Journal of Cellular Biochemistry 78:487–499 (2000)

Overexpression of a Secretory Form of FGF-1 Promotes MMP-1-Mediated Endothelial Cell Migration

Charles R. Partridge, James R. Hawker, Jr., and Reza Forough*

Department of Medical Physiology and Cardiovascular Institute, Texas A&M University, Health Science Center, College Station, Texas 77843

Abstract A coordinated interaction between fibroblast growth factors (FGFs) and matrix metalloproteinases (MMPs) is implicated in migration of microvascular endothelial cells (ECs), an early stage of angiogenesis. Specifically, we investigated microvascular ECs migration in vitro, which can be initiated by the overexpression of a secretory form of the angiogenic fibroblast growth factor-1 (FGF-1) and mediated through the enzymatic activity of matrix metalloproteinase-1 (MMP-1). MMP-1 is a member of the MMP family with a propensity for degradation of interstitial type I collagen. We stably overexpressed a chimeric FGF-1 construct composed of the FGF-4 signal-peptide gene, linked in-frame to the FGF-1 coding frame gene (sp-FGF-1), in cultured postcapillary venular ECs. The presence of the biologically active form of FGF-1 was readily detected in the conditioned medium of ECs transfected with sp-FGF-1 construct as demonstrated by DNA synthesis assay. The sp-FGF-1-, but not the plasmid vector alone-transfected ECs, exhibited an altered morphology as demonstrated by their conversion from a classic cobblestone form to a fibroblastlike shape that featured prominent neuritelike extensions. Addition of the anti-FGF receptor 1 antibody (FGFR1 Ab) reverted the transformed phenotype of sp-FGF-1 transfectants. This suggests that the resulting phenotypic transformation in sp-FGF-1 transfectants requires an uninterrupted interaction between the FGF-1 ligand and its receptor. We studied migration of cells through matrices of either highly pure collagen I or reconstituted basement membrane (matrigel) and found that sp-FGF-1-transfected cells migrated two times and six times faster than the vector control transfectants in the respective matrices. We further demonstrated that the enhanced migration rate of sp-FGF-1-transfected EC coincided with the induction of their MMP-1 mRNA level and increased enzymatic activity. The enhanced migratory activity of sp-FGF-1 could be blocked with a selective inhibitor of MMP-1. These results suggest that the multipotent FGF-1 plays a key role in the early stages of angiogenesis, by mediating MMP-1 proteolytic activity. J. Cell. Biochem. 78:487–499, 2000.

Key words: FGF-1; secretion; MMP-1; endothelial cells; migration

Migration, growth, and tube formation of microvascular endothelial cells (ECs) are the three major steps of angiogenesis [Folkman and Shing, 1992]. To migrate, proliferate, and differentiate into new capillary tubes, the sprouting ECs must be able to selectively degrade the surrounding matrix macromolecules without producing a general dissolution of the tissue. Being a mitogen, motogen, and morphogen, the growth factor fibroblast growth factor-1 (FGF-1) possesses all the major characteristics to influence angiogenesis [Burgess and Maciag, 1989; Godspodarowicz, 1990]. Additionally, FGF precursors have been shown to regulate the gene expression of collagen I and its respective protease, matrix metalloproteinase-1 (MMP-1), in smooth-muscle cells [Kennedy et al., 1997]. Therefore, we propose that during angiogenesis, there is active remodeling of the extracellular matrix (ECM) that requires coordinated interaction between the FGF-1 (growth factor) and the MMP-1 (protease). This hypothesis is supported by the work of Wang et al. [1994], in which application of synthetic inhibitors of MMPs to block MMP proteolytic activity has proven effective in restraining tumor growth in animals. MMP-1, also known as interstitial collagenase, is a major member of the MMP family, primarily because of its specificity for type I collagen, the prominent collagen

Grant sponsor: American Heart Association, Texas Affiliate; Grant number: 97G-804 and 98BG026.

*Correspondence to: Reza Forough, Department of Medical Physiology, Texas A&M University, Health Science Center, Room 350, Reynolds Medical Building, College Station, TX 77843. E-mail: forough@tamu.edu

Received 21 December 1999; Accepted 31 January 2000

of the interstitial matrix. In addition, MMP-1 can degrade collagen types II, III, VII, and X [Matrisian, 1992]. Regulation of MMP-1 can occur at three levels: 1) at the promoter level, 2) through activation of secreted proenzyme, and 3) through specific interaction with tissue inhibitors of matrix metalloproteinases, which are the natural inhibitors of MMPs. Recently, it has been shown that the FGF-2 (bFGF), the other primary member of the FGF family, can tightly regulate MMP-1 promoter activity [Aho et al., 1997]. These authors further suggested that FGF-2 induction of MMP-1 involves AP-1 transcription factors, because the deletion of the AP-1 binding site can abolish the MMP-1 promoter activity [Aho et al., 1997].

The polypeptide growth factor FGF-1 has the ability to induce metabolic and phenotypic changes in EC required to stimulate angiogenesis in both in vivo and in vitro models. FGF-1, also known as acidic FGF, is a prototypic member of the FGF family, which currently includes 18 members [Slavin, 1995; Smallwood et al., 1996; Emoto et al., 1997; Hartung et al., 1997]. Unlike the remaining members of this expanding family, the two prototypes FGF-1 and FGF-2 lack conventional signal peptides for secretion [Burgess and Maciag, 1989]. The addition of known signal peptide sequences to FGF-1 and FGF-2 results in the generation of modified versions of the growth factors that have potent transforming activities [Rogelj et al., 1988; Blam et al., 1988; Forough et al., 1993]. Presumably, this transforming activity is the result of a constitutive interaction between the modified growth factor and its cognate receptor because of establishment of an external or internal autocrine loop [Sporn and Roberts, 1985; Browder et al., 1989; Yayon and Klagsbrun, 1990]. In general, FGF-1 signal transduction is initiated by the binding of the natural form of the growth factor to its corresponding receptor at the cell surface and continues with an immediate activation of the receptor protein tyrosine kinase domains [Lee et al., 1989; Friesel et al., 1989; Ullrich and Schlessinger, 1990]. Therefore, the pleiotropic effects of most of the members of FGF's family have been studied in the angiogenic model systems, where exogenously added growth factor has been the sole angiogenic stimulus [Montesano et al., 1986; Cornelius et al., 1995]. Conversely, we have used an overexpression system of the secretory version of FGF-1 (sp-FGF-1) whereby a constant supply of the growth factor is synthesized and secreted by the transfectants. This system is advantageous in defining the molecular events that lead to FGF-induced/MMP-mediated-migration of ECs. In spite of the current knowledge concerning the activity of FGF and MMP, the intracellular FGF/MMP signaling mechanisms that regulate EC migration are less well understood. For example, it has been shown that although FGF-2 stimulates migration of low-density seeded microvascular ECs on type I collagen-coated plates, it reduces migration of these cells when seeded under the same conditions at a high cell density [Hoying and Williams, 1996]. In light of the foregoing observations, we have examined the effects of overexpression of sp-FGF-1 on EC migration in vitro. Our studies show that overexpression of FGF-1 protein stimulates EC migration, in part, through regulation of the MMP-1 gene induction and collagenolytic activity.

MATERIALS AND METHODS

Constructions of sp-FGF-1 Plasmid

We previously have described construction of the signal-peptide-containing FGF-1 [Forough et al., 1991, 1993]. Briefly, the signal-peptide used corresponds to the published sequence of one of the secretory members of the FGF family known as FGF-4 or human stomach tumor [Taira et al., 1987] or Kaposi sarcoma (KS3) [Delli-Bovi et al., 1987] growth factor. We chose the FGF-4 signal peptide sequence for our construct because the FGF-4 oncogene in its native form encodes a secreted growth factor with 30–40% homology to FGF-1. In addition, the polymerase chain reaction (PCR) was used to add a Kozak sequence (CCACCATGG) to the final construct for maximal eukaryotic translational efficiency [Kozak, 1986]. The expression vector pMEXneo containing murine sarcoma virus long terminal repeat and the SV40 polyadenylation site [Martin-Zanca et al., 1989] was chosen for the stable transfection of the sp-FGF-1 into microvascular ECs.

