Human Endothelial Cells are Chemotactic to Endothelial Cell Growth Factor and Heparin

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ABSTRACT The response of human endothelial cell migration to various extracellular matrix components and growth factors has been assessed. Human endothelial cells demonstrate increased chemotaxis and chemokinesis when placed in a modified Boyden chamber with endothelial cell growth factor (ECGF) used at a concentration of 10^{-9} M. Anti-ECGF antibody inhibits the chemotactic response. Heparin (10^{-8} to 10^{-10} M) was also chemotactic and was shown to potentiate the chemotactic activity of ECGF. Although laminin, fibronectin, the polypeptide (epidermal, fibroblast, and nerve) growth factors, and collagen types I, II, III, IV, and V demonstrate a chemotactic response, these activities were one third to one half less than observed with ECGF. These data suggest that ECGF and heparin may play a significant role as response modifiers of human endothelial cell migration which may be relevant to tumor metastasis, wound healing, and atherogenesis.

Chemotactic behavior is a property of a variety of cell types engaged in biological processes including inflammation, wound repair, organ development, neurite outgrowth, and tumor invasion (29). Factors that modify cellular chemotaxis are also important as modulators of cellular growth and differentiation (17, 26, 28).[1] Extracellular matrix proteins such as fibronectin and laminin have been shown to stimulate mammalian cell motility in a variety of cell types (1, 25, 28, 39)[2] and may play a prominent role in the process of differentiation (1, 3, 12, 15, 16, 22, 27, 36, 40, 41).[1] Endothelial cell growth factor (ECGF)[3], the principle polypeptide mitogen for human endothelial cells in vitro (20), has been implicated in neovascularization (19, 30). In addition, it has been demonstrated that the glycosaminoglycan, heparin, is a modulator of the biological activity of ECGF (30, 37). Specifically, heparin increases the activity of the polypeptide mitogen by a mechanism involving ECGF receptor occupancy (30). Furthermore, mast cell heparin has been shown to stimulate migration of bovine capillary endothelial cells but not endothelium from bovine aorta (4). Since heparin in combination with ECGF promotes cell growth (20, 30, 37) and mediates alterations in endothelial cell phenotype (37), we examined their chemotactic properties. We report here that ECGF and heparin are indeed chemotactic for human umbilical vein endothelial cells.

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[3] *Abbreviation used in this paper:* ECGF, endothelial cell growth factor.

MATERIALS AND METHODS

Human Endothelial Cell Cultures: Human endothelial cells derived from the umbilical vein were propagated as previously described (21). In all cases, early passage cells were used (equal to or less than 25 cumulative population doublings). The cells used for the chemotaxis assays were removed from the cell culture dishes by incubation in a divalent, cation-free, balanced salt solution containing 0.1% EDTA, 0.1% EGTA in 25 mM Hepes, 10 mM Na_2HCO_3, 6 mM K_2HPO_4, 100 mM NaCl and 60 mM mannitol, pH 7.4.

Chemotaxis Assay: Chemotaxis was assayed in the modified Boyden chamber as previously described (29). Briefly, human endothelial cells were suspended at a concentration of 2.5×10^5 cells/ml in Medium 199 containing 200 μg/ml bovine serum albumin and placed in the upper compartment of the Boyden chamber. The lower compartment, which contained the chemoattractant, was separated from the upper compartment by a nuclepore filter (Neuro Probe, Inc., Cabin John, MD; pore diameter, 8 μm). After a 4-h incubation at 37°C, filters were removed, washed, stained with hematoxylin, and mounted bottom side up on glass slides. Ten high power fields (× 400) were counted to determine the number of cells that had migrated entirely across the 100-μm width of the filter. Negative controls were the number of cells that had migrated in the absence of an attractant. In all cases, the SEM did not exceed 10%.

