

Inhibition of Endothelial Cell Proliferation by Gamma-Interferon

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Abstract. Endothelial cell growth factor (ECGF) is a potent polypeptide mitogen for endothelial cells and fibroblasts. The mitogenic effects of ECGF are inhibited by the lymphokine gamma-interferon (gamma-IFN) in a dose-dependent manner. Gamma-IFN also induces a unique change in endothelial cell morphology which is maximally expressed in the presence of ECGF. The antiproliferative and phenotypic modulatory effects of gamma-IFN on endothelial cells are reversible. Inhibition of ECGF-induced endothelial cell proliferation by gamma-IFN is accompanied by a

concentration- and time-dependent decrease in binding of ^{125}I -ECGF to the endothelial cell surface. Scatchard analyses of the binding data in the presence and absence of gamma-IFN demonstrate a decrease in the number of ECGF-binding sites rather than a decrease in ligand affinity for the receptor. Cross-linking experiments with disuccinimidyl suberate demonstrate a decrease in the 170,000 M_r cross-linked receptor-ligand complex. These data suggest that gamma-IFN inhibits endothelial cell proliferation by a mechanism which involves growth factor receptor modulation.

THE endothelial cell participates in neovascularization, a phenomenon which includes the formation of new blood vessels in response to a variety of normal and pathological situations (6, 7, 9, 16). Although the mechanisms of angiogenesis are not defined, an important component of the process is the migration and proliferation of the endothelial cell. The stimulation of neovascularization under a rather broad set of circumstances and conditions demonstrates the fundamental importance of this phenomenon to our understanding of human physiology and pathology. Thus, it is important to identify specific modulators of endothelial cell migration and proliferation since this behavior underlies the angiogenic process.

Alpha-endothelial cell growth factor (ECGF)¹ is a member of a family of endothelial cell polypeptide mitogens (27) which presently includes beta-ECGF, acidic fibroblast growth factor (30, 31), eye-derived growth factor II (5), and heparin-binding growth factor I (28). This family of endothelial cell polypeptide mitogens has also been shown to be angiogenic in vivo by the chick chorioallantoic membrane, rabbit corneal neovascularization (28, 31), and hamster cheek pouch assays (Schreiber, A. B., and T. Maciag, unpublished observation). The polypeptide, alpha-ECGF, is mitogenic (19) and chemotactic (29) for human endothelial cells in vitro and

represents the des 1-21 form of beta-ECGF (3). The gene encoding this polypeptide is localized on human chromosome 5 and encodes a polypeptide which does not contain a sequence equivalent to a traditional signal peptide (13). The mitogenic (8, 19, 32) and chemotactic (29) response of alpha-ECGF on endothelial cells is potentiated by the glycosaminoglycan, heparin, which binds the polypeptide mitogen (19, 26) and decreases the apparent K_d for receptor occupancy (26). The mitogenic signal which is transduced across the endothelial cell plasma membrane involves the noncovalent binding of alpha-ECGF to a cell surface-associated polypeptide with an apparent M_r of 150,000 (8). It is also known that ligand occupancy of the ECGF receptor induces down-regulation of the receptor (8, 26), an observation which is consistent with the behavior of other polypeptide mitogens (12).

In this report, we demonstrate that the lymphokine, gamma-interferon (gamma-IFN), inhibits ECGF-induced endothelial cell proliferation with a concomitant change in endothelial cell morphology. In addition, we demonstrate that the effects of gamma-IFN on ECGF-induced proliferation correlate with a decrease in the number of ECGF receptors on the endothelial cell surface.

Materials and Methods

Materials

Human native gamma-IFN (sp act 1.5×10^6 U/mg), human recombinant gamma-IFN (sp act 1.2×10^8 U/mg), and anti-human gamma-IFN mAb's were a gift of Dr. Nava Sarver (Meloy Laboratories, Inc.) and human fibronectin was a gift from Dr. Michael Hrinca (Meloy Laboratories, Inc.). Crude ECGF was prepared as described (17). Highly purified murine

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1. *Abbreviations used in this paper:* ChFBS, charcoal-treated fetal bovine serum; DSS, disuccinimidyl suberate; ECGF, endothelial cell growth factor; EGF, epidermal growth factor; HUVEC, human umbilical vein endothelial cells; IFN, interferon.

gamma-IFN (sp act 6.6×10^6 U/mg) was a gift of Dr. Sidney Grossberg (University of Wisconsin). Recombinant murine gamma-IFN (sp act 1.3×10^7 U/mg) was a gift of Dr. Dvord Samid (Uniformed Services University for Health Sciences). ECGF was prepared as previously described (2). Medium 199 and trypsin-EDTA were purchased from Gibco (Grand Island, NY). FBS was purchased from HyClone Laboratories, Sterile Systems, Inc. (Logan, UT); tissue culture plasticware was from Costar (Cambridge, MA); epidermal growth factor (EGF) was purchased from Collaborative Research, Inc. (Waltham, MA); ^{125}I -EGF was a gift of Dr. Towia Libermann (Biotechnology Research Center); and heparin was from Upjohn Co., (Kalamazoo, MI). Radioisotopes were purchased from New England Nuclear (Boston, MA), and disuccinimidyl suberate (DSS) was from Pierce Chemical Co. (Rockford, IL). All electrophoresis reagents were from British Drug House and other reagents were reagent grade.

Cell Culture

Human umbilical vein endothelial cells (HUVEC) were a gift from Dr. M. Gimbrone (Harvard Medical School, Boston, MA). HUVEC were cultured in Medium 199, 10% (vol/vol) FBS, 100 $\mu\text{g}/\text{ml}$ crude ECGF, and 5 U/ml heparin as previously described (17, 32). HUVEC were used between passages 5 and 11 (1:5 split ratio) for all experiments. Murine lung capillary endothelial cells (LEII) were obtained from Dr. A. Schreiber (Meyo Laboratories, Inc.) and cultured in DME supplemented with 5% (vol/vol) FBS and 5% (vol/vol) charcoal-treated fetal bovine serum (ChFBS).

