Hepatocyte Growth Factor Is a Potent Angiogenic Factor Which Stimulates Endothelial Cell Motility and Growth

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Abstract. Hepatocyte Growth Factor (HGF, also known as Scatter Factor) is a powerful mitogen or motility factor in different cells, acting through the tyrosine kinase receptor encoded by the *MET* proto-oncogene. Endothelial cells express the *MET* gene and expose at the cell surface the mature protein (p190^{MET}) made of a 50 kD (α) subunit disulfide linked to a 145-kD (β) subunit. HGF binding to endothelial cells identifies two sites with different affinities. The higher affinity binding site (K_d = 0.35 nM) corresponds to the p190^{MET} receptor. Sub-nanomolar concentrations of HGF, but not of a recombinant inactive precursor, stimulate the receptor kinase activity, cell proliferation and motility. HGF induces repairs of a wound in endothelial cell monolayer. HGF stimulates the scatter of endothelial cells grown on three-dimensional collagen gels, inducing an elongated phenotype. In the rabbit cornea, highly purified HGF promotes neovascularization at sub-nanomolar concentrations. HGF lacks activities related to hemostasis-thrombosis, inflammation and endothelial cells accessory functions. These data show that HGF is an in vivo potent angiogenic factor and in vitro induces endothelial cells to proliferate and migrate.

HEPATOCYTE growth factor (HGF)[1] and scatter factor were originally identified as distinct cytokines that promote, respectively, the growth or motility of epithelial cells. HGF was identified in the serum of partially hepatectomized rats as a potent mitogen for cultured rat hepatocytes (Nakamura et al., 1984; Michalopoulos et al., 1984). It has later been isolated from rat platelets (Nakamura et al., 1986), human plasma (Gohda et al., 1988), human serum (Zarnegar and Michalopoulos, 1989), and rat liver (Asami et al., 1991). The HGF cDNA has been cloned and sequenced (Miyazawa et al., 1989; Nakamura et al., 1989; Tashiro et al., 1990; Rubin et al., 1991). Scatter factor was originally described as a secretory product of fibroblasts that dissociates normal and malignant epithelial cells in vitro, increasing their motility and invasiveness (Stoker et al., 1987; Gherardi et al., 1989; Weidner et al., 1990). Recently, sequence analysis (Gherardi and Stoker, 1990) and cDNA cloning from fibroblasts, placenta, and liver showed that HGF and scatter factor are identical molecules (Weidner et al., 1991; Naldini et al., 1991*a*). HGF is a disulfide-linked heterodimer of a 55–65 kD (α) and a 32–36 kD (β) subunits (Nakamura et al., 1987; Godha et al., 1988; Zarnegar and Michalopoulos, 1989; Gherardi et al., 1989; Weidner et al., 1990). The α and β subunits originate from proteolytic cleavage of a single 92-kD inactive precursor (Miyazawa et al., 1989; Nakamura et al., 1989; Tashiro et al., 1990; Hartmann et al., 1992). The molecular identity of the two cytokines has been further proven by their interchangeable activities in promoting hepatocyte growth, epithelial cell dissociation, and matrix invasion (Naldini et al., 1991*a*; Weidner et al., 1991; Furlong et al., 1991).

Furthermore, HGF and scatter factor are indistinguishable ligands for the receptor encoded by the *MET* proto-oncogene (p190^{MET}), and both can stimulate the tyrosine kinase activity of the receptor (Naldini et al., 1991*a*,*b*). The p190^{MET} receptor is a transmembrane tyrosine kinase (Park et al., 1986) made of a 50 kD (α) subunit disulfide linked to a 145 kD (β) subunit (Giordano et al., 1988, 1989*a*,*b*). Ligand binding induces kinase activation (Bottaro et al., 1991; Naldini et al., 1991*b*), receptor phosphorylation at specific tyrosine sites (Ferracini et al., 1991) and recruitment intracellular signal transducers such as phosphatidyl inositol-3-kinase (Graziani et al., 1991).

The target cells responsive to HGF have not yet been fully identified. The cognate receptor, p190^{MET}, has been detected so far in a variety of epithelial cells (Chan et al., 1988; Giordano et al., 1989*b*; Naldini et al., 1991*b*; Di Renzo et al., 1991; Prat et al., 1991). HGF is expressed in several organs (Tashiro et al., 1990; Zarnegar et al., 1990) including vascular smooth muscle cells (Rosen et al., 1990*a*). HGF stimulates in vitro keratinocytes (Matsumoto et al., 1991)

1. *Abbreviations used in this paper*: GM-CSF, granulocyte-macrophage colony stimulating factor; HGF, hepatocyte growth factor; PAF, platelet-activating factor; PCA, procoagulant activity; PCR, polymerase chain reaction.

and melanocytes (Rubin et al., 1991; Kan et al., 1991) and determines the spatial arrangement of epithelial cells (Montesano et al., 1991). Induction of "scattering" and thymidine incorporation in cultured endothelial cells has also been observed (Rosen et al., 1990*b*; Rubin et al., 1991; Morimoto et al., 1991).

Because of its distinctive ability to act as a mitogen, a motogen and a morphogen, HGF is an ideal candidate to orchestrate the biological processes leading to the development of normal or pathological complex structures (organs or tumors). In both cases the formation of new blood vessels is required. Here, we report that HGF stimulates the proliferation and the migration of endothelial cells in vitro and has angiogenic properties in vivo.

Materials and Methods

Purification of Human and Murine HGF

Human HGF was purified to homogeneity from tissue culture medium conditioned by the human fibroblast line MRC5, grown on Cytodex beads (Pharmacia Fine Chemicals, Piscataway, NJ) in a 3 liter bioreactor (Applikon, Schiedam, Netherlands) equipped with automatic control of pH, CO_2, and O_2. The steps described by Weidner et al.(1990) were followed. Briefly, filtered medium (0.45-μm membrane; Millipore Continental Water Systems, Bedford, MA) was loaded onto a heparin-Sepharose column (Pharmacia Fine Chemicals) at 4°C. After washing with 0.5 M NaCl, 50 mM Tris-HCl, pH 7.5, HGF was eluted by a linear gradient (0.5–1.8 M) of NaCl, with a flow of 10 ml/h. Effluent aliquots were analyzed by SDS-PAGE, followed by silver staining. The biological activity was determined by the scatter assay described below. Further purification of pooled fractions containing HGF activity (0.9–1.3 M NaCl) was performed by cation exchange chromatography on Bio-Rex 70 column (Bio-Rad Laboratories, Cambridge, MA) in 50 mM Tris-HCl, pH 7.5. After dilution of the samples to reduce the salt concentration, scatter activity was eluted with a linear gradient of 0.2–1.2 NaCl in the same buffer. Biologically active fractions (0.7–0.8 M NaCl) were pooled.

Murine HGF was purified to homogeneity from medium conditioned by *ras* transformed NIH-3T3 cells by a two-step procedure described in details elsewhere (Coffer et al., 1991). The factor was precipitated by ammonium sulphate at 60% saturation, dissolved in 50 mM MES, 0.1 M NaCl, pH 6, loaded onto a Mono S column (Pharmacia Fine Chemicals) and eluted by a linear gradient of NaCl (0.1–1.0 M). The pooled biologically active material was adsorbed onto an hydroxyapatite column (Bio-Rad Laboratories) equilibrated with 3 mM sodium phosphate buffer, pH 7.4, and eluted by a linear gradient of sodium phosphate (0.1–0.6 M).

Purity of the final preparations was checked by SDS-PAGE electrophoresis after [^{125}I] labeling as described below. Both the human and the murine HGF showed the expected three bands corresponding to the β chain (33 kD), the α chain (62 kD), and the uncleaved 92-kD precursor. The biological activity was monitored by the scatter assay on MDCK cells as described in details by Weidner et al.(1990): one unit was defined as the highest dilution that clearly dissociates MDCK cells and corresponded to 0.3 ng of protein in a standard preparation.

Molecular Cloning, Expression, and Site-directed Mutagenesis of HGF cDNA

The cDNA coding the 723 amino acid form of HGF was cloned as described in details elsewhere (Naldini et al., 1991*a*). Briefly, amplification by polymerase chain reaction (PCR) of liver HGF sequences was performed on single stranded DNA templates obtained from human normal liver mRNA. For the amplification Vent polymerase (New England Biolabs, Beverly, MA), endowed with proof-reading activity, was used. 30 PCR cycles were performed in a Programmable Thermal Controller (MJ Research, Cambridge, MA) with a step cycle profile of 1 min at 92°C, 2 min at 50°C, and 2 min at 72°C. The PCR products were then purified and cloned in Bluescript plasmid vector (Stratagene, La Jolla, CA). DNA sequencing was performed on double-stranded DNA using the deoxynucleotide procedure (Sanger, 1977). Specific oligonucleotide primers were synthesized by standard phosphoramidite methods with a PCR-Mate 391 DNA Synthesizer (Applied Biosystem, Foster City, CA). The 2.2 kb BamHI-SalI HGF cDNA was inserted into the phagemid pSelect™1 vector and further processed to obtain the non cleavable (single chain) HGF mutant. The altered sites mutagenesis system (Promega Biotec, Madison, WI) was used to mutate a codon CGA (Arg) in CAA (Gln) within the sequence located between the nucleotide position 1467 and 1472. The sequence Arg^{489}-Val^{490} in the HGF primary translation product is recognized by a proteolytic processing system of the cell, leading to the dimer α-β.

