endothelial cells were quantified with a hemocytometer. The data are reported as means ± standard deviation of triplicate determinations. (C) In an independent run, the heparin-Sepharose column was eluted with 0.5*M* NaCl and 1.0*M* NaCl. These fractions were pooled, dialyzed against 10 m*M* sodium phosphate, *p*H 7.2, and processed for SDS-PAGE (*19*). Samples were subjected to electrophoresis and then silver-stained. (Lane a) Peak eluted from heparin-Sepharose column with 0.5*M* NaCl (360 ng per lane). No ECGF biological activity was present in this sample. (Lane b) Peak eluted from heparin-Sepharose column with 1*M* NaCl (800 ng per lane). (Lane c) Molecular weight protein standards (Pharmacia); the molecular weights of the individual standards are shown on the right.

IgM molecule and ECGF. These results indicate that the major promoter of endothelial cell growth in crude extracts of bovine brain is ECGF. The residual endothelial cell growth–promoting biological activity (less than 20 percent) present in crude brain extracts, which is not inhibited by monoclonal antibodies to ECGF, may be attributed to the presence of other brain-derived mitogens such as FGF (*8*).

The ability of ECGF, an acidic polypeptide, to bind to heparin, an anionic glycosaminoglycan, suggests that the interaction between the carbohydrate and polypeptide is independent of ion exchange. In addition, these results are in contrast to the use of the ion-exchange properties of immobilized heparin to purify cationic growth factors, including the FGF-like growth factor from chondrosarcoma (*14*) and platelet-derived growth factor (*15*). Although the chondrosarcoma-derived growth factor is a potent mitogen for murine 3T3 cells, the cationic polypeptide appears to be a weak endothelial cell mitogen (*14*).

The interaction between ECGF and heparin is of interest since heparin has intrinsic growth regulatory effects on vessel wall–derived cells (*16, 17*) and is a cofactor that potentiates the biological activity of ECGF (*5*). In principle, such an interaction may occur in vivo and, if so, it could have a bearing on the intricate interplay between mast cells and endothelium or to the effects of exogenous heparin administration on angiogenesis (*17, 18*). It is also well established that the biological activity of ECGF (*6*) and related growth factors (*9*) is labile in the presence of extreme variations in temperature and *p*H. It is therefore possible that when heparin binds to ECGF, the carbohydrate stabilizes the tertiary structure of the polypeptide in a configuration that either induces a more mitogenic conformation or renatures biologically inactive ECGF in crude extracts of bovine brain.

There is considerable interest in the use of crude preparations of ECGF for the routine maintenance and cultivation of endothelial cells in vitro for research purposes (*1, 5, 9, 11*). Since ECGF is the primary endothelial mitogen in neutral extracts of bovine brain, and heparin-Sepharose chromatography is a simple and efficient purification procedure, ECGF prepared by heparin-Sepharose chromatography may be a desirable alternative to the use of crude preparations of ECGF for studies utilizing human endothelial cells in vitro.

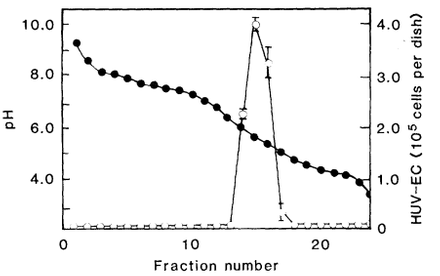

Fig. 2. Isoelectric focusing on heparin-bound ECGF. The preparation of ECGF eluted from the heparin-Sepharose column was dialyzed against distilled water and applied to a Biolyte matrix containing 5 percent Ampholine (*p*H 3 to 10; Bio-Rad) in a flatbed electrophoresis unit as described (*3*). The sample was focused at 7 W for 18 hours at 4°C. The granular bed was divided into 0.5-cm fractions, and the soluble content of the gel slices was eluted from the gel with 0.15*M* NaCl. The fractions were tested for ECGF biological activity as described (*3, 4*), and the *p*H of each fraction was accurately measured. The mean human umbilical vein endothelial cell number per dish (○) and *p*H (●) are given as a function of fraction number.