The HUVEC growth assay was performed as previously described (17). Briefly, HUVEC (2×10^4 cells per well) were plated on 35-mm wells previously coated with 10 $\mu\text{g}/\text{cm}^2$ human fibronectin and incubated for 3 d at 37°C with Medium 199 supplemented with 10% (vol/vol) FBS. The low seed density cultures were incubated with alpha-ECGF, gamma-IFN, and antibodies at the indicated concentrations and combinations for 10–12 d. The biological response modifiers were replaced every 2–3 d during this period. At the indicated intervals, duplicate culture dishes were harvested by treatment with trypsin-EDTA and the number of viable endothelial cells quantitated by counting with a hemocytometer.

The [^3H]thymidine assay was performed using LEII cells as previously described (19). Briefly, LEII cells were seeded into 48-well plates and grown to confluence in 0.5 ml DME, 5% FBS, 5% ChFBS. The cells were starved for 48 h in 0.5 ml DME supplemented with 0.5% ChFBS. Alpha-ECGF, with or without various concentrations of murine native or recombinant gamma-IFN, were added and the cells were incubated at 37°C for 18 h. The LEII cells were pulsed with [^3H]thymidine (0.5 μCi per well) for 4 h, after which the culture dishes were washed with PBS and DNA was precipitated with 10% TCA. TCA-precipitable material was solubilized in 0.1 N NaOH and radioactivity was measured by liquid scintillation counting as described (19).

ECGF Receptor Binding Assays

The binding assays were performed essentially as described (26) with minor changes. LEII cells were seeded into 24-well tissue culture plates and grown to confluence in DME, 5% (vol/vol) FBS, and 5% (vol/vol) ChFBS. The cells were starved in DME, 0.5% ChFBS for 24 h, after which the cells were treated with various concentrations of murine gamma-IFN at 37°C, with or without 6 nM alpha-ECGF for various time intervals. At the end of these treatments, the cells were washed three times with ice cold binding buffer (DME containing 25 mM Hepes and 5 mg/ml BSA, pH 7.4) and incubated at 4°C with 0.2-ml binding buffer containing ^{125}I -alpha-ECGF at a final concentration of 15 ng/ml unless otherwise noted. Incubations proceeded for 2 h at 4°C to allow binding to reach equilibrium after which the medium was aspirated and the cells were washed three times with cold binding buffer. The cell-associated radioactivity was determined by solubilizing the cells in 1.0 ml 0.1 M NaOH. Nonspecific binding was determined by the incubation of ^{125}I -alpha-ECGF with a 10^2 -fold molar excess unlabeled alpha-ECGF, and did not exceed 20% of the total binding. All reported values are corrected for nonspecific binding. Cell numbers were determined by harvesting cells by treatment with trypsin-EDTA solution and quantitated with a hemocytometer. The cell number after confluence did not change significantly over the time interval tested regardless of the addition of ECGF or gamma-IFN.

ECGF was iodinated as previously described (8). Briefly, 2–4 μg of alpha-ECGF (protein concentration determined by amino acid analysis) in 0.1 M sodium phosphate buffer, pH 8.0, received 50 μl of Enzymobead (BioRad Laboratories, Richmond CA) suspension, 0.5 mCi ^{125}I and 20 μl of 2 mg/ml Beta-D-glucose. The reaction was carried out at 22°C for 5 min. The enzymobeads were removed by centrifugation, and free iodine sepa-

rated from iodinated alpha-ECGF by binding and elution of ^{125}I -alpha-ECGF to a 0.5-ml heparin-Sepharose column equilibrated in 50 mM Tris-HCl, 10 mM EDTA, pH 7.3 as described (8). ^{125}I -Alpha-ECGF was eluted off heparin-Sepharose with 1.5 M NaCl containing 50 mM Tris-HCl, pH 7.5, and collected in BSA at a final concentration of 0.1% (wt/vol). ^{125}I -ECGF prepared in this manner possesses a specific activity of $\sim 1\text{--}2 \times 10^5$ cpm/ng, and is >95% precipitable with 10% (wt/vol) TCA. ^{125}I -Alpha-ECGF retains full biological activity as determined by [^3H]thymidine assay using LEII cells (8). Both native alpha-ECGF and ^{125}I -alpha-ECGF give half maximal stimulation of [^3H]thymidine incorporation between 0.5 and 1.0 ng/ml.

^{125}I -EGF binding assays were performed essentially as described for ^{125}I -ECGF binding assays. ^{125}I -EGF (sp act $6\text{--}8 \times 10^4$ cpm/ng) was added to a final saturating concentration of 20 ng/ml. Nonspecific binding was assessed in the presence of 10^2 -fold molar excess unlabeled EGF and did not exceed 10%.

ECGF Receptor Cross-linking Protocol

LEII cells were grown to confluence in 35-mm tissue culture plates and starved overnight in DME, 0.5% ChFBS. The cells were incubated with murine gamma-IFN, alpha-ECGF, or in combination for 20 h. The LEII cells were washed three times in cold binding buffer and incubated with 15 ng/ml ^{125}I -alpha-ECGF in 1.0 ml binding buffer. After 2 h, the incubation medium was aspirated and the cells washed twice with cold binding buffer, and once with cold PBS. The cells were incubated further at 4°C for 20 min with 1 ml of PBS containing 10 μl of 30 mM DSS in DMSO. The cross-linking reaction was quenched with 20 μl of 2.0 M Tris-HCl, pH 8.0, in the culture dish. The cells were scraped from the dish in 1 ml PBS and pelleted at 15,000 g for 10 s. The cell pellet was solubilized in 100 μl of 50 mM Tris, pH 7.3, containing 10 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 200 mM NaCl, 1.0% (wt/vol) Triton X-100 (extraction buffer) at 4°C, and insoluble material was removed by centrifugation at 15,000 g for 10 min at 4°C. The supernatants were subjected to electrophoresis using 7.5% (wt/vol) SDS polyacrylamide slab gels as previously described (14). The gels were fixed, stained, destained, dried, and subjected to autoradiography as previously described (8).