Cell Culture and Transfection

Endothelial cells isolated from 15-μm-diameter postcapillary venules of the bovine heart were a gift from Dr. H. Granger, Texas A&M University [Shelling et al., 1988]. They were grown in Dulbecco's Modified Eagle's Me-

dium (DMEM) (GIBCO/BRL, Grand Island, NY) containing high glucose and L-glutamine supplemented with 10% (vol/vol) fetal bovine serum (FBS) (HyClone, Logan, UT), 100 U/ml penicillin G, and 100 μg/ml streptomycin (GIBCO/BRL). This cell strain was chosen because postcapillary venules are the major sites for angiogenesis [Diaz-Flores et al., 1992]. Plasmids were amplified in *Escherichia coli* and purified using the Qiagen MaxiPrep kit (Qiagen; Chatsworth, CA). For stable transfections, approximately 24 h before transfection, ECs were seeded at 2×10^5 cells/9.6 cm^2 surface area of each well of a six-well tissue culture plate in 10% FBS/DMEM so that they were approximately 80% confluent the next day. TransIT-LT1 polyamine transfection reagent (PanVera, Madison, WI) was used for the delivery of sp-FGF-1 plasmids into the cells. For DNA/polyamine complex formation, 6 μl TransIT-LT1 was added dropwise to 100 μl serum-free and antibiotic-free DMEM in a sterile plastic tube and left at room temperature (RT) for 5 min. Subsequently, 1.5 μg plasmid DNA was added to the diluted TransIT, and incubated for an additional 5 min at RT. Next, media was removed from the wells and cells were washed with phosphate-buffered saline (PBS). The mixture of the DNA/polyamine complex was added to the cells and incubation continued at 37°C with 5% CO_2 for 5 h. Media were then replaced with fresh 10% FBS-DMEM for incubation overnight. The transfected cells were trypsinized and split at a 1:10 ratio in selection media made of 10% FBS-DMEM and 800 μg/ml G418 (GIBCO/BRL). The selection medium was changed three times a week and after 7 to 10 days, colonies of G418-resistant cells were recovered with cloning cylinders and grown separately. Furthermore, we demonstrated the authenticity of our stably transfected endothelial cells by their unique ability to uptake acetylated low-density lipoprotein, a specific marker for EC (data not shown).

For the experiments directed at reversing the sp-FGF-1 cells transformed phenotype, each sp-FGF-1 and control cells were seeded at densities of 1×10^5 cells/well of a six-well tissue culture plate in DMEM supplemented with 10% FBS (vol/vol). Cell were allowed to attach and spread for 5 h at 37°C with 5% CO_2. The culture media were then replaced with fresh 10% FBS/DMEM containing 5 μg/well of a commercial polyclonal antibody (Ab) made against a synthetic peptide corresponding to a region in the extracellular domain of the chicken high-affinity FGF receptor (Upstate Biotechnology, Lake Placid, NY). Culture media were replaced with fresh 10% FBS/DMEM containing the FGFR Ab every other day, and pictures were taken 7 days after the cell seeding and Ab treatment.

RNA Extraction and Northern Analysis

Total cellular RNA was extracted by using the protocol of Chomczynski and Sacchi [1987]. Aliquots of total RNA (20 μg) were fractionated on 1.2% agarose gel containing 2.2 M formaldehyde and transferred to Zeta probe nylon membranes (BioRad, Richmond, CA) and ultraviolet (UV) cross-linked using a Stratalinker UV crosslinker (Stratagene, La Jolla, CA). The membranes were prehybridized at 65°C for 2 h in a hybridization phosphate buffer containing 1% bovine serum albumin (BSA), 7% sodium dodecyl sulfate (SDS), 0.5 M NaH_2PO_4 pH 7.0, 1 mM EDTA, and 20% formamide supplemented with 100 μg/ml denatured salmon sperm DNA (5 Prime-3 Prime, Boulder, CO) [Church and Gilbert, 1984]. Subsequently, the prehybridized membranes were probed with FGF-1 cDNA labeled by [α-^{32}P]dCTP (Amersham, Arlington Heights, IL) using a random primer kit (GIBCO/BRL). After hybridization, the membranes were first washed twice for 15 min each in wash buffer A containing 0.5% BSA, 5% SDS, 40 mM NaH_2PO_4 pH 7.0, 1 mM EDTA and then washed twice for 15 min each in wash buffer B containing 1% SDS, 40 mM NaH_2PO_4 pH 7.0, 1 mM EDTA. The membranes were air dried and exposed to film for 24 h at −70°C. For quantification, X-ray films were scanned with the BioRad Gel-Doc 1000 using the Multianalyst version 1.0 program (BioRad).

Protein Extraction and Western Analysis

Cells from confluent 100-mm plates were washed with PBS and lysed in 1.0 ml lysis buffer made of 10 mM Tris HCl, pH 7.5, 10 mM $MgCl_2$, 1% Triton-X100 supplemented with the following protease inhibitors: 2 mM PMSF and 10 μg/ml aprotinin. The cell lysates were transferred into an Eppendorf tube and centrifuged for 5 min at 14,000 rpm at 4°C. The supernatant was incubated with 100 μl of a hydrated

suspension of heparin-Sepharose (GIBCO/BRL) overnight with rotation at 4°C. The mixture was centrifuged briefly and the pellet was boiled for 5 min in 25 μl of Laemmli sample buffer. After a brief centrifugation, the supernatant was subjected to a 12.5% SDS–polyacrylamide gel electrophoresis. The proteins were transferred onto a nitrocellulose membrane (BioRad), and the blot was incubated with 1 μg/ml of an affinity-purified polyclonal antibody made against bovine aFGF (R&D System, Minneapolis, MN). The blot was further processed for Western analysis following the manufacturer's recommendations using the Super Signal kit (Pierce, Rockford, IL).

Mitogenic Activity Assay

A 3T3 cell DNA synthesis assay using a modified enzyme-linked immunosorbent assay for bromodeoxyuridine (BrdU) incorporation [Muir et al., 1990] was used to test the biological activity of the sp-FGF-1 protein secreted into the EC culture medium. Briefly, NIH 3T3 were plated at a density of 1×10^4 cells/well in 96-well tissue culture plates in 0.5% FBS/DMEM and were starved for 72 h. The quiescent 3T3 cells were then stimulated with aliquots of 24 h serum-free conditioned media collected from confluent sp-FGF-1 or vector control transfected ECs for 18 h. After this period, BrdU (Sigma) was added to 10 μM final concentration, and incubation was continued for an additional 2 h. The assay was terminated by fixing the 3T3 cells with 70% ethanol for 20 min. Each well was incubated with 2 M HCl for 10 min at 37°C to denature the DNA, followed by the addition of 200 μl of the neutralizing solution of 0.1 M borate buffer pH 9. Each well was incubated with 50 μl of 2% goat serum (GIBCO/BRL) made in PBS for 15 min at 37°C to block nonspecific sites. Subsequently, a final concentration of 1 μg/ml of mouse anti-BrdU Ab (Dako, Carpinteria, CA) was added to each well and the incubation continued for 60 min at 37°C. After several washes in PBS containing 0.1% Triton X-100, 0.4 μg/ml of goat anti-mouse IgG peroxidase conjugate (Pierce) serving as secondary Ab was added. Several washes with the PBS/Triton solution were performed as before and were followed by the addition of 100 μl/well of tetramethylbenzidene (KPL, Gaithersburg, MD), an appropriate substrate for peroxidase. Absorbance of the samples was measured at 650 nm wavelength using a BioRad Model 550 microplate reader.