Wild type and the mutated constructs were cloned into the expression vectors pBAT (Frixen et al., 1991) or pMT2 (Kaufman et al., 1989): pBAT (kindly provided by Dr. W. Birchmeier) contains the promoter of the chicken β actin gene; pMT2 the major late adenovirus promoter. The recombinant plasmids were obtained by ligation of a SacI-SalI HGF 2.2 kb fragment (for pBAT) and of a KpnI HGF fragment of the same size (for pMT2). The plasmids were transfected into Neuro2A (pBAT) or COS cells (pMT2) by the lipofection procedure. Three d after transfection supernatants were collected and titrated in the scatter assay.

Antibodies against the HGF Receptor

Polyclonal rabbit antibodies were raised against a synthetic peptide corresponding to nineteen COOH-terminal amino acids (from Ser^{1372} to Ser^{1390}) of the *MET* sequence (Ponzetto et al., 1991; EMBL Data-Bank reference number X54559). The peptide was coupled to keyhole limpet hemocyanin and the conjugate used to immunize rabbits. Antipeptide antibodies were purified on an affinity column of synthetic peptide coupled to CH-Sepharose 4B (Pharmacia Fine Chemicals).

c-MET Probe and Northern Blot Analysis

Total cellular RNA was prepared from monolayer cultures, using the single-step method of extraction described by Chomczynski and Sacchi (1987). For Northern blot analysis RNA was fractionated by electrophoresis on 0.8% denaturing agarose gels and transferred to nitrocellulose. Hybridization was carried out at 42°C in the presence of 50% formamide. The filters were washed at high stringency. The entire *MET* cDNA was used as a probe. The full-size 4.3 kb HindIII fragment was prepared from clones selected from a λgt11 cDNA library obtained from the gastric carcinoma cell line GTL-16 mRNA.

Western Blotting and Tyrosine Phosphorylation

Cells were directly solubilized in boiling Laemmli buffer (Laemmli, 1970), with or without reducing agents. Proteins, separated by SDS-PAGE, were transferred to nitrocellulose sheets and probed with anti-*Met* antibodies as described elsewhere (Giordano et al., 1989*a*). The phosphorylation state of the HGF receptor was probed by anti-phosphotyrosine antibodies as described previously (Comoglio et al., 1984). Briefly, subconfluent cell monolayers were made quiescent by 20 h starvation in serum-free Iscove's medium. After incubation with HGF for 10 min at 37°C, cells were lysed in a 25 mM Hepes buffer pH 7.6, containing 150 mM NaCl, 1% NP-40, 10% glycerol, 5 mM EDTA, 1 mM EGTA and protease and phosphatase inhibitors (50 μg/ml pepstatin, 50 μg/ml leupeptin, 10 μg/ml aprotinin, 2 mM PMSF, 500 μg/ml soybean trypsin inhibitor, 100 μM $ZnCl_2$, 1 mM Na orthovanadate). Proteins were immunoprecipitated by anti-*Met* antibodies, as described below, separated by SDS-PAGE and analyzed in Western blots probed with anti-phosphotyrosine antibodies. Rabbit anti-mouse immunoglobulin conjugated to Horseradish-peroxidase and the Enhanced Chemiluminescence procedure (Amersham Corp., Arlington Heights, IL) were used.

Cell Surface Iodination and Immunoprecipitation

Monolayers of cells were incubated with 1 mCi of [^{125}I] (Amersham Corp.), and lactoperoxidase in the presence of exogenous H_2O_2 as described elsewhere (Naldini et al., 1991*a*). The reaction was arrested with cold PBS containing 0.02% sodium azide. After labeling, cells were extracted with ice cold buffer containing 10 mM Pipes, pH 7.6, 100 mM NaCl, 5 mM $MgCl_2$, 300 mM sucrose, 5 mM EGTA, 1% Triton X-100, and inhibitors of proteases as above. Cell extracts were precleaned on protein-A Sepharose and immunoprecipitated with rabbit antibodies. Immunocomplexes were collected on protein-A Sepharose, washed and eluted in Laemmli buffer with or without 2β-mercaptoethanol.

HGF Radioiodination and Binding Assay

Pure murine HGF (1 μg) was radio-labeled with carrier free [^{125}I] (2 mCi) (Amersham Corp.) and Iodogen (Pierce Chemical Co.). 200 μl of Iodogen at 50 μg/ml in chloroform were dried in a polypropilene vial under nitrogen flow. HGF and [^{125}I] were then added in phosphate buffer, pH 7.4. The reaction was allowed to proceed for 15 min at 4°C, and then the mixture was transferred to another vial and left on ice for 10 min. Carrier BSA was added to a final concentration of 0.1% in 0.4 M NaCl, 0.1% CHAPS, 20 mM PO_4 buffered to pH 7.4, and the labeled ligand was fractionated from the free [^{125}I] by affinity chromatography on a 1 ml heparin-Sepharose column (Pierce Chemical Co.) pre-equilibrated with the same buffer. After extensive washing, the column was eluted with 1.3 M NaCl in the same buffer and 0.5-ml fractions were collected. Fractions containing TCA-precipitable radioactivity were pooled, concentrated with a Centrisart (Sartorius Balances, Westbury, NY) diafiltration apparatus with a membrane cut off above 20 kD and stored at 4°C. The specific activity of the tracer was approximately 0.25 Ci/mg, corresponding to an I/HGF molar ratio of approximately 11/1. The radiolabeled factor retained its biological activity as scatter factor assayed on MDCK cell line.

For binding displacement studies, cell monolayers were seeded at low density on collagen-coated microwells, put on ice, rinsed four times with prechilled RPMI medium containing 20 mM Hepes, pH 7.4, 0.1% BSA, 100 μg/ml soybean trypsin inhibitor and bacitracin (binding medium), and incubated with 0.05 nM [^{125}I]-HGF in binding medium, without or with the indicated concentrations of unlabelled competitor, for 3 h at 4°C in a final volume of 200 μl/well. The monolayers were then washed five times with the binding medium and extracted with 2% SDS in PBS. The extracts were then counted in a Packard γ-counter. Total binding was below 10% of the added cpm and specific binding—calculated subtracting from the total the cpm bound after incubation with a hundredfold excess of unlabeled ligand—was ~75%. Specific ligand binding studies were performed by using increasing concentrations of [^{125}I]HGF in the presence of 50-fold excess of cold ligand at 4°C for 3 h in the same conditions described above. The K_d of affinity binding sites was estimated by Lineweaver-Burk plot.

Cell Growth Assay

2.5×10^3 human endothelial cells were plated in 96-well plates (code 3595; Costar, Cambridge, MA) coated with gelatin (Difco Laboratories, Detroit, MI; 0.05%, for 1 h at 22°C) in M199 medium containing 20% FCS (Irvine, Santa Ana, CA). After 24 h the medium was removed and replaced with M199 containing 5% FCS with or without factors. 1.2×10^3 and 1.8×10^3 tEnd.1 and eEnd.1 cells, respectively, were plated in DME containing 10% FCS. After 24 h the experiments were done in Iscove's medium (ICN Biomedical, Irvine, CA) supplemented with 6 mg/l transferrin, 5 mg/l insulin, 100 mg soybean lecithin, 6.73 μg/l sodium selenite, and 400 mg/l BSA. Endothelial cells number was estimated after staining with crystal violet by a colorimetric assay described by Kueng et al.(1989). Briefly, the cells were fixed for 20 min at room temperature with 2.5% glutaraldehyde and then stained with 0.1% crystal violet in 20% methanol. The stained cells were solubilized with acetic acid (10%); the wells were read at 595 nm in a Microplate Reader (model 3550; Bio-Rad). A calibration curve was set up with known number of cells. Proportionality between absorbance and cell counts exists up to 8×10^4 cells.

Chemotaxis Assay

Chemotaxis assays on human endothelial cells were performed as previously described (Dejana et al., 1985; Bussolino et al., 1989) with the Boyden chamber technique. Polycarbonate filters (5-μm pore size, polyvinylpyrrolidone—free; Nucleopore Corp., Pleasanton, CA) were coated with gelatin (Difco Laboratories; 0.1% for 6 h at room temperature). HGF in M199 containing 1% FCS were seeded in the lower compartment of the chamber, and 2×10^5 suspended cells in M199 containing 1% FCS were then seeded in the upper compartments. After 8 h of incubation at 37°C, the upper surface of the filter was scraped with a rubber policeman. The filters were fixed and stained with Diff-Quick (Harleco, Gibbstown, NJ), and 10 oil immersion fields were counted after coding samples.