Preparation of Substrates and Antibodies: Type I collagen was prepared from lathyritic rat skin (5); type II collagen from a rat chondrosarcoma (35); type III collagen from fetal calf skin (9); type IV collagen from the EHS tumor (18); and type V collagen from human placenta (6). ECGF was prepared

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as previously described (7, 21). Briefly, bovine brains were extracted at neutral pH in a low ionic strength buffer. The extract was next subjected to acid extraction and a series of ammonium sulfate precipitations. The resulting material was further purified by heparin-Sepharose affinity chromatography followed by reversed-phase high performance liquid chromatography. This material yields a single band on SDS PAGE, a single UV absorbing peak on reversed-phase high performance liquid chromatography, and a single NH_2-terminal residue as determined by automated Edman degradation. Laminin and fibronectin were purified as previously described (14, 18, 38), and antibodies to laminin and fibronectin were raised in rabbits (10). Their specificities were confirmed by immunoprecipitation and Western blot analysis. Antibody to purified ECGF was prepared as previously described (20). The polypeptide (epidermal, fibroblast, and nerve) growth factors were purchased from Collaborative Research, Inc. (Lexington, MA). Insulin was obtained from Sigma Chemical Co. (St. Louis, MO). Heparin was obtained from Armour Pharmaceutical Co. (Kankehee, IL).

RESULTS

ECGF is a Chemoattractant for Human Endothelial Cells

When human endothelial cells were placed in the upper compartment of a modified Boyden chamber at a density of 2×10^5 cells per chamber (42), we observed that ECGF and heparin, alone or in combination, stimulated human endothelial cell migration (Fig. 1). The optimal concentration of ECGF for directed cell movement was 10^{-9} M. Heparin also stimulated human endothelial cell migration at 10^{-9} M. The addition of both ECGF and heparin increased the chemotactic response of the cells (Fig. 1) relative to either the heparin or ECGF response. Although laminin and fibronectin stimulated endothelial cell migration at 10^{-9} M, this response was only 30 to 40% of the chemotactic response observed with ECGF and heparin at 10^{-9} M (Table I). In some cases ECGF, heparin, and collagen types III, IV, and V at increased concentrations revealed a decline in chemotaxis (Table I, Fig. 1). This decrease in response to higher concentrations of chemoattractants is commonly observed in assays of this type and is generally ascribed to either receptor desensitization or the possibility of a gradient breakdown (29).

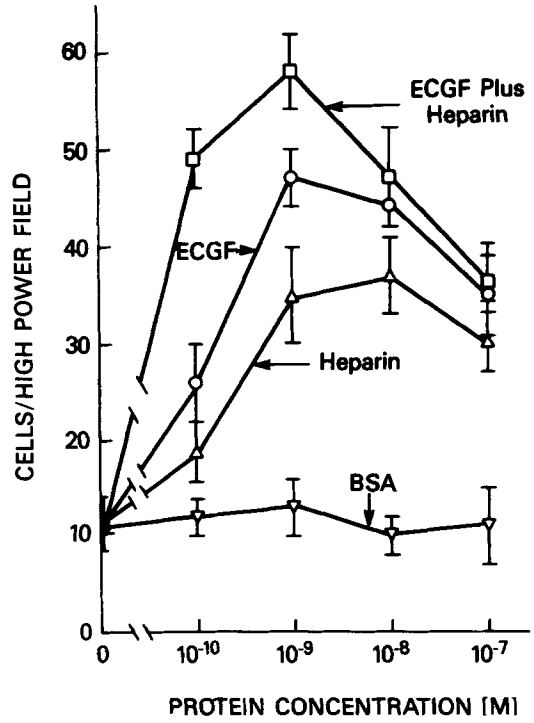

FIGURE 1 Chemotactic response of human endothelial cells to ECGF and heparin. Chemotaxis was assayed in the modified Boyden chamber. After a 4-h incubation at 37°C in a 100% humidified chamber containing 5% CO_2, the filters were removed, fixed in 100% methanol, and stained with hematoxylin and eosin. Because of the heterogeneous nature of heparin preparations, we assumed an average molecular mass of 2×10^4 daltons. Molar concentrations for heparin are based on this assumption. The data are expressed as the average number of migrated cells per high power field (× 400) ± one standard deviation. Each assay was performed in triplicate. The number of migrated cells per filter did not differ by more than 10%.