Results

Gamma-Interferon Inhibits Alpha-ECGF-induced Endothelial Cell Proliferation

HUVEC seeded at 2×10^3 cells/cm 2 in 35-mm dishes undergo a 15–20-fold increase in cell number over a 10–12 d period in the presence of 50 ng/ml alpha-ECGF and FBS (Fig. 1 A). ECGF acts synergistically with FBS to promote HUVEC proliferation since neither ECGF nor FBS can independently stimulate HUVEC growth (17). Human native gamma-IFN significantly inhibits alpha-ECGF-induced HUVEC proliferation at a concentration of 10^3 U/ml (Fig. 1 A). The antiproliferative effect is due specifically to gamma-IFN because an anti-human gamma-IFN mAb completely blocked the growth inhibiting activity of gamma-IFN. Experiments using recombinant human gamma-IFN produced similar results (data not shown).

The antiproliferative effects of gamma-IFN on alpha-ECGF-induced HUVEC growth were concentration dependent with maximum inhibition occurring between 10^2 and 10^3 U/ml (data not shown). The effects of gamma-IFN on HUVEC were reversible even at high concentrations of gamma-IFN. Human endothelial cell proliferation that had been inhibited by pretreatment with gamma-IFN for 4 d did resume alpha-ECGF-induced proliferation within 2–3 d after the removal of gamma-IFN from the culture medium (Fig. 2). Furthermore, the antiproliferative effects of gamma-IFN on alpha-ECGF-induced HUVEC growth are partially overcome by the addition of 5 U/ml of heparin (Fig. 1 B). These data demonstrate that gamma-IFN is an an-

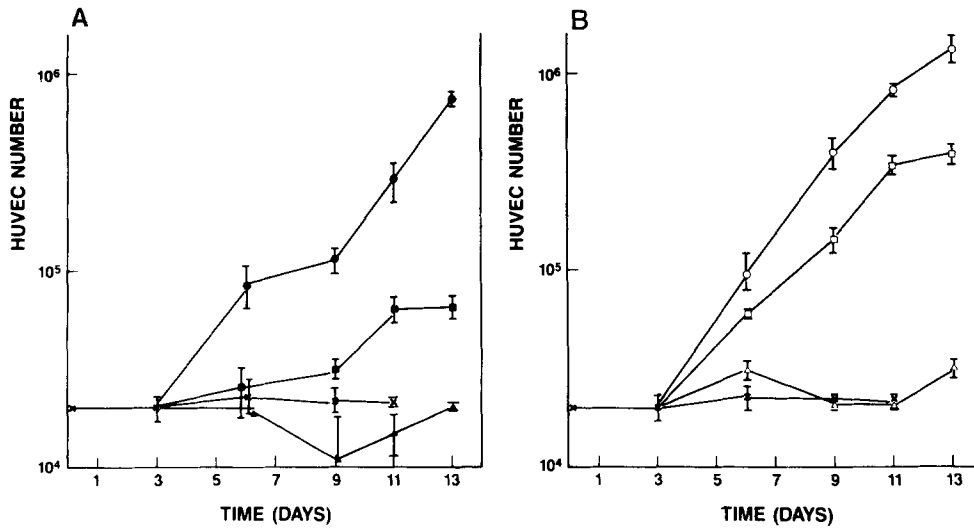


Figure 1. The antiproliferative effects of human gamma-IFN on alpha-ECGF-induced HUVEC growth and its reversal by heparin. HUVEC were seeded as described in Materials and Methods. After 3 d, cells were treated as indicated. (A) Cells received 50 ng/ml ECGF (solid circles) 50 ng/ml ECGF, and 10³ U/ml gamma-IFN (solid squares), 10³ U/ml gamma-IFN (solid triangles), or 10% FBS as a control (x). (B) 50 ng/ml ECGF and 5 U/ml heparin (open circles), 50 ng/ml ECGF, 10³ U/ml gamma-IFN, and 5 U/ml heparin (open squares), 10³ U/ml gamma-IFN, and 5 U/ml heparin (open triangles), or 10% FBS, and 5 U/ml heparin as a control (x). Cells were harvested and quantitated as described. All data are the mean of duplicate determinations ±1 SD.