Migration Assay

Migration of the transfectants was assayed by a modification of the Boyden chamber method using microchemotaxis chambers (Neuro Probe, Cabin John, MD) and PVP-free polycarbonate filters with pores of 10 μm (Nuclepore, Palo Alto, CA). Each filter was precoated with type I collagen (Vitrogen 100; Celtrix, Santa Clara, CA). A 40-μl volume of 10% FBS/DMEM serving as the chemoattractant was placed in each well of the lower chamber. Confluent transfectants were trypsinized and suspended in serum-free DMEM at a concentration of 2×10^4 cells/40 μl volume. A 40-μl volume of each cell suspension was placed in the upper chamber, and the chamber was incubated for 6 h at 37°C in a 5% CO_2 incubator. The filter was removed, and the cells on the upper side of the filter were scraped off. The cells that had migrated through the collagen layer to the lower side of the filter were fixed in methanol, stained with Diff-Quick staining solution (Baxter, Deerfield, IL), and counted under a microscope (×100) for quantitation of transfected ECs migration. Migration activity was expressed as the mean number of cells that had migrated per high-power field.

When matrigel was used for coating the filter membranes, the stock matrigel solution (Collaborative Biomedical Products, Bedford, MA) was diluted in serum-free DMEM (1:3) before being applied to the membrane. The coated matrigel-coated filter membrane was incubated for 1 h at 37°C for polymerization to proceed. Subsequent steps for the cell seeding and the migration assay were performed as described above.

RT-PCR

For RT-PCR, we have designed the following sense and antisense oligonucleotides corresponding to the published bovine MMP-1 sequence [Tamura et al., 1994], and the sequence of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (unpublished data; Mertens and Muriuki, GenBank Accession No. U85042) serving as the internal control: sense MMP-1 5′-TGCTCATGCTTTTCAACCAG-3′ and the antisense MMP-1 5′-TCCACTTCTGGGTACAAGGG-3′; sense GAPDH 5′-TTGTCAGC-

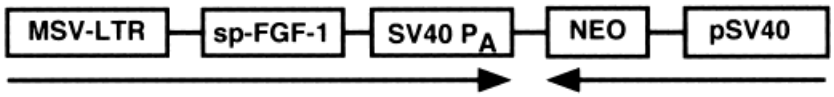

Fig. 1. Structures of the secretory version of fibroblast growth factor-1 (sp-FGF-1) expression plasmid. Signal-bearing FGF-1 is expressed from the long terminal repeat (LTR) of the murine sarcoma virus (MSV) promoter. G418 resistance (NEO) is expressed from the SV40 promoter.

ATGCCTCCTGCACC-3′ and the antisense GAPDH 5′-AACTGGTCCTCAGTGTAGCCT-AG-3′. All oligos for this study were synthesized by our core facility at Texas A&M University (Gene Technologies Laboratory; Texas A&M Core Molecular Biology Facilities, College Station, TX). We have successfully amplified MMP-1 and GAPDH using the "touch down" PCR conditions [Roux and Hecker, 1997]. For RT, 1.0 μg of total RNA was incubated with 0.5 μg of a 12-mer oligo dTTP serving as a primer in the presence of MuLV enzyme at 37°C for 1 h. For PCR, 1/20 volume of the cDNA generated by the RT was used in the following condition: cycle 1 was 95°C for 5 min, cycle 2 was 94°C for 1 min, 61°C for 1.5 min, and 72°C for 1 min, then cycle 2 repeated for five times, cycle 3 was 94°C 1 min 10 s, 65°C for 1 min 10 s, 72°C for 1 min, then cycle 3 repeated for 21 times. An additional 10-min extension at 72°C was added at the end of the last cycle of the PCR. We subsequently gel purified and cloned both amplified MMP-1 and GAPDH fragments and confirmed their authenticity by DNA sequencing using the Sanger method [Sanger et al., 1977].

MMP-1 Activity Assay

MMP-1 activity assays were performed essentially according to the protocol described by Netzel-Arnett et al. [1991]. Briefly, the 24-h serum-free conditioned media were harvested and dialyzed in 50 mM Tris HCl, pH 7.5 at 4°C overnight to remove the pH indicator present in DMEM. Subsequently, the media were concentrated using Centricon-10 (Amicon, Beverly, MA). Total protein concentration for each sample was measured using a BCA kit (Pierce Co., Rockford, IL). Each concentrated conditioned medium sample was treated with 1 mM PCMB [*p*-(chloromercuri)benzoate (Sigma, St. Louis, MO)] for 1 h at 23°C before the MMP-1 assays to activate the latent forms of MMP-1. Pure MMP-1 (a gift from Dr. H. Welgus, Washington University, St. Louis, MO) was used as a positive control. Assays for measuring the MMP-1 activity in each concentrated conditioned medium were performed in a buffer containing 50 mM tricine, pH 7.5, 0.2 M NaCl, 10 mM $CaCl_2$ at 23°C for 18 h using the fluorogenic peptide substrate DNP-Pro-Leu-Ala-Leu-Trp-Ala-Arg (Amersham) (12 μM per reaction) [Netzell-Arnett et al., 1991]. We measured the hydrolysis of the MMP-1 peptide substrate in each reaction (190 μl) at the indicated times using a Perkin-Elmer model LS-5 fluorometer using 280-nm excitation (slit width 3 nm) and 360-nm emission (slit width 10 nm).

Statistical Analysis

Student *t* tests (StatView, version 4.5; Abacus, Berkeley, CA) were used to determine statistical differences. A value of $P < 0.05$ was considered significant.

RESULTS

Overexpression of Signal Peptide-Containing FGF-1 in Cultured Postcapillary Venular EC

We used the sp-FGF-1 chimeric construct (Fig. 1) to stably transfect microvascular ECs grown in culture. We decided to use this particular microvascular EC as the host target because postcapillary venules are the major sites for angiogenesis, fluid exchange between the plasma and the interstitium, and tumor cell entry and exit [Diaz-Flores et al., 1992]. After the lipid-mediated transfection, several independent clones as well as pools of G418-resistant bovine ECs stably transfected with the sp-FGF-1 or vector alone were obtained. Total RNA obtained from these cells was analyzed by Northern blotting for recombinant FGF gene expression (Fig. 2A). The detected mRNA size of 500 bp for sp-FGF-1 recombinant construct was particularly advantageous because we could easily distinguish these transcripts from any possible endogenous bovine FGF-1 mRNA of 4.8 kb [Jaye et al., 1986, 1987; Burgess et al., 1986]. Western analysis of heparin-adsorbing proteins collected from the