TABLE I. *Chemotactic Response of Human Endothelial Cells to Biological Response Modifiers*

Polypeptide or Glycoprotein	Number of cells/high power field		
	10^{-10} M	10^{-9} M	10^{-8} M
BSA	12 ± 5	12 ± 2	10 ± 4
EGF	20 ± 4	24 ± 3	20 ± 5
FGF	13 ± 5	15 ± 5	14 ± 5
NGF	11 ± 3	12 ± 3	13 ± 6
Insulin	10 ± 2	10 ± 1	8 ± 2
Collagen I	18 ± 4	19 ± 2	22 ± 3
Collagen II	12 ± 4	13 ± 3	11 ± 4
Collagen III	25 ± 6	29 ± 4	20 ± 4
Collagen IV	25 ± 5	21 ± 3	22 ± 5
Collagen V	25 ± 6	27 ± 5	18 ± 5
Laminin	20 ± 4	22 ± 4	25 ± 5
Fibronectin	21 ± 5	23 ± 4	28 ± 6

Chemotactic assays were performed in triplicate as described in Materials and Methods. All factors were examined at concentrations between 10^{-8} and 10^{-10} M.

The concentration of ECGF in the upper and lower wells of the Boyden chamber was varied in a systematic fashion to distinguish chemotaxis from chemokinesis and also to distinguish positive from negative chemotaxis (42). We observed an increase in endothelial cell chemokinetic motility as a function of the concentration of ECGF (Fig. 2). We also observed a substantial chemotactic activity. The number of cells that had migrated in positive gradients was substantially greater than those that migrated in negative gradients. These data demonstrate that ECGF induces a true chemotactic response in human endothelial cells. An additional checkerboard assay was performed using heparin as the chemoattractant. In this experiment we observed positive chemotaxis and positive chemokinesis. No movement of human endothelial cells was noted when a negative gradient was established (Fig. 3).

Antibodies Against ECGF Inhibit ECGF-induced Chemotaxis

Human endothelial cells treated with an antibody directed against ECGF, which inhibits ECGF-induced endothelial cell proliferation and ECGF receptor occupancy (20), showed marked inhibition of the chemotactic response to ECGF. Antibodies against laminin (data not shown) or fibronectin had no effect on ECGF-induced human endothelial cell migration (Fig. 4). In a series of separate experiments, human endothelial cells were assayed for their chemotactic response to the various antibodies. We observed no movement of cells to either anti-ECGF, anti-laminin, or anti-fibronectin in the absence of ECGF (Fig. 5). In addition, when ECGF was added to the lower compartment, a maximal response was observed

ECGF CONCENTRATION (M) IN LOWER CHAMBER	ECGF CONCENTRATION (M) IN UPPER CHAMBER				
	0	10^{-10}	10^{-9}	10^{-8}	10^{-7}
0	12 ± 4	10 ± 6	9 ± 8	13 ± 7	12 ± 4
10^{-10}	26 ± 9	18 ± 4	19 ± 10	9 ± 5	17 ± 9
10^{-9}	59 ± 12	51 ± 8	30 ± 6	21 ± 6	17 ± 4
10^{-8}	49 ± 13	45 ± 12	36 ± 7	37 ± 5	18 ± 4
10^{-7}	31 ± 7	44 ± 14	33 ± 15	48 ± 11	17 ± 6

FIGURE 2 Checkerboard analysis of the motile response of human endothelial cells to ECGF. Chemotaxis assays are performed as described in Materials and Methods. Each assay was performed in triplicate. Numbers within the inner box express the number of migrated cells per high power field (× 400). The concentration of ECGF was varied in the upper and lower chambers as indicated. The response of human endothelial cells to the absence of a gradient (chemokinesis) are shown on the diagonal and to a negative gradient above the diagonal. Response to a positive gradient (chemotaxis) is shown below the diagonal.

HEPARIN CONCENTRATION (M) IN LOWER CHAMBER	HEPARIN CONCENTRATION (M) IN UPPER CHAMBER				
	0	10^{-10}	10^{-9}	10^{-8}	10^{-7}
0	11 ± 3	10 ± 3	9 ± 2	12 ± 1	11 ± 3
10^{-10}	19 ± 5	15 ± 6	11 ± 4	8 ± 2	10 ± 3
10^{-9}	35 ± 4	16 ± 4	20 ± 4	10 ± 2	12 ± 3
10^{-8}	36 ± 7	21 ± 5	20 ± 3	25 ± 4	13 ± 5
10^{-7}	30 ± 5	25 ± 4	24 ± 4	23 ± 6	27 ± 5

FIGURE 3 Checkerboard analysis of the motile response of human endothelial cells to heparin. Chemotaxis assays are performed as described in Materials and Methods. Each assay was performed in triplicate. Numbers within the inner box express the number of migrated cells per high power field (× 400). Heparin concentrations are varied as indicated. The response of human endothelial cells to the absence of a gradient (chemokinesis) are shown on the diagonal and to a negative gradient above the diagonal. Response to a positive gradient (chemotaxis) is shown below the diagonal.