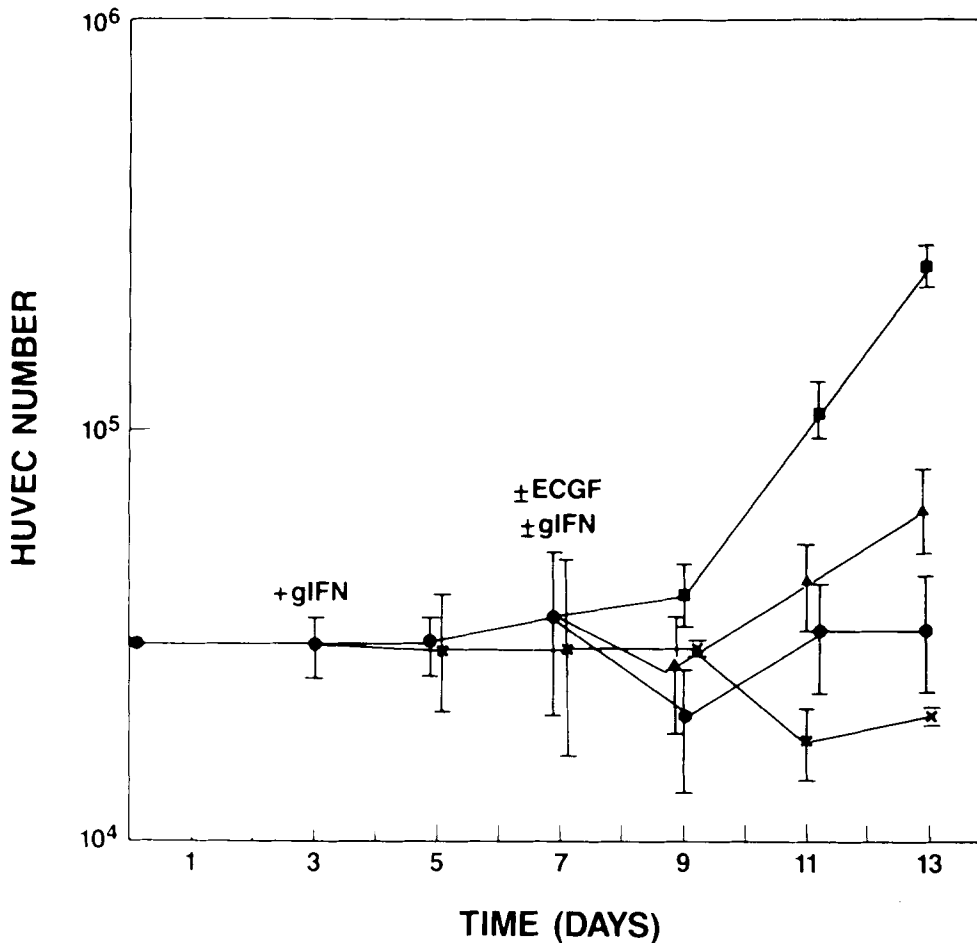


Figure 2. The antiproliferative effects of human gamma-IFN on alpha-ECGF-induced growth are reversible. HUVEC were seeded at 2×10^4 cells per well in 35-mm plates and incubated in Medium 199 and 10% (vol/vol) FBS for 3 d. Cells were then pretreated with 10³ U/ml gamma-IFN for 4 d with gamma-IFN added at 2-d intervals. On day 7, gamma-IFN was removed from the cultures by washing the cell culture dishes with Medium 199 and replaced with 50 ng/ml ECGF (solid squares), 50 ng/ml ECGF and 10³ U/ml gamma-IFN (solid triangles), 10³ U/ml gamma-IFN (solid circles), or a 10% FBS control (x). Cells were refed with these additions for an additional 6 d at 2-d intervals. Cells were harvested and quantitated at the indicated intervals as before. Data are the mean of duplicate determinations ±1 SD.

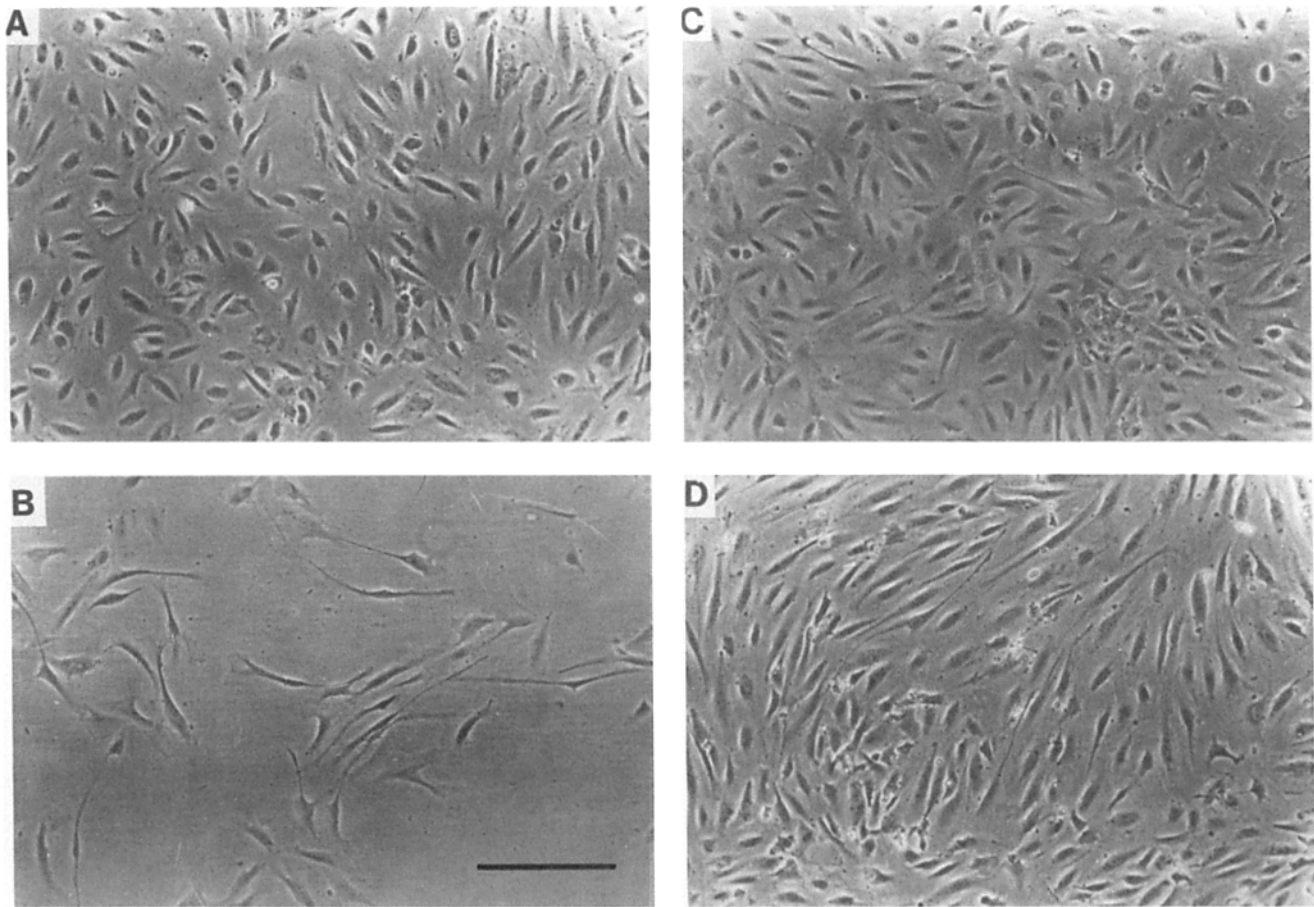


Figure 3. The gamma-IFN-induced HUVEC phenotype. (A) HUVEC seeded at 2×10^4 cells per well in 35-mm dishes (low seed density HUVEC) and grown for 8 d in the presence of 50 ng/ml of ECGF. (B) Low seed density HUVEC grown for 8 d in the presence of 50 ng/ml ECGF and 10^3 U/ml human gamma-IFN. (C) HUVEC grown to confluence, then treated for 2 d with 50 ng/ml alpha-ECGF. (D) HUVEC growth to confluence in 50 ng/ml ECGF, then exposed to 50 ng/ml ECGF plus 10^3 U/ml gamma-IFN for 2 d. Bar, 0.2 mm.