The directedness of HGF-stimulated migration was evaluated using checkerboard analysis (Zigmond et al., 1973). Increasing concentrations of HGF were placed in both top and bottom wells of the Boyden chamber in order to establish positive, absent, and negative concentration gradients across the filter barrier. Directed locomotion, chemotaxis, is the response to a net gradient of attractant; random stimulated migration, chemokinesis, is the response to attractant when no concentration gradient is present.

Culture of Human Endothelial Cells on Three-dimensional Collagen Gels

Collagen gels were prepared as described by Montesano et al. (1986). Briefly, a solution of rat collagen type I (2 mg/ml in 0.1% acetic acid) was dialyzed for 40 h at 4°C against 4 liters of DME. One ml of 10× DME and 2 ml of $NaHCO_3$ (0.37 M) were added to 7 ml of the cold collagen solution in an ice bath: 0.4 ml of this mixture were then dispensed into 24-well plates and allowed to gelify for 20 min at room temperature. 7×10^4 human endothelial cells were plated onto the gel layer and let to grow to confluence in M199 medium containing 20% FCS. The scattering assay was performed by adding HGF to the monolayer at the indicated concentrations. The cultures were fixed in isotonic formaldehyde (3%) for 30 min at room temperature and then observed under a Zeiss Axiophot microscope (Carl Zeiss, Oberkochen, Germany).

Wound Assay

Human endothelial cells were grown at confluence on 24-well plates (Falcon Plastics, Cockeysville, MD) coated with rat collagen (10 μg/ml). Monolayers were wounded with a razor blade as described (Bussolino et al., 1991*a*). After wounding, the cells were washed with PBS and incubated in M199 medium (ICN Biomedical) containing 5% FCS with or without HGF. Endothelial cells were fixed and stained with crystal violet as described above.

Procoagulant Activity, PAF Synthesis, and ELAM-1, VCAM-1, and Class II MHC Antigen Expression

Platelet activating factor (PAF) extracted from human endothelial cells was purified by TLC-HPLC, characterized, and quantified by aggregation of washed rabbit platelets as described (Bussolino et al., 1988). Procoagulant activity (PCA) was measured in a one-stage clotting assay on human endothelial cells monolayers grown on gelatin coated wells, according to Bevilacqua et al. (1984). 10^3 mU of standard PCA scored a clotting time of 23 s. The expression of endothelial-leukocyte adhesion molecule-1 (ELAM-1), vascular cell adhesion molecule-1 (VCAM-1), and class II MHC antigens (HLA-Dr) was measured by immunofluorescence after staining with specific mAbs. The cells were stimulated with HGF for 4 h before the PCA assay, 6 h before measuring PAF synthesis or ELAM-1 expression, 12 h before VCAM-1 and 96 h before HLA-Dr determination. These time points have been selected from preliminary time-course experiments lasting from 5 min to 96 h. IL-1β and TNF-α were used as positive controls for the production of PAF, PCA, and the expression of ELAM-1 and VCAM-1, both at 10 ng/ml, which is optimal concentration for these activities (Bussolino et al., 1988; Bevilacqua et al., 1984, 1989). The positive control for the expression of HLA-Dr antigens was INF-γ, used at 500 mg/ml (Pober et al., 1986).

In Vivo Angiogenic Assay

The angiogenic activity of HGF was assayed in vivo using the rabbit cornea assay described in details previously by some of us (Ziche et al., 1989; Bussolino et al., 1991*a*). Slow-releasing pellets ($1 \times 1 \times 0.5$ mm) were prepared in sterile conditions incorporating the dried HGF into a casting solution of a vinyl copolymer (Elvax 40; Dupont, Wilmington, DE), in 10% methylene chloride (10 μl/droplet). In the lower half of New Zealand white rabbit eyes (Charles River Breeding Laboratories, Wilmington, DE), anesthetized by pentobarbital (30 mg/Kg), a microsurgical pocket (1.5×3 mm), was produced using a pliable iris spatula 1.5-mm wide. The pellets were implanted in the micropockets, located into the transparent avascular corneal stroma. Subsequent daily observations of the implants were made with a slit lamp stereomicroscope without anesthesia. An angiogenic response was scored positive when budding of vessels from the limbal plexus occurred before 3–4 d and capillaries progressed to reach the implanted pellet according to the scheme previously reported (Bussolino et al., 1991*a*). Angiogenic activity was expressed as the number of implants exhibiting neovascularization over the total implants studied. Potency was scored by the number of newly formed vessels and by their growth rate.

Cells

Human endothelial cells from umbilical cord vein, prepared and characterized as previously described (Bussolino et al., 1989), were grown in M199 medium supplemented with 20% FCS (Irvine), endothelial cell growth fac-

tor (100 μg/ml) and porcine heparin (100 μg/ml), and used at early passages (I-IV). The mouse endothelioma cell lines eEnd.1, and tEnd.1 were obtained through the courtesy of Dr. E. F. Wagner (IMP, Wien; Williams et al., 1989). These cell lines, originally derived from a thymic (tEnd.1) and embryonal (eEnd.1) hemangioma, express the polyoma middle T antigen, and have morphological and functional features of microvascular endothelial cells (Bussolino et al., 1991*b*). The lines were maintained in DME (Gibco Laboratories, Grand Island, NY) containing 15% FCS and 750 mg/l G418 (Gibco Laboratories). The human diploid fibroblast MRC5 line was from American Type Culture Collection (Rockville, MD), and was grown in DME supplemented with 10% FCS. The human vascular smooth muscle cells were kindly provided by Dr. M. Trovati (University of Torino, Torino, Italy) and grown in DME supplemented with 20% FCS. The EA926 endothelial cell line (kindly provided by Dr. E. C. J. Edgell, University of North Carolina at Chapel Hill, Chapel Hill) was maintained in DME supplemented with 10% FCS.

Reagents

TNFα (1×10^6 U/mg protein) was obtained from Dr. P. Ralph (Cetus Corp., Berkeley, CA); basic-fibroblast growth factor (bFGF) from Farmitalia; human IFNγ (1×10^6 U/mg protein) from Biogen (Geneva, Switzerland); human IL-1β (1×10^8 U/mg protein) and human granulocyte-macrophage colony stimulating factor (GM-CSF) (1×10^7 U/mg) from Dr. Gillis (Immunex, Seattle, WA).

Phosphotyrosine antibodies were raised against *p*-amino-benzene-phosphonate and affinity-purified as previously described (Comoglio et al., 1984). mAbs anti-ELAM-1 and VCAM-1 were from British Bio-technology Limited; mAb antibody anti-class II-Dr antigens (AA3.84) were a gift of Dr. F. Malavasi (University of Torino, Torino, Italy). *S. Aureus* Protein A covalently coupled to sepharose was purchased from Pharmacia. Reagents for SDS-PAGE and nitrocellulose filters were from Bio-Rad. All other reagents were analytical grade from Sigma Chemical Co.

Results

Endothelial Cells Express the HGF Receptor

Total RNA was extracted from endothelial cells cultured from human umbilical cord veins. Northern analysis was performed using the full-size *MET* cDNA as a probe. A major transcript of 9 kb was observed (Fig. 1 *A*). To detect the protein, cell lysates were blotted and probed with polyclonal rabbit antibodies directed against a COOH-terminal peptide of the p190^{MET} β chain (Fig. 1 *B*). Proteins were extracted from human endothelial cells prepared from the umbilical cord vein and from a human endothelial cell line (EA926). In both cell lysates, analyzed under non-reducing conditions, a single protein band of the expected size (190 kD), corresponding to the αβ dimer, was detected. After reduction of disulfide bonds, the β chain alone (145 kD) was observed. In these conditions a 170-kD band was also detected, corresponding to the pr170 receptor precursor previously described (Giordano et al., 1989*b*). To visualize the mature α subunit exposed at the cell surface with the β subunit, the cells were labeled with [^{125}I]-lactoperoxidase detergent extracted and immunoprecipitated with the anti-*Met* antibody (Fig. 1 *C*). Human vascular smooth muscle cells, as well as a number of other cells of mesenchymal origin, were negative.

HGF Binds and Stimulates the Receptor Kinase in Endothelial Cells

Monolayers of human endothelial cells were incubated with 0.05 nM of [^{125}I]-HGF in the presence of increasing concentrations of unlabeled ligand. The ligand displacement curves at equilibrium showed specific binding of HGF to the cell surface and identified two classes of binding sites with differing affinities. A higher affinity HGF binding site was observed with a lower affinity, high capacity binding site (Fig. 2 *A*). The higher affinity binding site was further characterized by direct binding with increasing concentration of [^{125}I]HGF and the calculated K_d was 0.35 nM with 10^4 receptors per cell (b_{max} = 2.59 fmoles/cell) (Fig. 2 *B*).