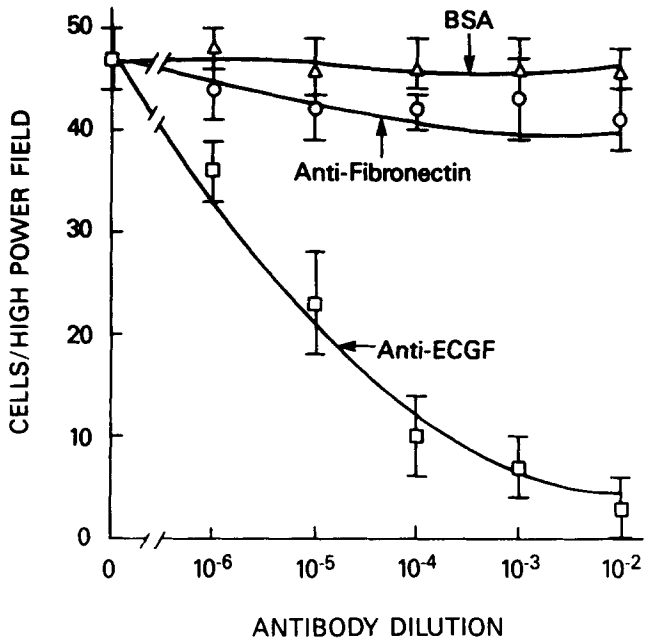

FIGURE 4 Inhibition of ECGF-induced endothelial cell chemotaxis. Chemotactic response of human endothelial cells to ECGF was measured in the presence and absence of antibodies. Anti-fibronectin (initial concentration of 100 μg/ml protein) and anti-ECGF (initial concentration of 100 μg/ml protein (20) were incubated with human endothelial cells in the upper compartment of the Boyden chamber. ECGF (10^{-9} M) was used as the chemoattractant in the lower well of the Boyden chamber. The chemotaxis assay was performed as described in Materials and Methods.

(Fig. 5). Furthermore, anti-ECGF antibody did not inhibit endothelial cell movement to either laminin or fibronectin (data not shown). These studies demonstrate the specificity of ECGF-induced endothelial cell migration and further suggest that the inhibition of cell motility by anti-ECGF was not due to a nonspecific antibody interaction with human endothelial cells.

Chemotactic Activity of Other Biological Response Modifiers on Human Endothelial Cells

Additional factors which, in general, modulate mammalian cell proliferation and differentiation were examined for their chemotactic activity with human endothelial cells. These included epidermal growth factor, basic pI-fibroblast growth factor, nerve growth factor, insulin, and collagen types I, II, III, IV, and V. The various factors were examined for chemotactic activity in the modified Boyden chamber at concentrations of 10^{-8}, 10^{-9}, and 10^{-10} M; the results obtained are shown in Table I. Collagen types I, III, IV, and V and EGF were capable of stimulating one third to one half maximal stimulatory capacity as compared to ECGF. Collagen type II was without effect in chemotactic stimulation. Both fibroblast and nerve growth factor were also without effect. These data

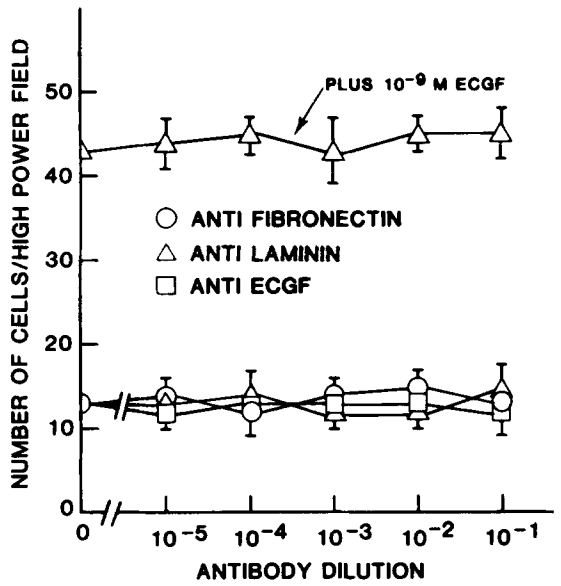

FIGURE 5 Antibodies against fibronectin, laminin, and ECGF are not chemotactic for human endothelial cells. The chemotactic response of human endothelial cells to anti-fibronectin, anti-laminin, and anti-ECGF (all initial protein concentrations adjusted to 100 μg/ml protein) was measured. Anti-laminin plus ECGF at 10^{-9} M was additionally tested. Assays were performed as described in Materials and Methods. All assays were performed in triplicate.