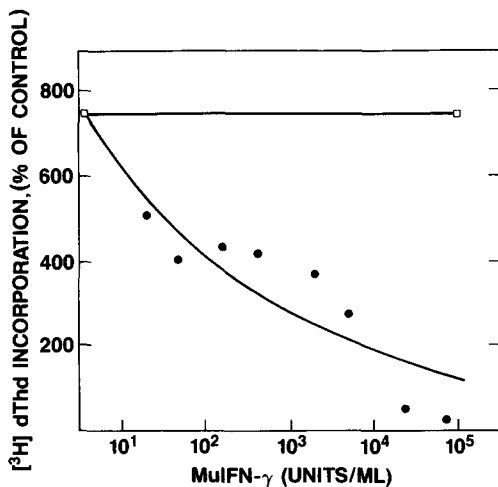


Figure 4. The effects of murine gamma-IFN on [3 H]thymidine (dThd) incorporation by LEII cells. LEII cells were grown to confluence in 48-well plates in DME containing 5% FBS and 5% ChFBS. At confluence, cells were starved for 2 d with DME with 0.5% ChFBS. After 2 d, cells received 20 ng/ml ECGF with (solid circles) or without (open squares) varying concentrations of native murine gamma-IFN. Cells were incubated for 18 h at 37°C. After 18 h, cells received 0.5 μ Ci/well [3 H]thymidine and were in-

tagonist of ECGF-induced HUVEC growth and that the process is reversible.

The Antiproliferative Effect of Gamma-IFN Is Accompanied by a Morphological Alteration of the Human Endothelial Cell Monolayer Phenotype

Human endothelial cells possess a characteristic cobblestone polygonal morphology when propagated in the presence of FBS and ECGF (17, 32). The incubation of gamma-IFN in either low cell density or confluent HUVEC cultures alters the morphology of the human endothelial cell monolayer. Human endothelial cells exposed to gamma-IFN assume an elongated fibroblast-like morphology (Fig. 3, B and D). The gamma-IFN-induced endothelial cell phenotype is apparent after 2 d, and persists for as long as gamma-IFN and alpha-ECGF are present (up to 12 d). Upon removal of gamma-IFN from the human endothelial cell culture and replacement with alpha-ECGF and FBS, the fibroblast-like HUVEC

incubated for an additional 4 h after which the radioactivity incorporated into cellular TCA-precipitable material was determined. The results shown are representative of five experiments performed with either native or recombinant murine gamma-IFN. Data are the mean of duplicate determinations.

phenotype reverts to the normal cobblestone morphology within 2 d (data not shown). These data suggest that the antiproliferative and morphological events may indeed be related.

The Antiproliferative Effects of Gamma-IFN Involve the Inhibition of Alpha-ECGF-induced DNA Synthesis

Murine lung capillary endothelial cells (LEII) incorporate [³H]thymidine into DNA in response to alpha-ECGF in a dose-dependent manner (2, 19). Half-maximum stimulation of [³H]thymidine incorporation occurs between 0.5 and 1.0 ng/ml of alpha-ECGF (2). The addition of murine gamma-IFN inhibits alpha-ECGF-induced [³H]thymidine incorporation by LEII cells (Fig. 4). The inhibitory effects of gamma-IFN is also concentration dependent. LEII cells incubated with a high concentration of alpha-ECGF (20 ng/ml) and varying concentrations of highly purified native murine gamma-IFN exhibit a 50% reduction in [³H]thymidine incorporation with as little as 5×10^2 U/ml. Recombinant murine gamma-IFN gave similar results (data not shown). The decrease in [³H]thymidine incorporation was not due to decreased cell viability since the viable cell number remained constant regardless of treatment. LEII cells pre-treated with gamma-IFN for up to 30 h before the addition of ECGF also showed a marked inhibition of [³H]thymidine incorporation (data not shown), suggesting that the decrease in [³H]thymidine incorporation is due to a decreased responsiveness of endothelial cells to ECGF rather than a delay

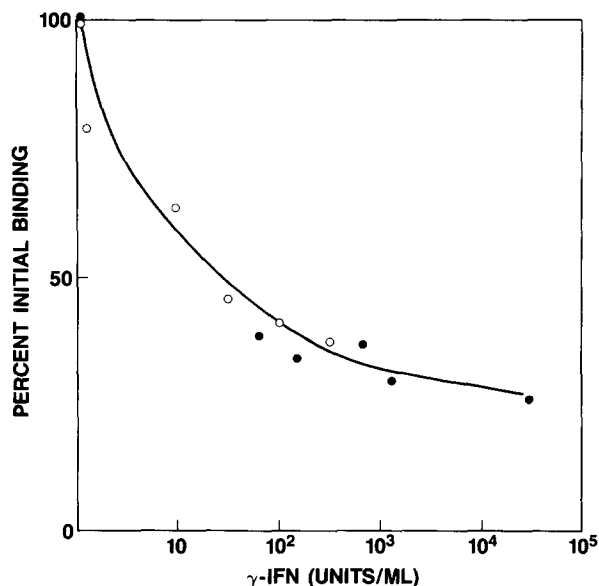


Figure 5. Decreased ¹²⁵I-ECGF binding to Gamma-IFN-treated LEII cells is concentration dependent. LEII cells were grown to confluence in 24-well plates and then starved for 24 h in DME containing 0.5% ChFBS. LEII cells were incubated in the presence of various concentrations of either native (open circles) or recombinant (solid circles) murine gamma-IFN at 37°C for 18 h. After 18 h, cells were washed three times with cold binding buffer, and incubated with 15 ng/ml ¹²⁵I-alpha-ECGF as described under Materials and Methods. All data are corrected for nonspecific binding in the presence of 100-fold excess unlabeled alpha-ECGF. Nonspecific binding did not exceed 15%. Data are the mean of duplicate determinations, and are representative of three experiments. The standard deviation did not exceed 10% of the mean.

