Jpn. J. Cancer Res. **90**, 647–654, June 1999

Roles of Extracellular Signal-regulated Kinase 1/2 and p38 Mitogen-activated Protein Kinase in the Signal Transduction of Basic Fibroblast Growth Factor in Endothelial Cells during Angiogenesis

Katsuhiro Tanaka, Mayumi Abe and Yasufumi Sato[1]

Department of Vascular Biology, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryou-machi, Aoba-ku, Sendai 980-8575

We examined the role of mitogen-activated protein (MAP) kinases in the signal transduction of basic fibroblast growth factor (bFGF)-mediated effects in endothelial cells (ECs). When MSS31 murine endothelial cells were stimulated with bFGF, three MAP kinase homologs, extracellular signal-regulated kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK) 1, and p38 MAP kinase were activated. The inhibition of the ERK1/2 pathway with PD98059, a specific inhibitor of MEK1, or of the p38 MAP kinase pathway with SB203580, a specific inhibitor of p38 MAP kinase, abrogated bFGF-mediated tube formation by MSS31 cells in type I collagen gel. Tube formation in type I collagen gel requires proliferation and migration of these cells, and degradation of the extracellular matrix by these cells. Both PD98059 and SB203580 inhibited bFGF-stimulated DNA synthesis as well as migration of MSS31 cells. Cell migration requires cytoskeleton reorganization and cell adhesion. bFGF induced actin reorganization and vinculin assembly in the focal adhesion plaque, both of which were inhibited by SB203580 but not by PD98059. bFGF induced the expression of the transcription factor ETS-1 in MSS31 cells. ETS-1 is responsible for the expression of proteases as well as integrin β3 subunit in ECs, and converts ECs to invasive phenotype. PD98059 inhibited this induction of ETS-1, whereas SB203580 did not. These results indicate that ERK1/2 and p38 MAP kinase are requisite for the signal transduction of bFGF in ECs. The roles of these two MAP kinase homologs are not identical, but these kinases work in a coordinated fashion.

Key words: bFGF — ERK1/2 — p38 MAP kinase — Angiogenesis

Angiogenesis is a process by which neo-vessels are formed from pre-existing ones. Although it is a physiological and fundamental process, especially in embryogenesis and reproduction, persistent angiogenesis plays an important role in the progression of certain pathological conditions, including malignant solid tumors. Recent studies reveal that tumor angiogenesis is indispensable for the growth of primary tumors, as well as distant metastasis.[1–3] Thus, angiogenesis has become an important subject in the research field of oncology.

Angiogenesis is thought to be controlled by the balance between angiogenic factors and angiogenesis inhibitors. A number of angiogenic factors have been identified to date, and basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) are recognized as representative angiogenic factors among them.[4, 5] VEGF is a specific mitogen for endothelial cells (ECs), and plays important roles in both physiological and pathological angiogenesis.[6, 7] In contrast, bFGF affects a broad spectrum of cell types including ECs.[2, 8] Although bFGF is not involved in the physiological angiogenic process of vascular development in the embryo, it is thought to play an important role in tumor angiogenesis.[9, 10] bFGF stimulates proliferation and migration of ECs, and also induces the transcription factor ETS-1, urokinase-type plasminogen activator (u-PA), and matrix metalloproteinase (MMP-1) in, and tube formation by, these cells.[11] However, the precise signal transduction system in ECs in relation to bFGF effects is ill-defined.

Members of the MAP kinase family, namely, extracellular signal-regulated kinase (ERK) 1/2, c-Jun N-terminal kinase (JNK) 1, and p38 MAP kinase, are central elements of post-receptor signal transduction pathways in mammalian cells including Ecs.[12] It is generally considered that growth factors primarily stimulate the ERK pathway, whereas many stress-related signals stimulate JNK and/or p38 MAP kinase pathways.[13] It has been reported that bFGF activates ERK1/2 in cells[14, 15] and that the ERK1/2 activation regulates the cell proliferation that is requisite for angiogenesis. p38 MAP kinase is involved in modulating the migration of human umbilical vein endothelial cells (HUVECs) through actin reorganization.[16]

In the present study, we investigated the roles of ERK1/2 and p38 MAP kinase in the signal transduction of bFGF effects in a murine endothelial cell line, MSS31. Our

[1] To whom correspondence should be addressed.
E-mail: y-sato@idac.tohoku.ac.jp

results indicate that both ERK1/2 and p38 MAP kinase are required for the signals of bFGF in angiogenesis. Although the roles of these two MAP kinases are not identical, they work in a coordinated fashion.