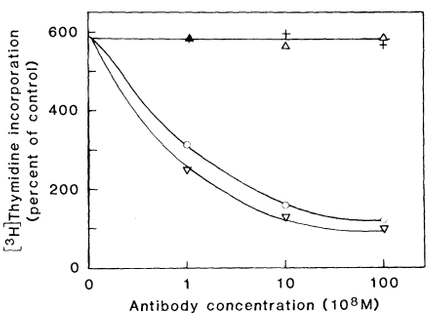

Fig. 3. Inhibition of endothelial cell DNA synthesis by monoclonal antibodies to ECGF. BALB/c mice were each given a subcutaneous (in a footpad injection of 5 μg of ECGF in 10 μl of phosphate-buffered saline (PBS) mixed with 10 μl of Freund's complete adjuvant. The ECGF preparation was obtained by a modification of a purification procedure (*10*). At 2 and 4 weeks later, the mice were each given a booster injection (footpad) 5 μg of ECGF mixed with Freund's incomplete adjuvant. Three weeks later the mice were each bled from the retroorbital plexus and given a final injection (intravenously) containing 2 μg of ECGF. Three days later, the mice were killed, and the splenocytes were fused to P3 myeloma cells with polyethylene glycol as the fusing agent (*17*). The hybrid cells were plated at 5×10^4 cells per well in Costar trays in Dulbecco's minimal essential medium (DMEM) containing 20 percent fetal bovine serum (FBS) and hypoxanthine, aminopterin, and thymidine. Hybridoma cultures secreting antibodies to ECGF were screened by a solid-phase ELISA procedure. Costar ELISA plates were coated with 50 ng of ECGF in PBS at 4°C for 18 hours. Plates were countercoated with a PBS solution containing 1 percent bovine gamma globulin for 2 hours at 37°C. The plates were washed with 0.1 percent bovine serum albumin (BSA) in PBS (PBS-BSA) and incubated at 37°C for 2 hours with 50 μl of hybridoma cell supernatant per well. After extensive washing with PBS-BSA, the plates were incubated at 25°C with affinity-purified peroxidase coupled to rabbit antibody to mouse IgM (Pel-Freez). After being washed with PBS-BSA, the plates were incubated at 25°C for 10 minutes with orthophenylenediamine substrate (1 mg/ml) in citrate buffer at *p*H 4.5 in the presence of 0.03 percent H_2O_2. Absorbance was determined on a Dynatech plate reader at 630 nm. Hybridoma cultures that were positive in the solid-phase ELISA were subcloned three times. Antibodies to IgM were prepared from murine ascitic fluid by 40 percent ammonium sulfate precipitation, extensive dialysis against PBS, and Ultrogel AcA 22 (LKB) chromatography. The H9, H18, and H22 monoclonal antibodies to ECGF do not cross-react with various polypeptide growth factors, including epidermal growth factor, fibroblast growth factor, nerve growth factor, and platelet-derived growth factor (*12*). Furthermore, the H9 and H22 preparations inhibited the ability of purified ECGF, but not FGF, to stimulate DNA synthesis in quiescent cultures of bovine and murine capillary endothelial cells while the H18 antibody preparation had no effect on the mitogenic activity of ECGF (*12*). Murine lung capillary endothelial cells (LE II) were grown to confluency in 48-well Costar culture dishes in DMEM containing 10 percent FBS. The cells were starved for 48 hours in DMEM containing 0.5 percent FBS. A crude preparation of bovine brain ECGF (*6*) was added at 100 μg per milliliter of medium for 18 hours in the presence or absence of increasing concentrations of antibodies H9 (○), H22 (▽), H18 (+), or M63 (△) prior to a 4-hour pulse with [^{3}H-methyl]thymidine (Amersham) (1 μCi/ml). The culture dishes were washed with DMEM, and the radioactivity was measured in a scintillation counter after precipitation with trichloroacetic acid. Results are reported as the percentage of DNA synthesis stimulated by ECGF.