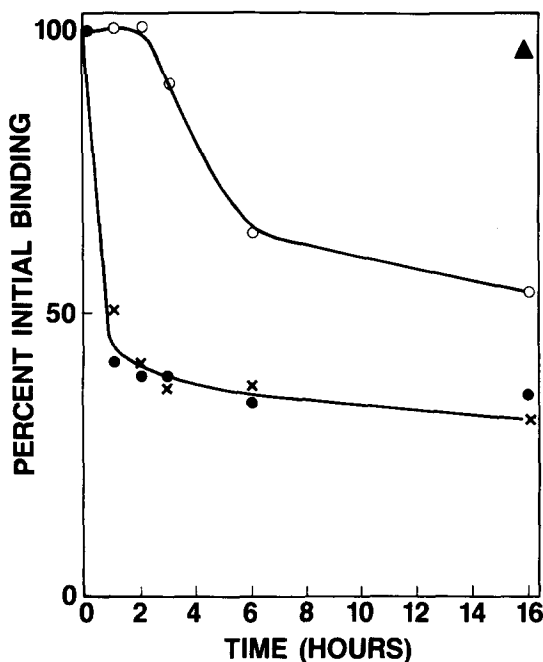


Figure 6. Time course of ¹²⁵I-alpha-ECGF binding activity to LEII cells. LEII cells were grown to confluence in 24-well plates with DME containing 10% ChFBS and incubated for 24 h with DME and 0.5% ChFBS. Cells were treated with 5×10^3 U/ml murine-gamma-IFN (open circles), 100 ng/ml ECGF (x), or both (solid circles) for the indicated time intervals. The cells were washed three times with binding buffer at the end of these incubation periods and ¹²⁵I-alpha-ECGF or ¹²⁵I-EGF binding were performed as described in Materials and Methods. The data are the mean of duplicate determinations and are representative of four experiments. The standard deviation did not exceed 10% of the mean. The solid triangle represents the binding of ¹²⁵I-EGF after 16 h exposure of the endothelial cell population to murine gamma-IFN.

in the mitogenic response. These data are in good agreement with the antiproliferative data obtained with human endothelial cells by measuring viable cell number.

Gamma-IFN Decreases the Binding of ECGF to LEII Cells

Endothelial cells possess a high affinity receptor for ECGF (26, 27) which is rapidly down-regulated by ligand occupancy (8, 26). We examined the ability of LEII cells treated with gamma-IFN at 37°C to bind ¹²⁵I-alpha-ECGF. The binding of ¹²⁵I-alpha-ECGF to gamma-IFN-treated and control LEII cells was carried out at 4°C to minimize the effects of postreceptor binding events (internalization, degradation, etc.) on the quantitation of ¹²⁵I-alpha-ECGF receptor binding. Incubation at 37°C for 20 h with murine gamma-IFN decreased the ability of LEII cells to bind ¹²⁵I-alpha-ECGF at saturating concentrations of ligand (Fig. 5). The effect of gamma-IFN on alpha-ECGF binding to LEII cells was concentration dependent with a 50% reduction in binding occurring at 10–10² U gamma-IFN per ml. This effect was obtained with either native murine gamma-IFN or recombinant murine gamma-IFN. Both preparations were equally potent in their ability to decrease alpha-ECGF binding to LEII cells (Fig. 5). These data correlate with the

inhibition of the gamma-IFN-induced antagonism by monoclonal anti-gamma-IFN and together demonstrate that the effect of gamma-IFN on alpha-ECGF binding is due to gamma-IFN and not a minor contaminant of these preparations.

The effect of gamma-IFN on alpha-ECGF binding to LEII cells was also time dependent (Fig. 6). Decreases in ^{125}I -alpha-ECGF binding at saturation did not begin until at least 3 h after exposure of LEII cells to gamma-IFN at 37°C. Beginning at 3 h post-exposure to gamma-IFN, there was a rapid decline in ^{125}I -alpha-ECGF binding for 3 h and then a more gradual decline for up to 16 h at which time binding decreased by 40–50%. These data contrast with the decrease in binding of ^{125}I -alpha-ECGF to cells pretreated with 100 ng/ml alpha-ECGF. As demonstrated in Fig. 6, down-regulation of the ECGF receptor occurs very rapidly with a 50% decrease in binding apparently within the first hour after exposure to alpha-ECGF. In contrast, the binding of ^{125}I -EGF is not altered after exposure of endothelial cells to gamma-IFN for 16 h (Fig. 6). In addition, gamma-IFN did not affect the ability of EGF to down-regulate its own receptor (data not shown).

Gamma-IFN did not influence the rate or degree of alpha-ECGF-induced receptor down-regulation within the first hour. Upon prolonged incubation, (up to 48 hours) LEII cells treated with gamma-IFN did not significantly recover the ability to bind control levels of ^{125}I -alpha-ECGF (data not shown). In addition, LEII cells treated with both alpha-ECGF and gamma-IFN also did not appreciably recover ^{125}I -alpha-ECGF binding after 48 h of incubation. No effect on ^{125}I -alpha-ECGF binding was observed when LEII cells were exposed to gamma-IFN at 4°C instead of 37°C (data not shown). In contrast, when endothelial cells were treated with alpha-ECGF alone, binding decreased rapidly but returned to control values after 30 h of the initial exposure of alpha-ECGF (data not shown). In addition, gamma-IFN does not compete with ^{125}I -alpha-ECGF for cross-linking to its receptor on LEII cells (8).