The high affinity HGF receptor is the p190^{MET} tyrosine kinase (Naldini et al., 1991*a*). To show that in human endothelial cells the kinase activity is controlled by binding to the specific ligand, studies were carried on using phosphotyrosine antibodies. The receptor was solubilized from cells made quiescent by serum starvation and stimulated by

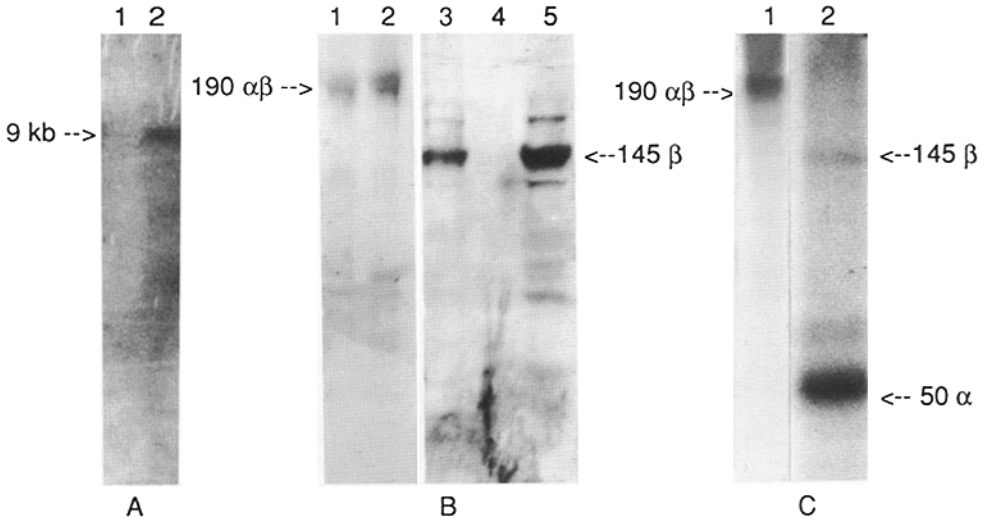

Figure 1. Expression of HGF receptor in human endothelial cells. (*A*) Northern blot analysis of total RNA extracted from control vascular smooth muscle cells (lane *1*) and endothelial cells prepared from umbilical cord vein (lane *2*). The cDNA probe encompassing the entire *MET* coding sequence, labeled with [^{32}P], was hybridized to 20 μg of RNA. X-ray films were exposed for 14 d with intensifying screens. (*B*) Western blot analysis of total protein solubilized from endothelial cells prepared from umbilical cord vein (lanes *2* and *5*), EA926 endothelial cell line (lanes *1* and *3*), or vascular smooth muscle cells (lane *4*). Proteins (200 μg per well) were run under non-reducing (lanes *1–2*) or reducing conditions (lanes *3–5*) and probed with anti-*Met* antibodies directed against the 145-kD β chain. (*C*) Immunoprecipitation of membrane proteins from endothelial cells, labeled with [^{125}I] under non-permeating conditions. Proteins extracted by non-ionic detergent were precipitated by anti-*Met* antibodies and separated under non-reducing (lane *1*) and reducing conditions (lane *2*).

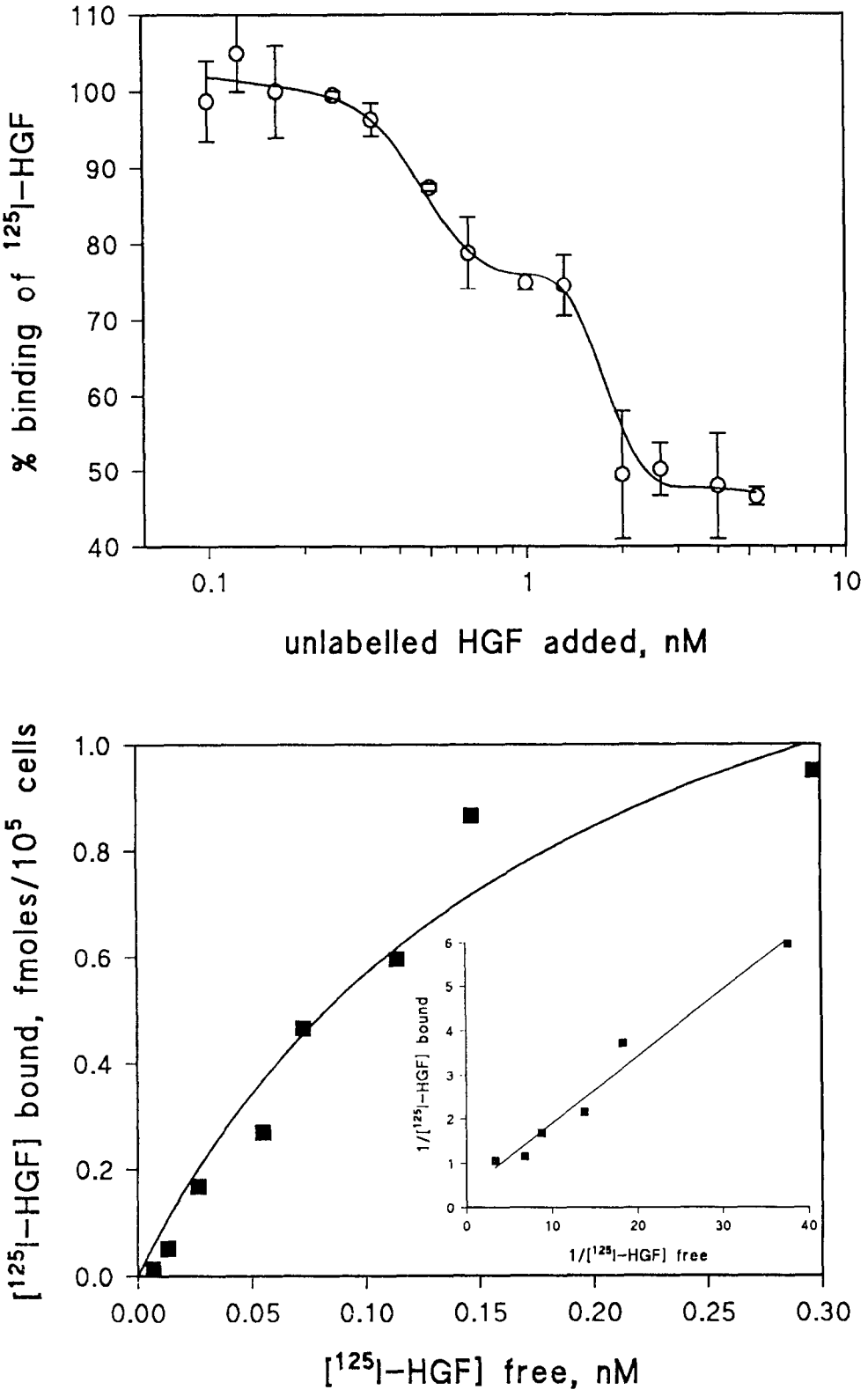

Figure 2. Binding of radiolabeled murine HGF to human endothelial cells. (*Top*) Ligand displacement curve. Monolayers were incubated with 0.05 nM [^{125}I]-HGF in the presence of the indicated concentrations of unlabeled HGF to equilibrium binding at 4°C. The points are means ± SD of triplicate determinations from a representative experiment. (*Bottom*) Specific ligand binding curve. Monolayers were incubated with indicated concentrations of [^{125}I]-HGF in the presence of 50-fold excess of cold HGF. The insert shows the analysis of the data by Linewear-Burk plot.

HGF. After immunoprecipitation and electrophoresis, the receptor was blotted onto nitrocellulose filters and probed with anti-phosphotyrosine antibodies. The kinase activation was assessed by measuring the tyrosine auto-phosphorylation of the β subunit. Specific phosphorylation of p190^{MET} was observed after stimulation with natural human HGF purified from MRC5 cell supernatants or with recombinant HGF expressed in Neuro-2A cells (Fig. 3). The specificity of the reaction was tested by using a recombinant HGF molecule with an inappropriate conformation. The HGF cDNA expressed in Neuro-2A cells was mutated to convert Arg^{489} into a Gln. As described in details elsewhere (Hartmann et

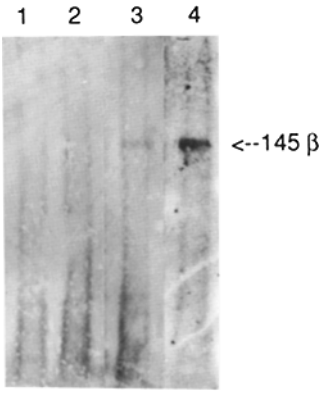

Figure 3. Tyrosine phosphorylation of the HGF receptor in human endothelial cells. Monolayers prepared from umbilical cord vein were incubated for 10 min at 37°C in serum-free culture medium supplemented as follows. (Lane *1*) Spent medium conditioned by Neuro-2A cells transfected with the vector alone. (Lane 2) Spent medium conditioned by Neuro-2A cells expressing the Gln^{489}-HGF mutant. (Lane *3*) Supernatant of Neuro-2A cells expressing the wild type HGF (12 ng/ml). (Lane *4*) Purified human HGF (30 ng/ml). Proteins extracted by non-ionic detergent were immunoprecipitated by anti-*Met* antibodies, separated under reducing conditions, blotted onto nitrocellulose, and probed with antiphosphotyrosine antibodies.