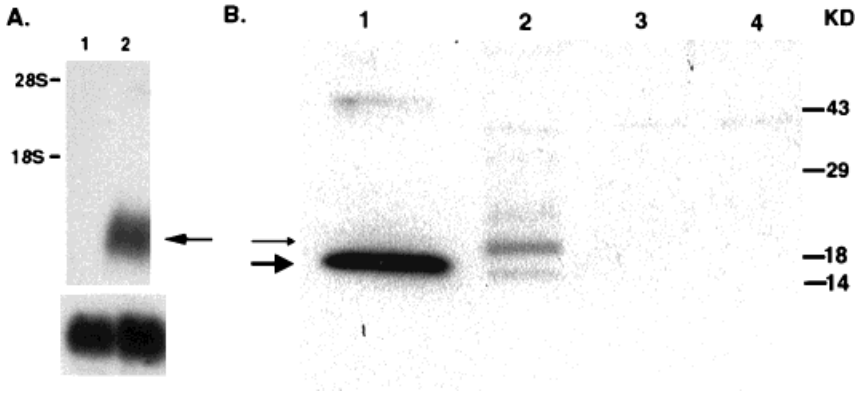

Fig. 2. Expressions of secretory version of fibroblast growth factor-1 (sp-FGF-1) RNA and protein in transfected bovine endothelial cells. **A:** Northern blot analysis of total RNA isolated from vector alone- (**lane 1**) and sp-FGF-1- (**lane 2**) transfected cells, probed for human FGF-1. Arrow points to recombinant sp-FGF-1 mRNA. The FGF mRNA signal was normalized to AEBP1 mRNA levels (bottom insert). **B:** Heparin-adsorbing proteins collected from the cell extracts processed for the Western analysis using a polyclonal antibody specific for FGF-1. **Lane 1:** 20-ng recombinant FGF-1 serving as a positive control; **lane 2:** sp-FGF-1-transfected endothelial-cell (EC) extracts; **lane 3:** vector alone-transfected EC extracts; **lane 4:** nontransfected bovine EC extracts. Upper arrow indicates unprocessed FGF-1 retaining its heterologous signal peptide. Lower arrow indicates the final processed form of FGF-1.

transfected cell extracts demonstrated the presence of sp-FGF-1 gene products (Fig. 2B).

Furthermore, the serum-free conditioned media from sp-FGF-1 but not the vector control transfected ECs stimulated DNA synthesis in NIH 3T3 cells, suggesting that the secreted extracellular form of the FGF-1 has retained its biological activity (Fig. 3).

Postcapillary Venular ECs Transfected With Signal Sequence-Bearing FGF-1 Exhibit Altered Phenotype in Culture

Cultured bovine postcapillary venular ECs with 15-μm diameter were used for the overexpression of sp-FGF-1 construct. Our immediate observation was the striking shift in EC morphology from a cobblestoned cell shape in normal parental cells to a highly elongated fibroblast-like morphology with prominent neuritelike extensions in sp-FGF-1 overexpressing cells (Fig. 4). Assumption of this altered phenotype is what we have termed the "angiogenic phenotype," consistent with loss of the side-by-side organization of the normal cells reported in more invasive cells of various cancers [Darnell et al., 1986]. Interestingly, exogenous addition of the FGFR1 Ab to sp-FGF-1 cell cultures reverted the transfected cells to a nontransformed phenotype. This is presumably because of blocking the autocrine stimulation through inhibition of the interaction between FGF and its cognate cell surface receptor (Fig. 5).

Postcapillary Venular ECs Transfected With Signal Sequence-Bearing FGF-1 Exhibit Increased Migration Property

The process of angiogenesis and tumor cell invasion share functional similarities, including alteration in adhesion and enhanced proteolysis of the matrix, which are required for cell migration [Stetler-Stevenson and Corcoran, 1997]. We wanted to determine whether the "angiogenic phenotype" could be correlated with enhanced cell migration. We measured the migration behavior of sp-FGF-1 transfectants, because the migration of ECs is indicative of the start of angiogenesis. This behavior was measured by comparing migration capacity of sp-FGF-1 and vector-alone transfected ECs through type I collagen- and matrigel-coated filter membrane of 10 μm pore size using a Neuro Probe 48-well chemotaxis chamber. sp-FGF-1 cells migrated two and six times faster than vector-alone transfected ECs through thin layers of the collagen and matrigel matrices, respectively (Fig. 6). The migration assay could not be performed in the absence of this minimal layer of matrix because the ECs fail to adhere to an uncoated polycarbonate filter membrane (data not shown). Because the migration assay is performed within a 6-h period, this eliminates the possibility that the increase in the number of migrated cells is

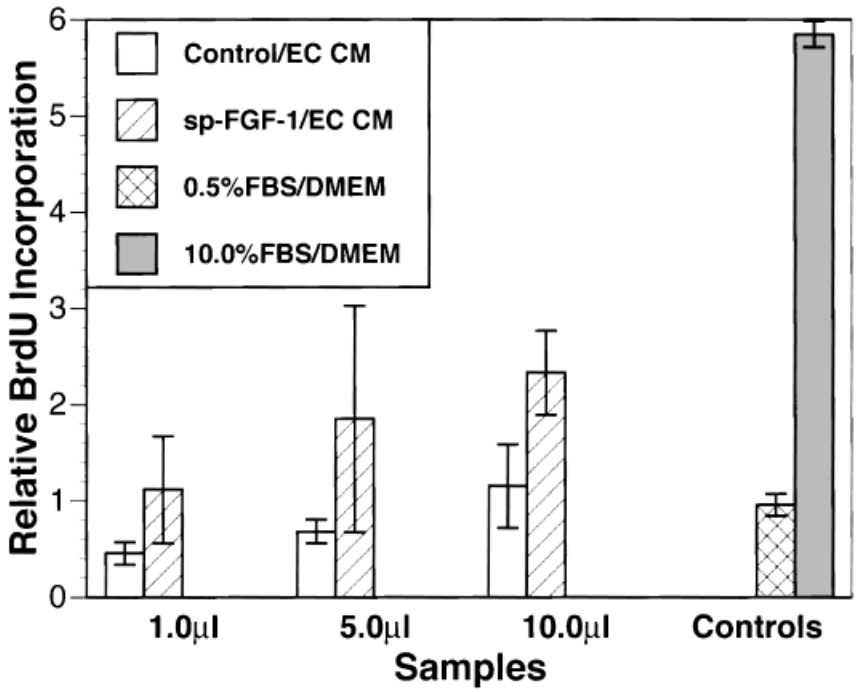

Fig. 3. DNA synthesis assay. NIH 3T3 cells were used to test the mitogenic activity of the conditioned media (CM) obtained from endothelial cells (ECs) transfected with secretory version of fibroblast growth factor-1 (sp-FGF-1) or vector alone. Each data point for relative bromodeoxyuridine (BrdU) incorporation into 3T3 cells represents the mean ± SE of three determinations. FBS, fetal bovine serum; DMEM, Dulbecco's Modified Eagle's Medium.

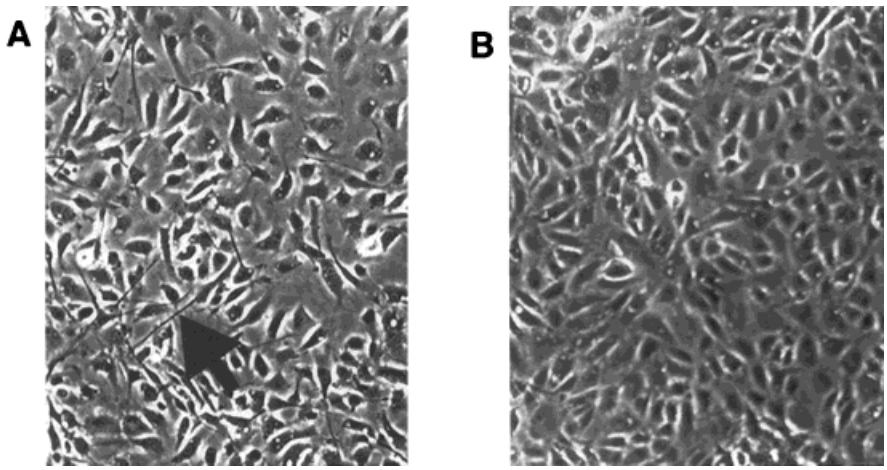

Fig. 4. The morphology of the transfected endothelial cells. **A:** Secretory version of fibroblast growth factor-1 (sp-FGF-1)-transfected cells. **B:** Vector alone-transfected cells. Arrow (A) indicates the neuritelike extensions present in sp-FGF-1-transfected bovine endothelial cells (×100 magnification).

merely caused by an increase in cell doubling.