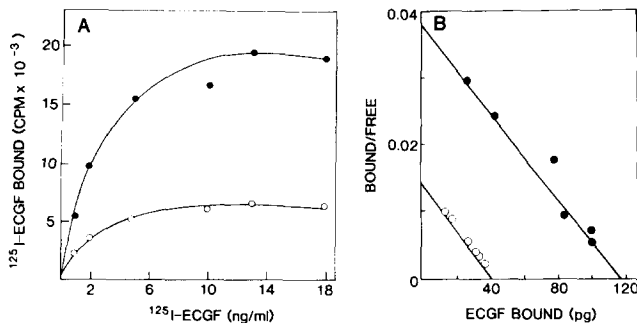


Figure 7. Gamma-IFN decreases ^{125}I -ECGF binding sites on LEII cells. (A) Confluent cultures of LEII cells in 24-well plates were incubated for 24 h in DME and 0.5% ChFBS. Cells received 600 U/ml recombinant gamma-IFN (open circles) or no additions (solid circles) and were further incubated for 18 h at 37°C. The cells were washed three times with binding buffer and incubated at 4°C for 2 h with various concentrations of ^{125}I -alpha-ECGF with or without 100-fold excess unlabeled ECGF. Data are the mean of duplicated determinations from a representative experiment. (B) Scatchard analysis of the binding data with (open circles) or without (solid circles) murine gamma-IFN.

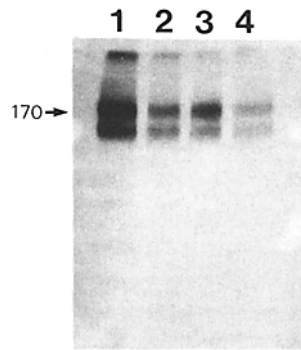


Figure 8. Gamma-IFN induces a decrease in the affinity cross-linking of the ECGF receptor on LEII cells. Confluent monolayers of LEII cells in 35-mm dishes were starved for 24 h as previously described. Cultures received no treatment (lane 1), 500 U/ml recombinant murine gamma-IFN (lane 2), 50 ng/ml ECGF (lane 3), or 500 U/ml IFN and 50 ng/ml ECGF (lane 4). After 20 h at 37°C, cells were washed, cross-

linked, solubilized, and electrophoresed on 7.5% (vol/vol) SDS polyacrylamide gels as described in Materials and Methods. An autoradiogram of the stained, dried gel is shown.

Gamma-IFN Decreases the Number of ECGF Receptors

To determine the basis for the decrease in alpha-ECGF binding to gamma-IFN-treated LEII cells, the concentration dependence of ^{125}I -alpha-ECGF binding to control and gamma-IFN-treated cells was determined (Fig. 7 A). Scatchard analysis of the binding data reveals that gamma-IFN treatment results in a reduction in the total number of available receptors rather than a change in the affinity for the receptor (Fig. 7 B). Treatment of LEII cells with 6×10^2 U/ml gamma-IFN for 20 h at 37°C resulted in a decrease from 2.3×10^4 to 1.0×10^4 high affinity binding sites per cell (Fig. 7 B). Analysis of the binding data demonstrated an absence of a significant change in the affinity of ^{125}I -alpha-ECGF for its receptor. The dissociation constants for control versus gamma-IFN-treated LEII cells were 0.2 nM and 0.25 nM, respectively.

Gamma-IFN Modulates the 150-kD Receptor for Alpha-ECGF

Alpha-ECGF binds to a major M_r 150,000 polypeptide receptor species on the cell surface of HUVEC and LEII cells which possess an apparent M_r of $\sim 170,000$ when measured as cross-linked ligand-receptor complex (8). To determine whether gamma-IFN modulates the ECGF receptor, DSS-mediated cross-linking of bound ^{125}I -alpha-ECGF was performed on LEII cells exposed to either gamma-IFN, ECGF, or both for 20 h. As shown in Fig. 8, the treatment of LEII cells for 20 h with gamma-IFN results in an $\sim 50\%$ reduction in the M_r 170,000 ^{125}I -alpha-ECGF-ECGF receptor cross-linked species. Pretreatment of the LEII cells with 100 ng/ml alpha-ECGF also resulted in a significant decrease in the cross-linked ligand-receptor polypeptide complex. Particularly noteworthy is the fact that the combined treatment of LEII cells with gamma-IFN and ECGF resulted in the greatest decrease in specific ^{125}I -alpha-ECGF cross-linking (Fig. 8). Also of interest is the presence of a minor M_r 150,000 polypeptide ^{125}I -ECGF cross-linked species which has been observed previously (8). This polypeptide may result either from proteolysis of the larger M_r 170,000 ligand-receptor complex or may indeed represent a second receptor (8). No other components were specifically labeled by ^{125}I -alpha-ECGF on either control or gamma-IFN-

treated cultures. These data suggest that the M_r 150,000 ECGF receptor polypeptide is a target for modulation by gamma-IFN on LEII cells.

Discussion

Gamma-IFN is a potent antiproliferative agent for normal and transformed cells in vitro (24, 33). In an attempt to understand the role of ECGF as a promoter of angiogenesis and elucidate its mechanism of action, we have investigated the role of gamma-interferon as an antagonist of ECGF-induced mitogenic activity in vitro. Gamma-IFN is shown to significantly inhibit ECGF-induced proliferation of HUVEC even at high concentrations of the polypeptide mitogen. In addition, gamma-IFN also inhibits the incorporation of [3 H]-thymidine into DNA from ECGF-stimulated murine lung capillary endothelial cells in vitro. We attribute the antiproliferative properties of gamma-IFN on ECGF-induced mitogenesis specifically to gamma-IFN since mAb's to gamma-IFN block the antagonism, and recombinant gamma-IFN had the same effect.