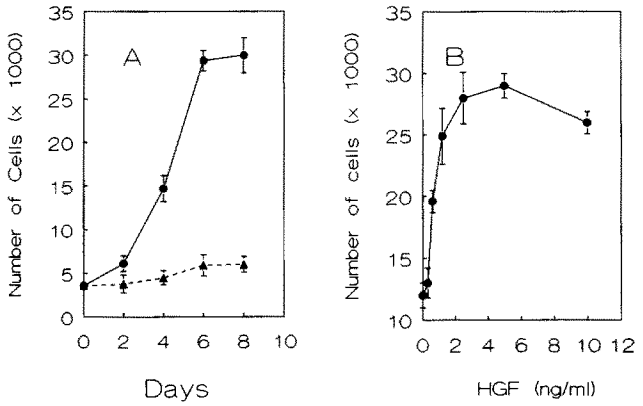

Figure 4. Proliferation of human endothelial cells stimulated by HGF: time-course (*A*) and dose dependence (*B*). Low density cultures of endothelial cells (2.5×10^3 per 0.32-cm^2 well) were incubated in M199 supplemented with 5% FCS in the presence (●) or in the absence (▲) of human HGF. Fresh factor was added every two days. *B*, cells were counted after 8 d. The data shown are the means of six determinations ± SD in a representative experiment.

al., 1992), this point mutation eliminates the specific proteolytic site in the HGF precursor, leading to the secretion of an uncleaved inactive molecule. The Arg^{489}-HGF mutant was almost completely inactive in the phosphorylation assay (Fig. 3).

HGF Stimulates the Growth of Endothelial Cells In Vitro

The mitogenic activity of HGF was studied using cultures of human endothelial cells and two murine cell lines derived from embryonal (eEnd.1) or microvascular (tEnd.1) endothelium. Human endothelial cells, seeded at 2.5×10^3 per 0.32-cm^2 well, undergo a 16-fold increase in cell number over an 8 d period when stimulated every 2 d with 5 ng/ml of natural human HGF (Fig. 4 *A*). Challenging endothelial cells cultures with a unique pulse of the factor (5 ng/ml) doubled the cell number within 6 d (not shown). The stimulatory activity was concentration dependent, with a maximum effect occurring between 2.5 and 5 ng/ml. Higher concentrations were uneffective (Fig. 4 *B*). The same growth stimulatory activity was measured when human recombinant HGF was tested. Both the natural and the recombinant factors were inactive after heat denaturation. The uncleaved Gln^{489} mutant was as well nearly devoid of biological activ-

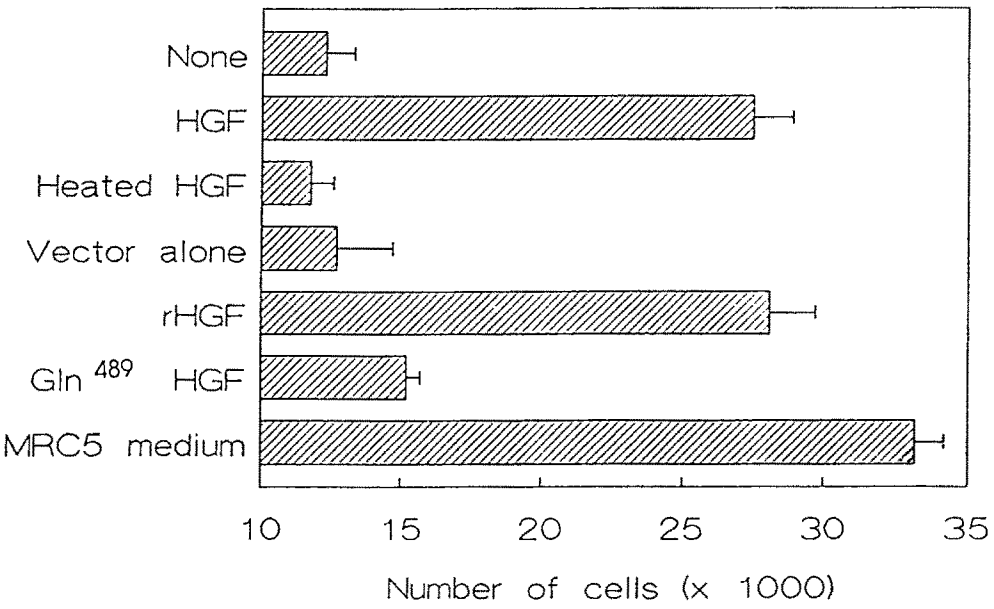

Figure 5. Effect of natural or recombinant HGF on the proliferation of human endothelial cells. Cell proliferation, assayed in the conditions described in the legend to Fig. 4, was measured 8 d after the addition of the following factors: *None*, fresh medium; *HGF*, purified human HGF; *Heated HGF*, the same reagent boiled for 3 min; *Vector alone*, spent medium conditioned by Neuro-2A cells transfected by the empty vector; *rHGF*, spent medium conditioned by Neuro-2A cells expressing recombinant HGF; *Gln^{489} HGF*, spent medium conditioned by Neuro-2A cells expressing the uncleaved mutant HGF; *MRC5 medium*, spent medium conditioned by MRC5 cells. Spent media were diluted to contain ~5 ng/ml of HGF. The data shown are the means of six determinations ± SD in a representative experiment.

ity (Fig. 5). The conditioned medium harvested from the human diploid fibroblast line MRC5, which is known to produce active HGF (Gherardi et al., 1989), seems to be more potent in stimulating endothelial cells growth than the purified human HGF, either natural or recombinant (Fig. 5). This higher effect on growth was observed when identical "scatter" units of factor were tested (see Materials and Methods), suggesting a possible cooperation with other factor(s) present in the conditioned medium.

We then compared the growth stimulatory activity of HGF with that induced by known angiogenic factors used at optimal concentration, namely GM-CSF (10 ng/ml) and basic-FGF (5 ng/ml). In the same experimental conditions HGF (5 ng/ml) (HGF: 4.0 ± 0.3 × 10^4 cells; control: 1.3 ± 0.2 × 10^4 cells) was approximately twofold more active than GM-CSF (2.3 ± 0.2 × 10^4 cells), while less potent than bFGF (7.4 ± 0.3 × 10^4).

The growth stimulatory effect of HGF is not restricted to endothelial cells of human origin. Murine HGF stimulate the growth of two murine endothelial cell lines originated by thymic (tEnd.1) and embryonic (eEnd.1) tissues (Williams et al., 1989). The stimulatory effect of HGF is detectable at 0.6 ng/ml, and the optimal concentration is 2.5 ng/ml, which increases of about twofold the growth rate (tEnd.1: control, 3.1 ± 0.2 × 10^4 cells; HGF, 5.8 ± 0.3 × 10^4 cells, eEnd.1: control, 2.2 ± 0.1 × 10^4 cells; HGF, 3.2 ± 0.1 × 10^4 cells). No stimulation was observed at higher concentrations. The factor is partially species-specific since human HGF is twice as potent on human endothelial cells than on mouse cell lines. The same kind of species-specificity was observed for the murine HGF tested on mouse and human endothelial cells (Table I).

HGF Enhances Endothelial Cell Motility

The effect of HGF on endothelial cell motility was evaluated in Boyden chamber. The motility response was exhibited with stimulated migration peaking at HGF concentration of 2.5 ng/ml (Fig. 6). At this concentration, the response to HGF is similar to that elicited by fibrinogen (1 mg/ml fibrinogen: 148 ± 18 migrated cells; control 66 ± 9 migrated cells) used as positive control (Dejana et al., 1985). Heating HGF removed the migration-stimulating activity (not shown). Checkerboard analysis shows that HGF induced not only chemokinesis, but also chemotaxis (Table II).