Upregulation of MMP-1 in sp-FGF-1 Transfectants

The migration assay results show that downstream regulators of enhanced cell migration must be 1) subjected to regulation by FGF-1 protein, and 2) able to specifically degrade type I collagen as well as matrigel, which constitutes the ECM of the system. MMP-1, also known as interstitial collagenase, is a member of the MMP family, which is known to possess both of these requisites. We initially predicted that the en-

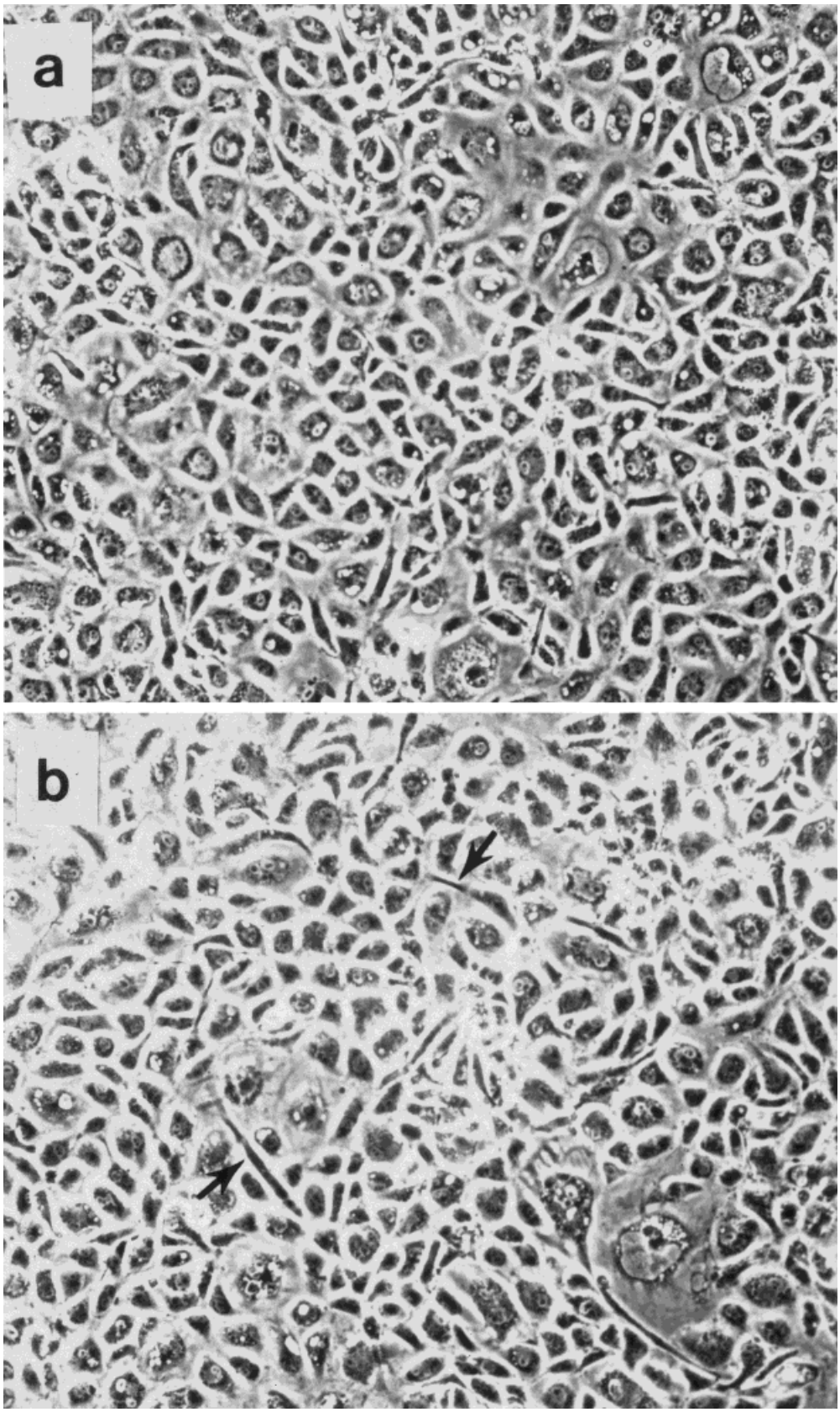

Fig. 5. Effect of FGFR1 antibody (Ab) on secretory version of fibroblast growth factor-1 (sp-FGF-1)-transfected endothelial-cell morphology. Sp-FGF-1-transfected cells were plated in a six-well tissue culture dish and treated (**a**) with 5 μg/well of the FGFR1 Ab or (**b**) without the Ab during a 7-day period as described in Materials and Methods. Arrows (b) indicate the neuritelike extensions present in sp-FGF-1 transfectants (×100 magnification).