The antiproliferative effects observed with gamma-IFN on endothelial cells in vitro persists for as long as gamma-IFN is present, and upon its removal, the endothelial cells again become responsive to ECGF. The effects of gamma-IFN on ECGF-induced HUVEC proliferation could be partially overcome by the addition of 5 U/ml of heparin. Although the mechanism of glycosaminoglycan reversion is not known, gamma-IFN, like ECGF, does bind to heparin (1). Thus, further experiments on the structural interaction between gamma-IFN and heparin are required since it is not clear whether the same glycosaminoglycan in the heparin preparation is responsible for the structural interaction between lymphokine and the growth factor.

Recently, gamma-IFN has been shown to have a variety of biological effects on human endothelial cells. Gamma-IFN has been demonstrated to (a) induce elevated levels of major histocompatibility antigens on endothelial cells (4, 23), (b) modulate the expression of a unique extracellular matrix (20), and (c) induce a unique morphological endothelial cell phenotype in vitro (22). Here, we confirm the observation that gamma-IFN alters the endothelial cell monolayer phenotype, by inducing an elongated fibroblast-like morphology which contrasts with the normal "cobblestone" appearance of the endothelial cell in vitro. The gamma-IFN-induced phenotype is reversible upon removal of gamma-IFN from the culture medium. It is of interest to note that the gamma-IFN-induced phenotype resembles the Stage I phenotype which human endothelial cells assume before the formation of the organized or nonterminal-differentiated phenotype in vitro (7, 16, 18, 21). This correlation between phenotypes may be significant since the differentiated phenotype and the gamma-IFN-induced phenotype are nonproliferative and both phenotypes are refractory to the mitogenic attributes of ECGF (16, 18). These observations argue that some stages in neovascularization may not involve the proliferation of endothelial cells, but rather involve endothelial cell migration and organization.

The exposure of endothelial cells to low concentrations of gamma-IFN results in an inhibition of alpha-ECGF-induced endothelial cell proliferation and [3 H]thymidine incorpora-

tion. The inhibition of ECGF-induced endothelial cell proliferation correlates with a decrease in the number of ECGF receptors present on the surface of the endothelial cell with no significant change in receptor affinity. Gamma-IFN also modulates the M_r 150,000 ECGF receptor polypeptide which is present on the endothelial cell surface (8). The exposure of the endothelial cell to gamma-IFN results in an ~50% decrease in the amount of the cross-linked receptor-ligand complex. The decrease in 125 I-ECGF binding and cross-linking to endothelial cells upon exposure to gamma-IFN is not due to a general inhibition of protein synthesis since endothelial cells exposed to gamma-IFN have increased levels of protein synthesis above those for control quiescent endothelial cells (Friesel, R., and T. Maciag, unpublished observations). The correlation between the modulation of the ECGF receptor on the endothelial cell surface by gamma-IFN in a concentration-, time-, and temperature-dependent manner with the kinetics of the gamma-IFN-induced antagonism confirms our initial observation which demonstrated that the presence of the ECGF receptor on the endothelial cell surface is essential for the stimulation of endothelial cell proliferation (26).

Several lines of evidence demonstrate that the modulation of the ECGF receptor by gamma-IFN does not result from a direct interaction of gamma-IFN with the ECGF receptor. First, we have demonstrated previously that gamma-IFN cannot compete either for 125 I-alpha-ECGF binding or cross-linking to the M_r 150,000 ECGF receptor (8). Other studies (25, 35) have demonstrated that murine gamma-IFN and human gamma-IFN bind to unique and specific high affinity membrane receptors (25, 35). Furthermore, affinity cross-linking of 125 I-gamma-IFN to sensitive cell lines demonstrate that the gamma-IFN cross-linked receptor complex possesses an M_r of ~110,000 on both human and murine cells (25, 35) and is therefore, distinct from the M_r 150,000 ECGF receptor polypeptide (8). Finally, the kinetics of ECGF receptor down-regulation by gamma-IFN in the absence of ECGF is unique. ECGF-induced receptor down-regulation results in a 50% reduction in 125 I-ECGF binding within the first hour after the addition of ECGF, whereas gamma-IFN treatment requires 16–20 h to yield a 50% reduction in 125 I-ECGF binding. Because the mechanism of gamma-IFN-induced down-regulation of the ECGF receptor does not involve the interaction of gamma-IFN with the ECGF receptor, it is reasonable to suggest that the mechanism may involve gamma-IFN-induced signal transduction mediated by the gamma-IFN receptor and directed, in part, toward uncoupling the mitogenic signal induced by ECGF. This is consistent with the kinetics of the down-regulation of the transferrin receptor on mouse peritoneal macrophages by gamma-IFN which involves the activation of protein kinase C (10, 34). Although it is not known whether the antagonist effects initiated by gamma-IFN on endothelial cells involve protein kinase C, it is of interest to note that phorbol esters, which are potent stimulators of intracellular protein kinase C, (a) inhibit endothelial cell proliferation, (b) induce an endothelial cell phenotype in vitro which is similar to the gamma-IFN-induced phenotype (Friesel, R., R. Lyall, and T. Maciag, unpublished observations), and (c) stimulate the organizational behavior of endothelial cells in vitro (21).

It is difficult to eliminate the possibility that the effect of

gamma-IFN as an antagonist of endothelial cell proliferation also involves the down-regulation of other growth factor receptors present on the endothelial cell surface. However, in this regard, we have not observed any significant alteration in the binding of ¹²⁵I-EGF to endothelial cells after treatment with gamma-IFN. Similarly, beta-TGF, a potent inhibitor of mink lung epithelial cell proliferation, has recently been shown to exert an antiproliferative effect on these cells without an alteration of EGF receptor binding or phosphorylation (15). In addition, beta-TGF has recently been shown to inhibit endothelial cell proliferation in vitro (11). Therefore, it appears that endothelial cell proliferation is tightly regulated by antiproliferative factors that exert their effects on cells at various levels which may include the modulation of growth factor receptors.

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