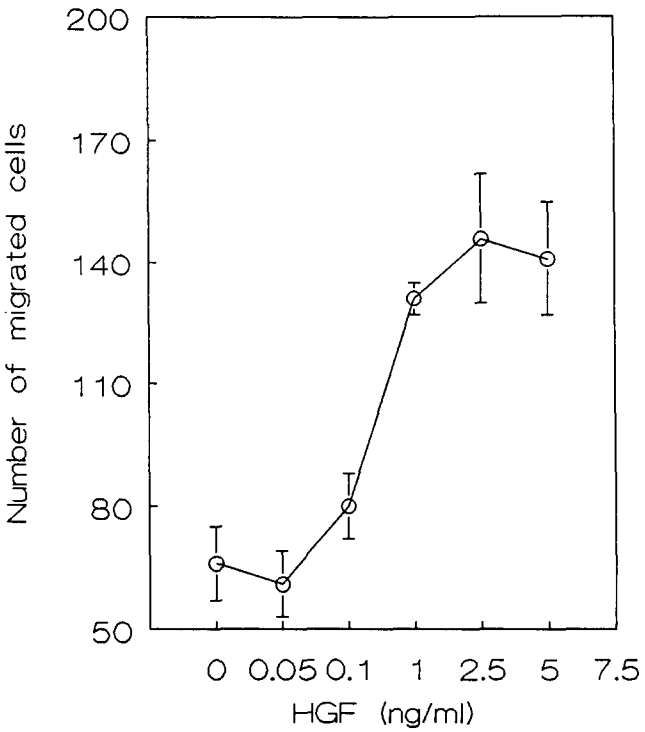

Figure 6. Migration of human endothelial cells stimulated with HGF. The migration of cells was measured by the modified Boyden chamber technique, as described in Materials and Methods. HGF in M199 containing 1% FCS were seeded in the lower compartment of the chamber, and 2 × 10^5 endothelial cells in the same medium were seeded in the upper compartment. Cells migrated after 8 h incubation to the lower surface of the filter were counted after coding samples. The numbers are the mean ± SD of three fields counted, in a representative experiment.

*Table I. Effect of Human and Murine HGF on Endothelial Cells**

	HUVEC	eEnd.1	tEnd.1
1. Control	8.2 ± 0.3	17.3 ± 1.1	19.0 ± 2.1
2. human HGF	22.2 ± 1.2	24.5 ± 2.3	23.1 ± 0.8
3. murine HGF	14.2 ± 2.0	38.2 ± 1.6	37.3 ± 3.1

* Number of cells (1 × 10^3).
2.5 × 10^3 human umbilical cord vein endothelial cells (HUVEC) were seeded in 96-well microtiter plates (0.32 cm^2) in 0.2 ml of M199 medium, containing 5% FCS. 1.8 × 10^3 murine endothelial cells (eEnd.1) and 1.2 × 10^3 (tEnd.1) were seeded in 0.2 ml of Iscove's medium without serum modified as detailed in Materials and Methods. The cultures were stimulated with different HGFs as indicated; fresh factor was added every two days. The cell number was scored at the plateau (8th day for human cells or 6th day for murine cells). Numbers are the mean (± SD) calculated from six duplicates of one representative experiment. For each data column, p was calculated by ANOVA and was always <0.0001. Then Student-Neuwam-Keuls test was applied for more specific comparisons. The following comparisons gave $p < 0.05$: human endothelial cells: 1 versus 2, 1 versus 3, 2 versus 3; eEnd.1: 1 versus 2, 1 versus 3, 2 versus 3; tEnd.1: 1 versus 2, 1 versus 3, 2 versus 3.

HGF Promotes Wound Repair in Endothelial Cell Monolayers

Wound healing assays in vitro, i.e., the ability of filling artificial gaps created in cell monolayers, requires both cell growth and activation of cell movements. HGF induced repair, in a dose-dependent manner, of mechanical wounds generated in human endothelial cells monolayers (Fig. 7). After 24 h of stimulation, endothelial cells moved into the denuded area, and completely filled the free space available. Movement was induced by as little as 5 ng/ml; the maximum effect was reached at concentrations around 50 ng/ml. When endothelial cells were treated for 60 min at 37°C with mitomycin C (10 μg/ml) and then stimulated with HGF, the repair process observed after 24 h was significantly reduced. This shows that the process requires both cell migration and DNA synthesis (data not shown). The heat-inactivated and the Gln^{489} mutant HGF were inactive (data not shown).

Table II. Checkerboard Analysis of Induction of Endothelial Cell Migration by HGF

	Upper chamber			
Lower chamber	Medium	HGF 1 ng/ml	HGF 2.5 ng/ml	HGF 5 ng/ml
Medium	61 ± 7	58 ± 7	51 ± 3	49 ± 10
HGF 1 ng/ml	80 ± 4*	67 ± 2	74 ± 3*	65 ± 3
HGF 2.5 ng/ml	92 ± 3*	81 ± 2*	83 ± 4*	71 ± 5*
HGF 5 ng/ml	129 ± 3*	117 ± 4*	100 ± 6*	77 ± 3*

Different concentrations of purified natural HGF were placed in the upper and/or lower compartments of the Boyden chamber. Results are expressed as the number of migrated cells (mean ± SD of three samples for each experimental point).
* $P < 0.05$ versus medium control, by *t*-test.

HGF Stimulates Endothelial Cell Scattering on Collagen Three-dimensional Gel

Endothelial cells plated onto plastic surface do not constitute colonies as epithelial cells. This characteristic does not permit study of the scatter activity of HGF as dispersion of cell colonies. To investigate whether HGF can induce scatter, human endothelial cells were plated on three-dimensional collagen gels. After the attainment of confluence the monolayers were stimulated with sub-nanomolar concentrations of the factor. Scatter along this three-dimensional structure was examined by observing scattered cells whose plane of focus was obviously within or beneath the layer of the collagen gel (Fig. 8). The treated cells monolayer started to penetrate through the matrix within 24 h. After 4 d of culture in the presence of HGF, the matrix was filled by endothelial cells showing the characteristic elongated phenotype sprouting cytoplasmic extensions and ruffles (Fig. 8 *d*). HGF also induced a network of branched and associated elongated cells, often anastomosed with one another (Fig. 8, *e* and *f*). Endothelial cells maintained in control medium during the same time retained their characteristic cobblestone appearance and did not scatter along the underlying matrix (Fig. 8, *a* and *b*).

HGF Does Not Affect Activities Related to Thrombosis, Inflammation, and Other Endothelial Cells Functions

The ability of HGF to modulate endothelial cell functions related to thrombosis, inflammation and the so called accessory functions was investigated by measuring the following parameters: (*a*) induction of procoagulant activity (PCA) and of platelet activating factor (PAF); (*b*) modulation of expression of cell adhesion molecules (e.g., ELAM-1 and VCAM-1); and (*c*) expression of HLA-Dr antigens. Interleukin-1β, Tumor Necrosis Factor α, IFNγ, were used as reference standards. HGF did not affect with any one of the listed activities (Table III).

In Vivo Angiogenic Effect of HGF

To explore the real relevance of HGF to the angiogenesis in vivo, we studied the formation of new capillary vessels in the rabbit cornea. Purified HGF was incorporated into a vinyl copolymer pellet and implanted in a corneal pocket 2 mm from the limbus. The factor is released in the avascular trans-

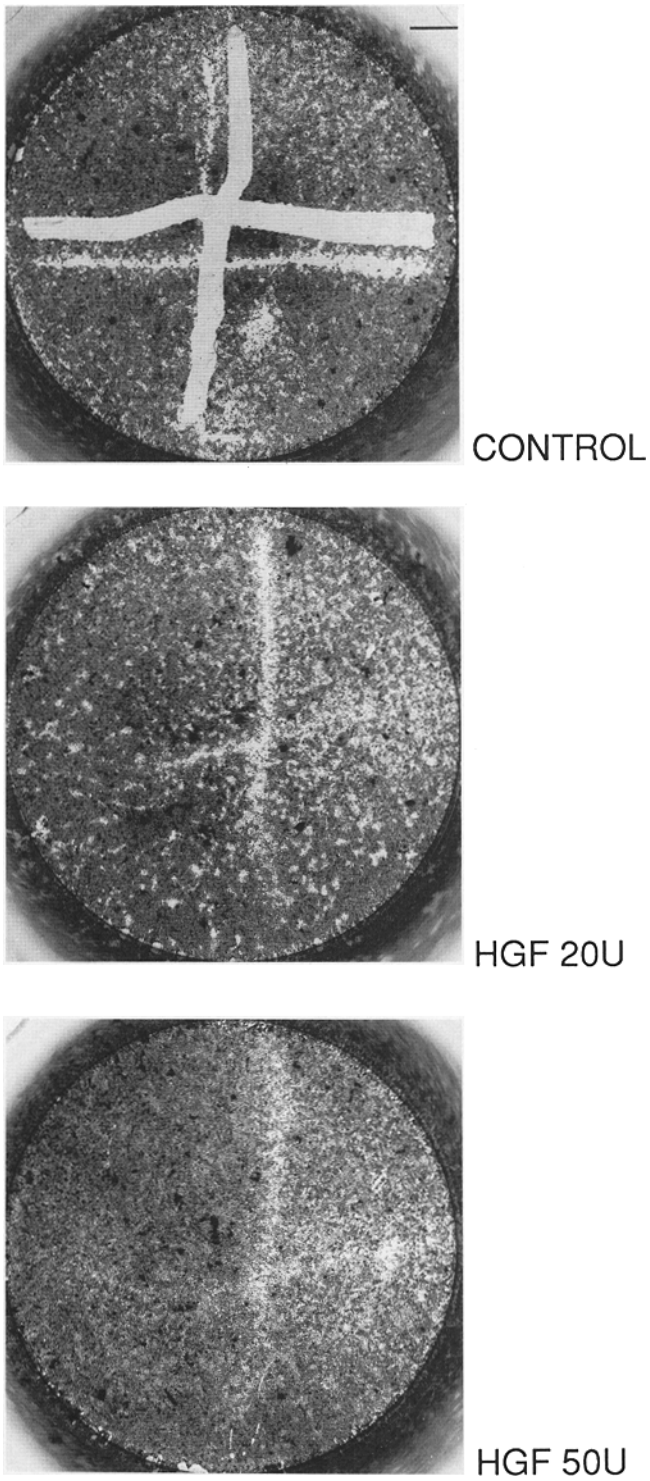

Figure 7. Wound healing of human endothelial cells monolayer induced by HGF. Confluent stationary endothelial cell monolayers were wounded with two cross-shaped scratches of different width. After a 24-h recovery, monolayers were incubated in the absence (*top*) or in the presence of HGF 5 ng/ml (*middle*) or 12 ng/ml (*bottom*), corresponding to the indicated Scatter Units (*U*). The thinner scratch was filled completely at both concentrations.