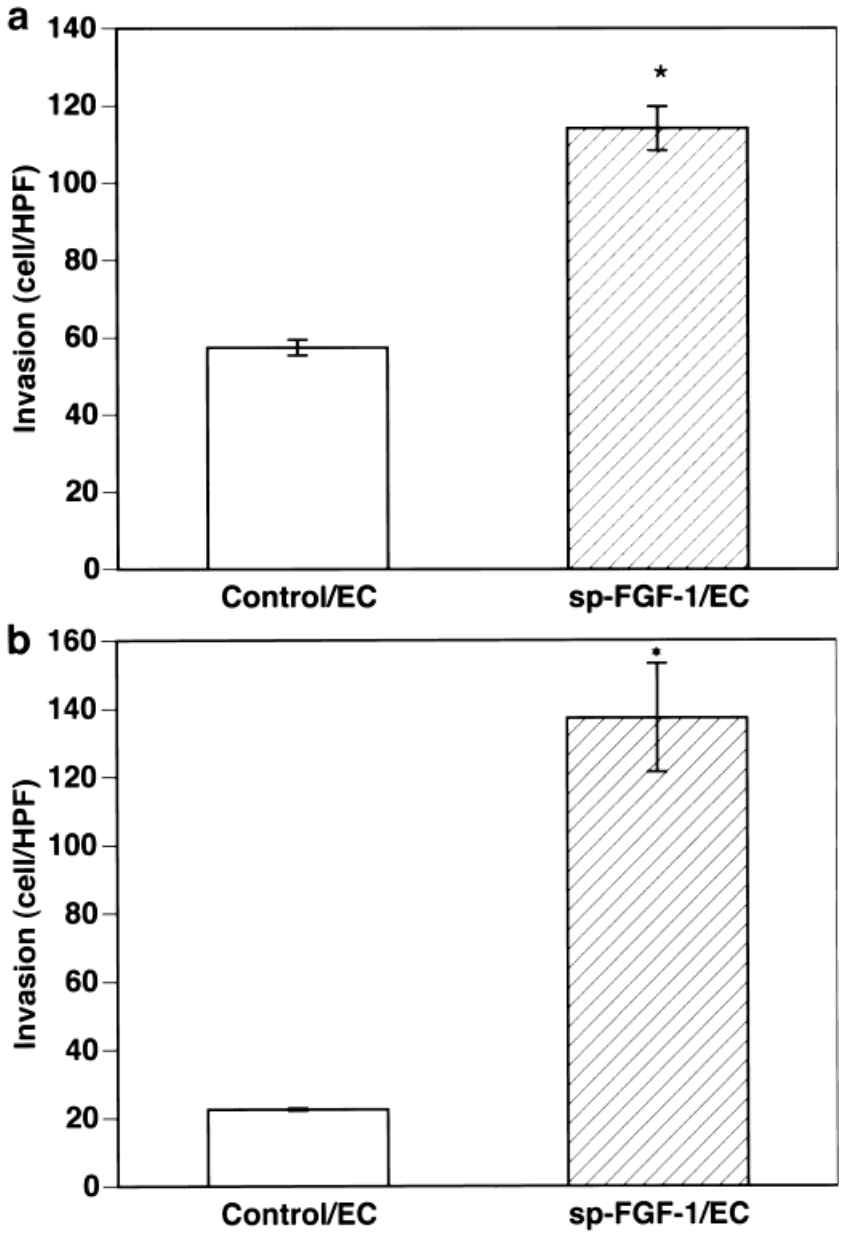

Fig. 6. Invasion of control and secretory version of fibroblast growth factor-1 (sp-FGF-1) cells through collagen and matrigel matrices using a modified Boyden Chamber assay. 2×10^4 of each transfectant was placed in the upper chamber, and media containing 10% serum was placed in the lower chamber. Number of cells migrated through a 10-μm pore-size polycarbonate filter precoated with (**a**) pure type I collagen and (**b**) matrigel after 6 h incubation at 37°C under 5% CO_2 was determined. HPF, high-power field. EC, endothelial cells. Results are shown as mean ± SD for triplicate determinations. $^*P < 0.05$, student *t* test. The data are representative of three independent experiments.

hanced migration of the sp-FGF-1-transfected ECs through type I collagen is caused by the shift toward an excessive proteolytic activity of MMPs. Consequently, we have demonstrated an increase in the MMP-1 mRNA expression in sp-FGF-1 compared to vector-alone transfected ECs using the RT-PCR method (Fig. 7). The collagenolytic activities of the bovine MMP-1 secreted in the conditioned media of transfected ECs were confirmed by a fluorescent assay using a synthetic substrate peptide for MMP-1 [Netzel-Arnett et al., 1991]. Briefly, in the unhydrolyzed state, the Trp fluorescence is efficiently quenched by the dinitrophenol (DNP) residing on the

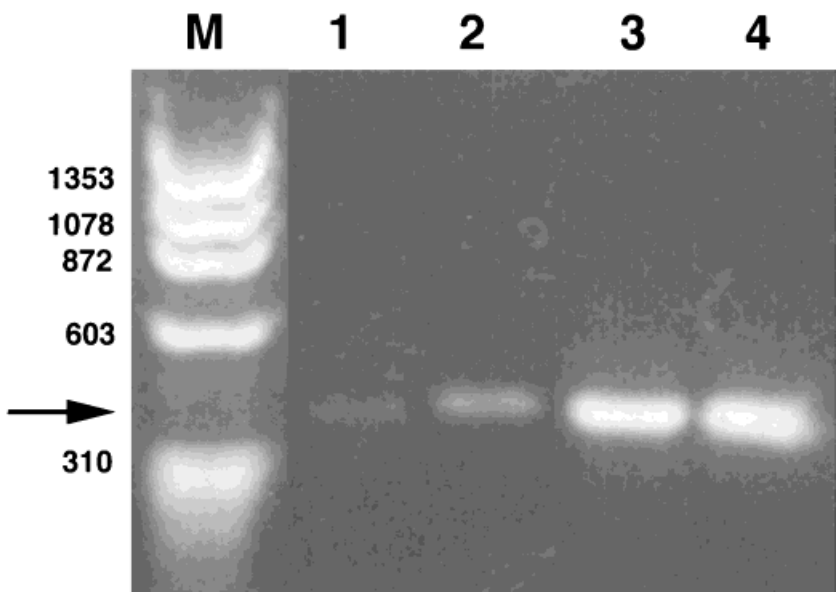

Fig. 7. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis of changes in matrix metalloproteinase-1 (MMP-1) mRNA levels in the transfected endothelial cells. Total RNA was isolated from confluent vector-alone-transfected cells (**lanes 1 and 4**); secretory version of fibroblast growth factor-1-transfected cells (**lanes 2 and 3**). RT-PCR was performed simultaneously on these RNAs for MMP-1 (**lanes 1 and 2**) and the internal control glyceraldehyde-3-phosphate-dehydrogenase (**lanes 3 and 4**) expression. M is the size marker phage $\phi\times174$ RF DNA digested with HaeII enzyme. The final PCR products were analyzed on a 1.2% agarose gel and stained with ethidium bromide. The data are representative of five independent experiments.

N-terminus of the peptide substrate (see Materials and Methods), presumably through resonance energy transfer [Stack and Gray, 1989]. Addition of MMP-1 cleaves the peptide substrate; therefore, DNP will no longer remain in the correct proximity and orientation to accept energy transfer from Trp. The final result will be a proportional increase in fluorescence emission as the hydrolysis of substrate peptide proceeds. By using this assay, we demonstrated significant increased MMP-1 collagenolytic activity in the conditioned media harvested from sp-FGF-1 transfectants when compared to the vector-alone groups (Table I). Furthermore, MMP-1 enzymatic activity was eliminated when concentrations of 50 μM of the metal chelating agent EDTA were incorporated into the assay reactions. Thus, these results indicated that FGF-1 can induce MMP-1 activity, which can in turn promote EC migration. To further confirm MMP-1 involvement in the migration of sp-FGF-1-transfected cells, we blocked the increased invasiveness of sp-FGF-1 transfectants using 1 μM RS-113456, a synthetic MMP inhibitor (a gift from Roche Bioscience, Palo Alto, CA) [Lovejoy et al., 1999], and demonstrated that the blockade of MMP-1 activity can reverse the invasion profile of sp-FGF-1-transfected ECs (Fig. 8).

TABLE I. Matrix Metalloproteinase-1 (MMP-1) Activity as Demonstrated by Hydrolysis of a Specific Fluorogenic Peptide Substrate[a]

Transfectant condition media	MMP-1 activity
Vector alone	315.06 ± 25.70
Signal peptide FGF-1	422.72 ± 9.35*

[a]Values are mean ± SE. n = 4 per transfectant. *$P < 0.05$, student t test. MMP-1 activity was determined by increased fluorescence accompanied hydrolysis of the peptide substrate by MMP-1 in the condition media. (λ_{em} = 280 nm, λ_{ex} = 360 nm). FGF-1, fibroblast growth factor-1.
Note: Incorporation of 50 μM metal chelator EDTA blocked MMP-1 activity ($P < 0.01$); data not shown.

DISCUSSION

Migration of ECs from the preexisting microvessels is a critical first step in angiogenesis. Both polypeptide growth factors and matrix-degrading proteases take part in the regulation of angiogenesis. Therefore, an understanding of how signaling is coordinated between these different entities helps us to better define the molecular mechanisms that underlie

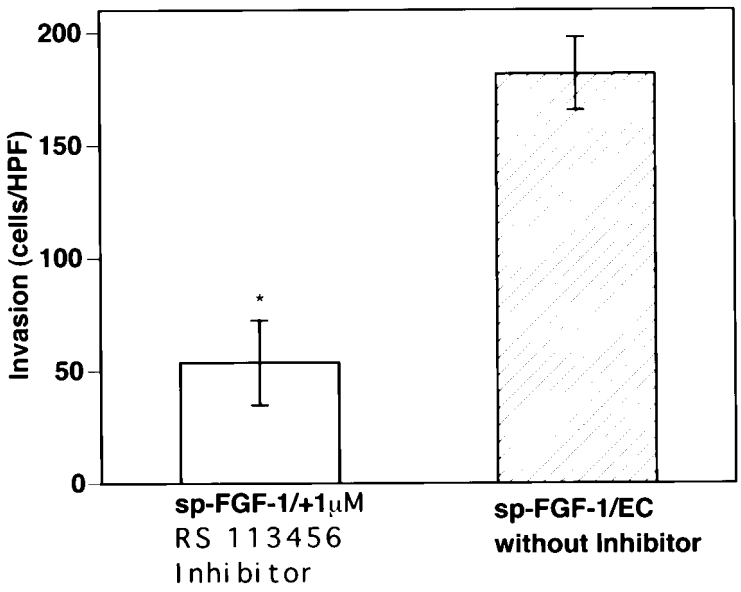

Fig. 8. Effect of matrix metalloproteinase (MMP) inhibitor on secretory version of fibroblast growth factor-1 (sp-FGF-1)-transfected endothelial cell (EC) invasion of collagen matrix using a modified Boyden Chamber assay. MMP inhibitor, RS113456, was added to a suspension of sp-FGF-1 transfectant before the addition to the upper chamber, and media containing 10% serum was placed in the lower chamber. Number of cells migrated through a 10-μm pore-size polycarbonate filter precoated with pure type I collagen after 6 h incubation at 37°C under 5% CO_2 was determined. HPF, high-power field. Results are shown as mean ± SD for six determinations. $^*P < 0.05$, student *t* test. The data are representative of two independent experiments.