TABLE II. *Chemotactic Response of Human Endothelial Cells to Various Biological Response Modifiers with and without 10^{-9} M Heparin*

Polypeptide or Glycoprotein	Number of cells/high power field	
	+ Heparin	− Heparin
EGF	26 ± 3	25 ± 4
FGF	14 ± 3	14 ± 4
Laminin	25 ± 3	22 ± 2
Fibronectin	25 ± 2	23 ± 3

Endothelial cell chemotaxis was assayed using 10^{-9} M polypeptide or glycoprotein with and without the addition of 10^{-9} M heparin. Results are the mean of four experiments plus and minus one standard deviation.

demonstrate that ECGF is a more potent chemoattractant for human endothelial cells in vitro relative to other known chemoattractants. In addition, we tested the ability of heparin in combination with epidermal growth factor, fibroblast growth factor, laminin, and fibronectin to stimulate directed movement of endothelial cells. We observed no increase (or decrease) in chemotactic responsiveness when heparin (at 10^{-9} M) was included (Table II).

DISCUSSION

These data demonstrate that human endothelial cells exhibit a chemotactic response to both ECGF and heparin. Since the chemotactic activity of ECGF is potentiated by heparin at suboptimal concentrations, these data suggest an interaction between the polypeptide and the glycosaminoglycan. This proposed interaction is consistent with the ability of heparin to bind ECGF (20) and modulate the mitogen activity of the polypeptide (30, 37). Previous studies have demonstrated that the modulation by heparin occurs at the receptor level (30). The interaction between ECGF and heparin increases the dissociation constant for ECGF receptor occupancy and may involve heparin-induced changes in the conformation of the polypeptide mitogen (30). The demonstration of heparin and ECGF as chemoattractants for endothelial cells further extends these observations and suggests that these components may be important in the development of blood vessels.

Other biological response modifiers have been shown to be chemotactic for various cell types. For example, the extracellular matrix glycoproteins, laminin and fibronectin, are known chemoattractants. Murine melanoma cells, Schwannoma cells, and neutrophils will move towards laminin (24, 25, 34),[2] while fibronectin stimulates directed migration of Schwann cells, endothelial cells, fibroblasts, and certain tumor cells (8, 11, 24, 33, 39, 41). Furthermore, fibroblasts as well as smooth muscle cells move to platelet-derived growth factor (13, 32). In addition to eliciting a chemotactic response, platelet-derived growth factor is well recognized as a potent stimulator of mesenchymal cell proliferation (31). Additionally, mast cell heparin has been shown to stimulate random movement of bovine capillary endothelium (4). In these studies, bovine aortic endothelium did not move to any factors.

Endothelial cells are capable of organization which results in the formation of three-dimensional tubular structures in vitro (19). The generation of the differentiated endothelial cell phenotype in vitro can be accelerated by extracellular matrix components (19, 23), and involves endothelial cell migration during the early stage of the organizational process (15, 22, 30). Since extracellular cell culture environments which limit endothelial cell proliferation augment the process of endothelial cell differentiation (15, 22, 23, 30), it is not clear whether the chemotactic properties of ECGF participate in the organizational pathway in vitro. However, Senior et al., have demonstrated that the chemotactic and proliferative attributes of platelet-derived growth factor can be separated (31). Therefore, we suggest that the chemotactic and proliferative properties of ECGF may also be distinct.

Neovascularization, which occurs during wound healing as well as in tumor growth, is initiated by a variety of biological effectors which cause the induction of endothelial cell migration (19, 22). This multitude of diverse biochemical stimuli reveals an apparent generalized, rather than specific, response of the endothelial cell and supports a model for angiogenesis which assumes a number of points of entry and control (19). This is especially evident from the chemotactic activity noted for many of the biological response modifiers (Table I) which would be localized at sites of endothelial cell migration. Our data suggest that ECGF and heparin play a specific role in the control of human endothelial cell migration and may ultimately be involved in the cascade of biochemical events which control neovascularization.

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