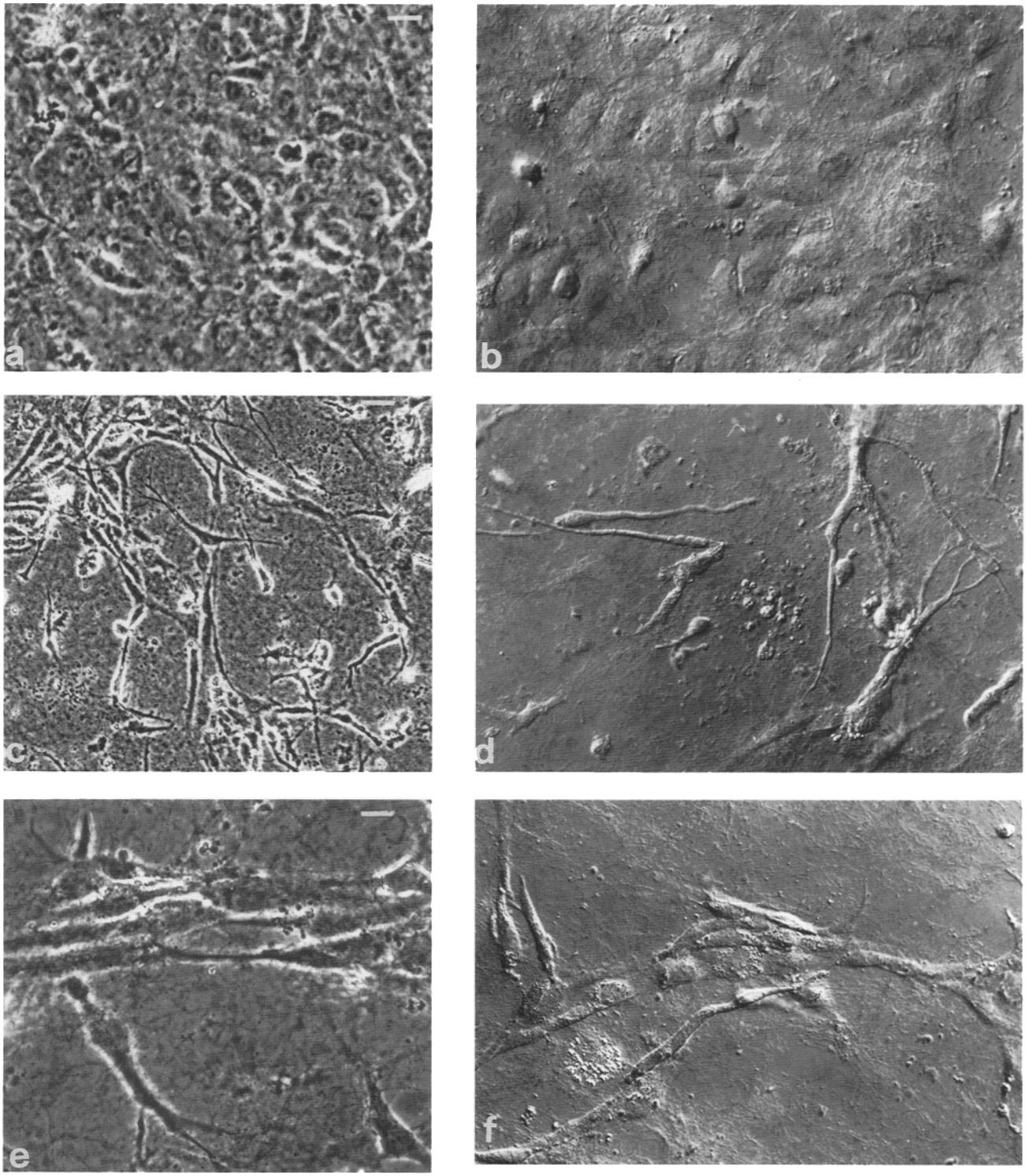

Figure 8. Scattering within three-dimensional collagen gel by human endothelial cells stimulated with HGF. (Phase contrast microscopy in *a*, *c*, and *e*; Nomarsky interference microscopy in *b*, *d*, and *f*). Untreated cells formed cobblestone-like monolayer at the surface of collagen gels after 4 d of culture. Cells treated with HGF (5 ng/ml) for 4 d scattered acquiring a dendritic or irregular shape (the plane of focus is beneath that of the monolayer). In some instances cells formed an incomplete network of branching cell cords. Bars: (*a* and *b*) 20 μm; (*c* and *d*) 30 μm; (*e* and *f*) 17 μm.

parent cornea around the pellet and generates a gradient. The neo-formation of capillaries from the limbus was monitored (Fig. 9). HGF promoted capillary outgrowth in a significant number of animals ($p < 0.05$). The angiogenic effect was dose-dependent and seen within a sub-nanomolar range of concentrations (Table IV). The newly formed vessel network consisted approximately of 30–40 capillaries that reached and surrounded the pellet implant by d 8. No signs

Table III. Stimulation of PAF Synthesis, Procoagulant Activity, ELAM-1, VCAM-1, and Class II MHC Antigen Expression in Human Endothelial Cells

	Growth*	PAF pmoles	PCA mU	% positive cells		
				ELAM-1	VCAM-1	HLA-Dr
Control	3.3 ± 1.1	0.4 ± 0.2	6 ± 4	0	0–2	0
HGF	29.2 ± 0.4	0.5 ± 0.2	11 ± 5	0–4	0–2	0
IL-1β	12.2 ± 1.3	1.2 ± 0.1	116 ± 7	43–67	25–52	0–3
TNFα	14.3 ± 2.0	2.9 ± 0.7	167 ± 18	50–61	40–55	0
INFγ	9.8 ± 1.6	0.7 ± 0.4	10 ± 4	0–3	0	28–61

* Number of cells × 10^3 scored in the growth assay.
The growth assay was performed as described above, using optimal concentrations of the tested factors, as described in Materials and Methods. The production of PAF, PCA, and the expression of ELAM-1, VCAM-1 and HLA-Dr antigen was measured in cells stimulated for 4 h (PCA), 6 h (PAF and ELAM-1), 12 h (VCAM-1), and 96 h (HLA-Dr) with HGF (5 ng/ml), IL-1β and TNFα (10 ng/ml), and INFγ (500 ng/ml) as described under Materials and Methods. A range of concentrations of HGF (from 0.1 to 100 ng/ml) and exposure times (from 5 min to 96 h) have been studied with results consistent with those selected for display in this table. Numbers are the mean (± SD) calculated from triplicates of one representative experiment or ranges of three experiments done in duplicate.

of accompanying inflammatory reaction were observed as shown by the persistence of corneal transparency for all the duration of the experiment. When compared with the well-established angiogenic factor bFGF, HGF showed equal potency on molar basis (Table IV).

Discussion

The data reported here prove that HGF is a powerful angiogenic factor stimulating endothelial cell proliferation and migration.