the overall angiogenic process. For this purpose, we chose to overexpress a signal peptide-containing form of FGF-1, a known angiogenic growth factor with the ability to bring about metabolic and phenotypic changes in EC. The sp-FGF-1 was specifically chosen to determine the mechanism by which the autocrine/paracrine stimulation by sp-FGF-1 influences the EC migration. A retroviral-based plasmid vector (pMEXneo) was used for introducing the sp-FGF-1 gene into ECs because of its high efficiency and the persistence of gene expression.

Altered Phenotypes of sp-FGF-1 Overexpressors

We have observed that sp-FGF-1 overexpression in ECs alters the morphology of these cells in culture. The sp-FGF-1 transfectants assumed a fibroblastlike morphology with prominent neuritelike extensions and the loss of cell–cell contacts. This transformed phenotype resembles those of invasive tumor-derived cell lines. Similarly, others have shown that NIH-3T3 cells transfected with FGF-2 fused to IgG heavy chain exhibited a phenotype change and an enhanced motility [Rogelj et al., 1988]. Interestingly, on addition of the FGFR1 Ab into the culture media, sp-FGF-1 cells reverted to the same appearance as the nontransformed phenotype. It is possible that this Ab abrogated the growth factor/receptor interactions and thereby inhibited autocrine transformation.

Characterization of Migration Properties of Transfected ECs

EC migration is an early step in angiogenesis. Migratory ECs assume an angiogenic phenotype, a characteristic appearance that is also present in invasive cells. Because our bovine ECs assumed an angiogenic phenotype after transfection with the sp-FGF-1 construct, we tested the migratory behavior of sp-FGF-1-transfected ECs across a filter membrane precoated with type I collagen or matrigel using a chemotaxis assay. Concomitant with the morphological changes, sp-FGF-1 cells demonstrated a twofold and sixfold increase in migration in response to DMEM containing 10% FBS over vector alone cells in the respective matrices. Thus, the present study shows that targeting of FGF-1 for secretion, via the addition of a signal peptide, increases EC migration. We do not yet know how the addition of a signal peptide confers such a profound effect on cell migration. One explanation is that the presence

of signal peptide on FGF-1 generates a constitutive interaction of the FGF-1 and its cell surface receptor in similar fashion to the autocrine/paracrine loop described for malignant cell transformation [Sporn and Roberts, 1985].

Effect of sp-FGF-1 Overexpression on MMP-1

Much of what we know about cell migration comes from the study of malignant cells, where it is known that tumor invasion has an absolute requirement for focal dissolution of the matrix. Given also the knowledge that 1) most components, if not all, of ECM can be degraded by MMPs [Matrisian, 1990]; 2) FGF-2, which functions similarly to FGF-1, has the ability to stimulate MMP-1 synthesis in fibroblast cells [Chua et al., 1991]; and 3) the type I collagen matrix used in our study is a specific substrate for MMP-1, and furthermore its expression can be influenced by FGF [Tan et al., 1993], we focused on investigating FGF-induced MMP-1-mediated EC migration to identify downstream mediators of the FGF signaling pathway. In support of our hypothesis, MMP-1 mRNA and activity levels were greatly upregulated in sp-FGF-1 transfectants. Furthermore, the incorporation of RS113456, a strong and specific MMP inhibitor with an inhibitory constant (Ki) of 70 nM for MMP-1 [Lovejoy et al., 1999], significantly reduced the invasion rate of sp-FGF-1 transfectants. We propose that MMP-1 may facilitate FGF-driven EC migration by one or all of the following three mechanisms: 1) by lysis of collagenous matrix components and thus removal of the mechanical barrier at the leading edges of migrating ECs [Unemori et al., 1992]; 2) through release of ECM-bound growth factor release [Ruoslahti and Yamaguchi, 1991]; and 3) via cleavage of parental matrix peptides and the generation of new angiogenic matrix peptide fragments [Sage, 1997]. We are currently testing these three possibilities. One major contribution of this study is the demonstration of a coordinated interaction between FGF-1 and MMP-1 and its impact on the migration of ECs. Our observations suggest that FGF controls the proteolytic balance, which is critical to angiogenesis. Thus, in pathological angiogenesis, the FGF/MMP signaling pathway may regulate the extent of the nascent EC response to the migration and differentiation signals. Deliberate intervention in the growth factor/protease signaling pathway may be a plausible method to suppress pathological angiogenesis.

ACKNOWLEDGMENTS

We thank Dr. Nancy Dawson, Texas A&M University, for helpful comments on the manuscript and Dr. Robert Gray, University of Louisville School of Medicine, for helpful advice on MMP-1 activity assay. This work was supported by Research Grants 97G-804 (R.F.) and 98BG026 (J.H.) from the American Heart Association, Texas affiliate.

REFERENCES

Aho S, Rouda S, Kennedy SH, Qin H, Tan EM. 1997. Regulation of human interstitial collagenase (matrix metalloproteinase-1) promoter activity by fibroblast growth factor. Eur J Biochem 247:503–510.

Blam SB, Mitchell R, Tischer E, Rubin JS, Silva M, Silver S, Fiddes JC, Abraham JA, Aaronson SA. 1988. Addition of growth hormone secretion signal to basic fibroblast growth factor results in cell transformation and secretion of aberrant forms of the protein. Oncogene 3:129–136.

Browder TM, Dunbar CE, Nienhuis AW. 1989. Private and public autocrine loops in neoplastic cells. Cancer Cells 1:9–17.

Burgess WH, Maciag T. 1989. The heparin-binding (fibroblast) growth factor family of proteins. Annu Rev Biochem 58:575–606.

Burgess WH, Mehlman T, Marshak DR, Fraser BA, Maciag T. 1986. Structural evidence that endothelial cell growth factor beta is the precursor of both endothelial cell growth factor alpha and acidic fibroblast growth factor. Proc Natl Acad Sci USA 83:7216–7220.

Chomczynski P, Sacchi N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159.

Chua CC, Chua BH, Zhao ZY, Krebs C, Diglio C, Perrin E. 1991. Effect of growth factors on collagen metabolism in cultured human heart fibroblasts. Connect Tissue Res 26:271–281.

Church GM, Gilbert W. 1985. The genomic sequencing technique. Prog Clin Biol Res 177:17–21.

Cornelius LA, Nehring LC, Roby JD, Parks WC, Welgus HG. 1995. Human dermal microvascular endothelial cells produce matrix metalloproteinases in response to angiogenic factors and migration. J Invest Dermatol 105:170–176.

Darnell J, Lodish H, Baltimore D. 1986. Molecular cell biology. New York: Scientific American Books, 1035 p.

Delli Bovi P, Curatola AM, Kern FG, Greco A, Ittmann M, Basilico C. 1987. An oncogene isolated by transfection of Kaposi's sarcoma DNA encodes a growth factor that is a member of the FGF family. Cell 50:729–737.