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fractionation with 50 percent and 95 percent (weight to volume) ammonium sulfate. The ammonium sulfate precipitate was resuspended in distilled water and dialyzed against 10 m*M* sodium phosphate buffer, *p*H 7.2. This preparation of ECGF is designated low molecular weight-ECGF and is the starting material for the chromatographic studies with heparin-Sepharose.
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Small DNA Deletions Creating Avirulence in *Streptococcus pyogenes*

Abstract. *The M protein is the antigen on the surface of group A streptococci that allows these bacteria to resist phagocytosis. DNA encoding the M12 protein was cloned into* Escherichia coli *and used as an isotopically labeled hybridization probe to compare genomic DNA's isolated from M^+ and M^- isogenic cultures in an effort to elucidate the genetic basis of this variation. DNA's from two spontaneous, independent M^- variants contained small (approximately 50 base pairs) deletions which were mapped to identical restriction fragments within or adjacent to the M protein coding sequence. Taken together with the pleiotropic nature of these deletions, this suggests that they define a regulatory switch.*

Antigenic variation of microbial virulence has been observed in numerous pathogenic organisms. The group A streptococci (*Streptococcus pyogenes*), responsible for many human infections, have a number of characteristics that exhibit such variation—including the M protein (*1*, *2*), hyaluronic acid capsule (*2*), and streptococcal chemotactic factor inhibitor (SCFI) (*3*). The M proteins, a class of antigenically distinct but functionally similar molecules, are responsible for the ability of these streptococci to resist phagocytosis by human polymorphonuclear leukocytes (*4*–*6*). Nearly 90 different M serotypes are now recognized, although a given strain will generally have only one M protein type (*4*). Two types of variation have been observed: the inability to express an M^+ phenotype and the shift from synthesis of one M protein to a second (antigenically distinct) molecule. The phenotypic loss of M protein has been known since glossy morphological variants were first isolated (*1*) and shown to be M^- and avirulent for mice (*7*). The throats of healthy carriers gradually, over time, yield M^- streptococci (*8*). Strains with new M proteins have arisen in populations experiencing frequent infections, where initially epidemic strains were replaced by other related strains that had antigenically distinct M proteins (*9*). We now show that M^- variant streptococci have acquired a DNA rearrangement responsible for reduced synthesis of M protein by these avirulent forms.

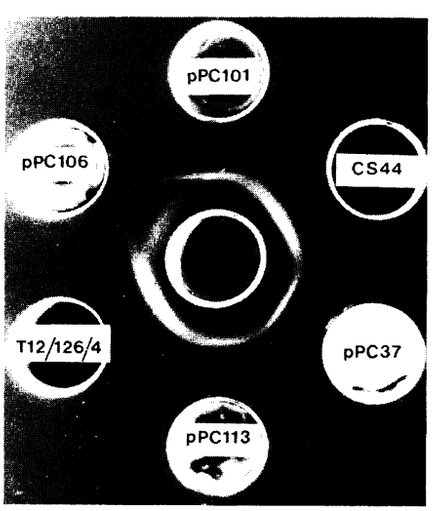

Fig. 1. Immunodiffusion analysis of *E. coli* clones that produce M protein. Center well contains antiserum to M12 prepared by inoculation of rabbits with strain CS44, a whole-cell vaccine (*23*). Peripheral wells contain acid extracts of streptococci prepared as described by Lancefield (*24*) or sonicated extracts of recombinant *E. coli* strains. Cultures of *E. coli* in the late log phase of growth were washed in phosphate-buffered saline (*p*H 7.2) and sonicated until they clarified. Cellular debris was removed by centrifugation (10,000 rev/min, 15 minutes), and the extracts were sterilized by passage through a filter (0.45 μm). The extract wells are labeled with the appropriate streptococcal strain or *E. coli* plasmid designations. Immunodiffusion agar was made as described (*15*); plates were incubated at 25°C for 15 hours.

A genomic library of the group A streptococcal strain CS44 (M12, T12) (*2*) was constructed as follows. Partially digested (Mbo I) bacterial DNA was sized on a 10 to 40 percent sucrose gradient (*10*), and 20- to 30-kilobase (kb) fragments were ligated to the alkaline phosphatase–treated, single Bam HI restriction site of the 12.9-kb cosmid vector pSF5, a derivative of pSF1 (*11*). Recom-