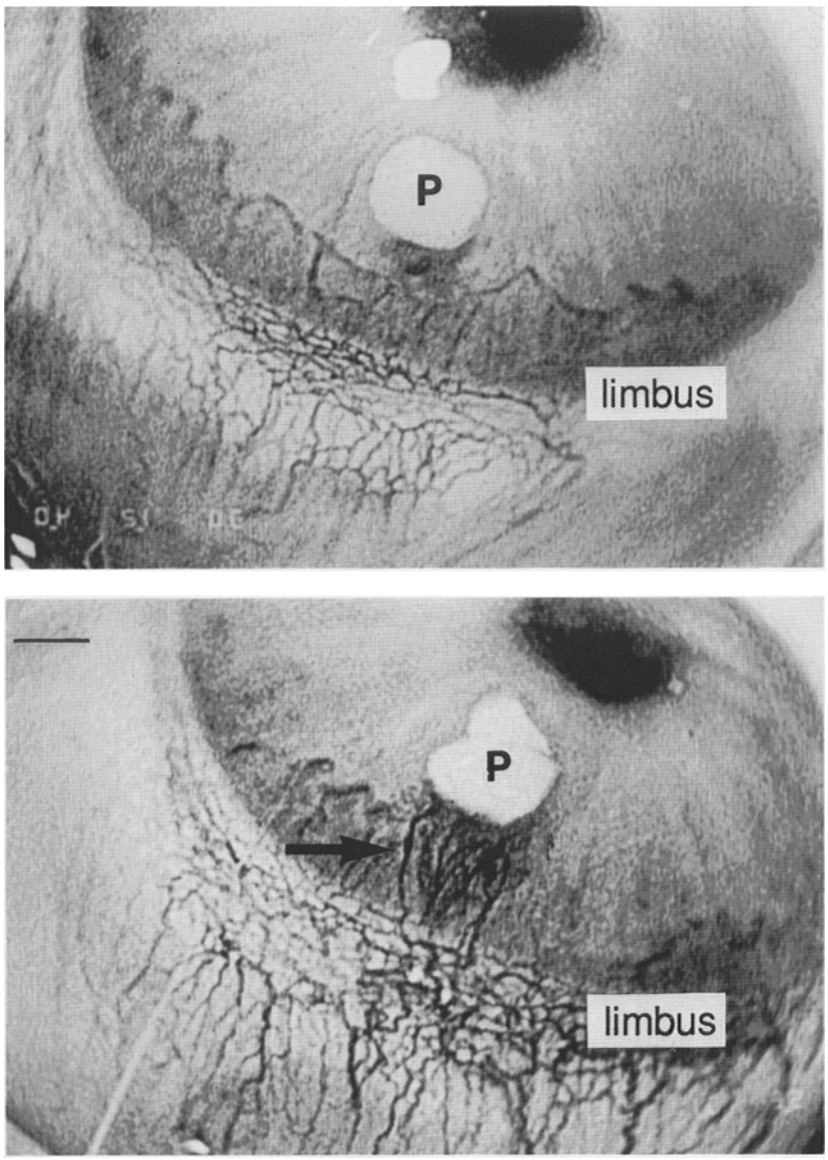

Figure 9. Induction of angiogenesis in vivo by HGF. A vinyl copolymer pellet (*P*) was implanted in the avascular cornea of rabbit eyes. The upper picture shows a control implant lacking HGF. The lower picture shows a pellet containing 50 ng of HGF. A network of newly formed blood vessels (*arrow*) sprouting from the limbus was observed. The neoformed vessels invaded the corneal stroma and reached the pellet releasing HGF. Photographs were taken 8 d after the implant. Bar, 1,000 μm.

Table IV. HGF Induces Angiogenesis In Vivo

Factor	ng/pellet	Positive implants	Potency
HGF	0	0/8	−
	20	3/8	+
	50	6/8	+++
bFGF	0	0/10	−
	20	2/10	++
	50	8/10	+++

Slow-releasing polyvinyl pellets containing different amounts of pure HGF or bFGF were implanted into the avascular cornea of rabbits. Angiogenic activity was expressed as the number of pellet implants inducing neovascularization of the peripheral stroma within 10 d. The potency of the angiogenic response was scored on the basis of the number of the newly formed vessels observed without magnification. (−): no vessel growth; (+) vessel growth up to 0.6 mm from the limbus; (++) new vessel formation extending midway to the corneal pocket; (+++) new vessel formation reaching the corneal pocket. $p < 0.05$ for both factors (50 ng/pellet) by Fischer's exact test.

This effect is mediated by a direct interaction with the specific receptor (p190^{MET}), a transmembrane tyrosine kinase encoded by the *MET* oncogene (Park et al., 1986; Gonzatti et al., 1988; Giordano et al., 1989*a*). Endothelial cells express the *MET* protooncogene and expose at the cell surface the mature protein with the same α-β heterodimeric structure found in epithelial cells (Giordano et al., 1989*b*; Di Renzo et al., 1991; Prat et al., 1991). HGF binds to the endothelial cell surface through a high affinity site with a K_d comparable to that previously measured in epithelial cells (Naldini et al., 1991*a*; Higuchi and Nakamura, 1991; Zarnegar et al., 1991; Masumoto et al., 1991). A distinct lower affinity, higher capacity binding site was also detected.

The binding of HGF to p190^{MET} at the endothelial cell surface is followed by the activation of the receptor tyrosine kinase domain and phosphorylation of the β subunit. Autophosphorylation of tyrosine kinase receptors is known to trigger intracellular signals leading to the biological responses induced by the specific ligands (reviewed in Ullrich and Schlessinger, 1990). Sub-nanomolar concentrations of HGF induce the proliferation of endothelial cells derived from human large vessels and from murine microvascular capillaries. This biological effect is highly specific and requires the correct conformation of the α and β chains of HGF, since a single amino acid substitution abolishes activity. In this respect it is also intriguing that, in spite of the homology between human and rodent HGF being higher than 90% (Tashiro et al., 1990), cross-stimulations performed on human endothelial cells with murine HGF and viceversa suggest some degree of species specificity. A similar observation has been reported studying the effect of HGF on human and rat hepatocytes (Strain et al., 1991).

HGF markedly stimulates directed (chemotaxis) and random (chemokinesis) migration of endothelial cells. Proliferation and motility are involved in the repair of mechanically wounded endothelial cells monolayers induced by HGF. The wound healing is an in vitro model mimicking in vivo conditions where activation of cell motility is required (Heimark et al., 1986). In vitro wound healing induced by HGF was partially prevented by mitomycin C, an inhibitor of cell proliferation. This shows that the repair mechanism induced by HGF is mediated by stimulation of both migration and growth of endothelial cells.

A crucial step in the sequence of events that leads to the angiogenic response is the invasion of the perivascular matrix by sprouting endothelial cells. The process includes motility and proliferation and production of lytic enzymes enabling extracellular matrix penetration (reviewed in Folkman and Klagsbrun, 1987; Ingberg and Folkman, 1989; Sporn and Roberts, 1990).

A model, known as "angiogenesis in vitro" (Montesano et al., 1986), mimics these steps of the angiogenesis. Angiogenic factors, like FGF and phorbol esters stimulate endothelial cells grown on collagene three-dimensional gels to invade and form capillary-like structure (Montesano et al., 1986). HGF promotes the scattering of endothelial cells monolayer within the underlying collagen matrix, suggesting that this factor is able to activate endothelial cells also in vitro conditions mimicking the in vivo microenvironment.

Unlike other cytokines active on endothelial cells, such as IL-1β, TNFα, and IFNγ (reviewed in Pober and Cotran, 1990; Butcher, 1991; Osborn, 1990; Mantovani et al., 1992), HGF does not modulate functions related to hemostasis-thrombosis (synthesis of procoagulant activity and platelet activating factor), inflammation (expression of the cell adhesion molecules ELAM-1 and VCAM-1), or the so called accessory functions (expression of HLA-Dr antigens).

Having shown that HGF induces endothelial cell migration and proliferation in several in vitro assays, it was important to investigate the in vivo relevance of these properties. HGF has a clear angiogenic activity in the rabbit cornea, comparable to that promoted by bFGF. In view of the effects induced in vitro, it is reasonable to assume that the angiogenic effect of HGF in vivo reflects a direct interaction with endothelial cells. However, we cannot exclude that the angiogenic activity of HGF involves other circuits mediated by epithelial cells activated by this cytokine. Thus, HGF is to be included in the group of factors that induce angiogenesis via modulation of endothelial cell locomotion and growth (reviewed in Folkman and Klagsbrun, 1987; Rifkin and Moscatelli, 1989; Sporn and Roberts, 1990).

HGF is a potent mitogen and motogen for epithelial cells (reviewed in Gherardi and Stoker, 1991) and promotes their three-dimensional organization (Montesano et al., 1991). We now show that HGF is also a potent angiogenic factor. The generation of new capillaries is a necessary event concomitant to the assembly of multicellular structures in physiological conditions and is implicated in several diseases, including development of solid tumors and metastases. The multiple effects on epithelial cells matched to the angiogenic properties, point to the critical role of HGF in these processes.

We thank W. Birchmeier for suggesting the use and for providing the pBAT vector and Neuro-2A cell line, G. C. Panzica for the assistance in the evaluation of scattering experiments, C. Ponzetto for reading the manuscript. The excellent secretarial help of Antonella Cignetto is gratefully acknowledged.

This work was supported by the Associazione Italiana Ricerche sul Cancro (AIRC), the Consiglio Nazionale delle Ricerche (CNR: target project ACRO), by the Istituto Superiore di Sanita' (V AIDS Project) and by Telethon Foundation. E. Bochietto and M. Olivero are recipient of an AIRC and a Ghirotti Foundation fellowship.

Received for publication 2 April 1992 and in revised form 7 July 1992.

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