Diaz-Flores L, Gutierrez R, Varela H. 1992. Behavior of postcapillary venule pericytes during postnatal angiogenesis. J Morphol 213:33–45.

Emoto H, Tagashira S, Mattei MG, Yamasaki M, Hashimoto G, Katsumata T, Negoro T, Nakatsuka M, Birnbaum D, Coulier F, Itoh N. 1997. Structure and expression of human fibroblast growth factor-10. J Biol Chem 272:23191–23194.

Folkman J, Shing Y. 1992. Angiogenesis. J Biol Chem 267:10931–10934.

Forough R, Engleka K, Thompson JA, Jackson A, Imamura T, Maciag T. 1991. Differential expression in Escherichia coli of the alpha and beta forms of heparin-binding acidic fibroblast growth factor-1: potential role of RNA secondary structure. Biochim Biophys Acta 1090:293–298.

Forough R, Xi Z, MacPhee M, Friedman S, Engleka KA, Sayers T, Wiltrout RH, Maciag T. 1993. Differential transforming abilities of non-secreted and secreted forms of human fibroblast growth factor-1. J Biol Chem 268:2960–2968.

Friesel R, Burgess WH, Maciag T. 1989. Heparin-binding growth factor 1 stimulates tyrosine phosphorylation in NIH 3T3 cells. Mol Cell Biol 9:1857–1865.

Gospodarowicz D. 1990. Fibroblast growth factor and its involvement in developmental processes. Curr Top Dev Biol 24:57–93.

Hartung H, Feldman B, Lovec H, Coulier F, Birnbaum D, Goldfarb M. 1997. Murine FGF-12 and FGF-13: expression in embryonic nervous system, connective tissue and heart. Mech Dev 64:31–39.

Hoying JB, Williams SK. 1996. Effects of basic fibroblast growth factor on human microvessel endothelial cell migration on collagen I correlates inversely with adhesion and is cell density dependent. J Cell Physiol 168:294–304.

Jaye M, Howk R, Burgess W, Ricca GA, Chiu IM, Ravera MW, O'Brien SJ, Modi WS, Maciag T, Drohan WN. 1986. Human endothelial cell growth factor: cloning, nucleotide sequence, and chromosome localization. Science 233: 541–545.

Jaye M, Burgess WH, Shaw AB, Drohan WN. 1987. Biological equivalence of natural bovine and recombinant human alpha-endothelial cell growth factors. J Biol Chem 262:16612–16617.

Kennedy SH, Rouda S, Qin H, Aho S, Selber J, Tan EM. 1997. Basic FGF regulates interstitial collagenase gene expression in human smooth muscle cells. J Cell Biochem 65:32–41.

Kozak M. 1986. Selection of translational start sites in eukaryotic mRNAs. In: Current communications in molecular biology. New York: Cold Spring Harbor, p 35–41.

Lee PL, Johnson DE, Cousens LS, Fried VA, Williams LT. 1989. Purification and complementary DNA cloning of a receptor for basic fibroblast growth factor. Science 245: 57–60.

Lovejoy B, Welch AR, Carr S, Luong C, Broka C, Hendricks RT, Campbell JA, Walker KA, Martin R, Van Wart H, Browner MF. 1999. Crystal structures of MMP-1 and -13 reveal the structural basis for selectivity of collagenase inhibitors. Nat Struct Biol 6:217–221.

Martin-Zanca D, Oskam R, Mitra G, Copeland T, Barbacid M. 1989. Molecular and biochemical characterization of the human trk proto-oncogene. Mol Cell Biol 9:24–33.

Matrisian LM. 1990. Metalloproteinases and their inhibitors in matrix remodeling. Trends Genet 6:121–125.

Matrisian LM. 1992. The matrix-degrading metalloproteinases. Bioessays 14:455–463.

Montesano R, Vassalli JD, Baird A, Guillemin R, Orci L. 1986. Basic fibroblast growth factor induces angiogenesis in vitro. Proc Natl Acad Sci USA 83:7297–7301.

Muir D, Varon S, Manthorpe M. 1990. An enzyme-linked immunosorbent assay for bromodeoxyuridine incorporation using fixed microcultures. Anal Biochem 185:377–382.

Netzel-Arnett S, Mallya SK, Nagase H, Birkedal-Hansen H, Van Wart HE. 1991. Continuously recording fluorescent assays optimized for five human matrix metalloproteinases. Anal Biochem 195:86–92.

Rogelj S, Weinberg RA, Fanning P, Klagsbrun M. 1988. Basic fibroblast growth factor fused to a signal peptide transforms cells. Nature 331:173–175.

Roux KH, Hecker KH. 1997. One-step optimization using touchdown and stepdown PCR. Methods Mol Biol 67:39–45.

Ruoslahti E, Yamaguchi Y. 1991. Proteoglycans as modulators of growth factor activities. Cell 64:867–869.

Sage H. 1997. Pieces of eight: bioactive fragments of extracellular proteins as regulators of angiogenesis. Trends Cell Biol 7:182–186.

Sanger F, Nicklen S, Coulson AR. 1992. DNA sequencing with chain-terminating inhibitors. Biotechnology 24: 104–108.

Sanger F, Nicklen S, Coulson AR. 1977. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467.

Schelling ME, Meininger CJ, Hawker JR Jr, Granger HJ. 1988. Venular endothelial cells from bovine heart. Am J Physiol 254:H1211–H1217.

Slavin J. 1995. Fibroblast growth factors: at the heart of angiogenesis. Cell Biol Int 19:431–444.

Smallwood PM, Munoz-Sanjuan I, Tong P, Macke JP, Hendry SH, Gilbert DJ, Copeland NG, Jenkins NA, Nathans J. 1996. Fibroblast growth factor (FGF) homologous factors: new members of the FGF family implicated in nervous system development. Proc Natl Acad Sci USA 93: 9850–9857.

Sporn MB, Roberts AB. 1985. Autocrine growth factors and cancer. Nature 313:745–747.

Stack MS, Gray RD. 1989. Comparison of vertebrate collagenase and gelatinase using a new fluorogenic substrate peptide. J Biol Chem 264:4277–4281.

Stetler-Stevenson WG, Corcoran ML. 1997. Tumor angiogenesis: functional similarities with tumor invasion. EXS 79:413–418.

Taira M, Yoshida T, Miyagawa K, Sakamoto H, Terada M, Sugimura T. 1987. cDNA sequence of human transforming gene hst and identification of the coding sequence required for transforming activity. Proc Natl Acad Sci USA 84:2980–2984.

Tamura M, Shimokawa H, Sasaki S. 1994. Primary structure of bovine interstitial collagenase deduced from cDNA sequence. DNA Seq 5:63–66.

Tan EM, Rouda S, Greenbaum SS, Moore JH Jr, Fox JW, Sollberg S. 1993. Acidic and basic fibroblast growth factors down-regulate collagen gene expression in keloid fibroblasts. Am J Pathol 142:463–470.

Ullrich A, Schlessinger J. 1990. Signal transduction by receptors with tyrosine kinase activity. Cell 61:203–212.

Unemori EN, Ferrara N, Bauer EA, Amento EP. 1992. Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells. J Cell Physiol 153:557–562.

Wang X, Fu X, Brown PD, Crimmin MJ, Hoffman RM. 1994. Matrix metalloproteinase inhibitor BB-94 (batimastat) inhibits human colon tumor growth and spread in a patient-like orthotopic model in nude mice. Cancer Res 54:4726–4728.

Yayon A, Klagsbrun M. 1990. Autocrine transformation by chimeric signal peptide-basic fibroblast growth factor: reversal by suramin. Proc Natl Acad Sci USA 87:5346–5350.