MATERIALS AND METHODS

Reagents Anti-JNK1 and anti-p38 MAP kinase antibodies and GST-ATF-2 (1-96) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); and myelin basic protein (MBP) and heat shock protein 25 (HSP-25), from Sigma Chemical Co. (St. Louis, MO). PD98059 was from New England Biolabs, Inc. (Beverly, MA) and SB203580, from Calbiochem (La Jolla, CA). Sheep polyclonal anti-MAP kinase activated protein (MAPKAP) kinase-2 antibody was from Upstate Biotechnology (Lake Placid, NY) and [γ-^{32}P]ATP, from Amersham Life Sciences Inc. (Arlington Height, IL). GST-c-Jun (1-79) expression plasmid was a generous gift from Dr. Masahiko Hibi (University of Osaka).[17] Human recombinant bFGF was provided by Takeda Chemical Industries, Ltd. (Osaka).

Cell culture MSS31, an endothelial cell line of mouse spleen, was kindly provided by Dr. Nobuaki Yanai (Tohoku University)[18] and grown in α-minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) (Summit Biotechnology, Ft. Collins, CO) and 100 μg/ml kanamycin.

Determination of ERK1/2 activity ERK1/2 activity was determined by an in-gel kinase assay as described.[19] Briefly, cells were extracted with lysis buffer (10 m*M* Tris-Cl [pH 7.5], 1% Triton X-100, 0.15 *M* NaCl, 1 m*M* EDTA, 50 m*M* NaF, 5 m*M* β-glycerophosphate, 1 m*M* Na_3VO_4, 2 m*M* phenylmethylsulfonyl fluoride). Samples containing equal amounts of protein were eluted in 4× sodium dodecyl sulfate (SDS) sample buffer and subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) with 0.25 mg/ml MBP co-polymerized in the gel under reducing conditions. After electrophoresis, the gel was washed with 20% 2-propanol, 50 m*M* Tris-Cl [pH 8.0] for 1 h. The gel was then denatured in 6 *M* guanidine chloride for 1 h and renatured in 50 m*M* Tris-Cl [pH 8.0] containing 0.04% Tween 40 and 5 m*M* 2-mercaptoethanol at 4°C overnight. The renatured gel was incubated with reaction buffer (40 m*M* HEPES [pH 7.5], 0.1 m*M* EGTA, 20 m*M* $MgCl_2$, 25 μ*M* ATP, and 10 μCi/ml of [γ-^{32}P]ATP) at room temperature for 1 h. Finally, the gel was washed with 5% trichloroacetic acid and 1% sodium pyrophosphate at room temperature several times, followed by drying and autoradiography.

Determination of JNK1, p38 MAP kinase, and MAPKAP kinase-2 activities JNK1, p38 MAP kinase, and MAPKAP kinase-2 activities were determined by an immune-complex kinase as described.[19] Briefly, cells were extracted in the same way as described for the in-gel kinase assay. For determination of JNK1 activity, cell lysates were incubated at 4°C overnight with 1 μg of rabbit antibody that recognizes the carboxyl terminus of human and murine JNK1. This was followed by the addition of protein A Sepharose 4 Fast Flow (Pharmacia Biotech AB, Uppsala, Sweden) and incubation for 1 h at 4°C. The resultant precipitates were washed 4 times with lysis buffer, and then twice with kinase buffer (20 m*M* HEPES [pH 7.5], 25 m*M* β-glycerophosphate, 0.1 m*M* Na_3VO_4, 2 m*M* dithiothreitol (DTT), 20 m*M* $MgCl_2$). The samples were incubated with 4 μg of GST-c-Jun (1-79) fusion protein, 20 μ*M* ATP, and 15 μCi of [γ-^{32}P]ATP for 15 min at 25°C in 30 μl of kinase buffer. The reaction was terminated by the addition of 10 μl of 4× SDS sample buffer, followed by SDS-PAGE under reducing conditions and autoradiography. For determination of p38 MAP kinase activity, cell lysates were immunoprecipitated with rabbit antibody recognizing the carboxyl terminus of human and murine p38 MAP kinase, and protein A Sepharose 4 Fast Flow. Two micrograms of GST-ATF-2 (1-96) fusion protein was used as a substrate. For determination of MAPKAP kinase-2 activity, cell lysates were immunoprecipitated with sheep antibody recognizing human, rat, and rabbit MAPKAP kinase-2, and protein G Sepharose 4 Fast Flow (Pharmacia Biotech AB). HSP-25 was used as a substrate.

Tube formation by MSS31 cells in type I collagen gel Tube formation by MSS31 cells in type I collagen gel was examined as described.[11] Briefly, MSS31 cells were seeded onto the surface of type I collagen gel (Nitta Gelatin, Osaka). After having reached confluence, the monolayers were incubated in α-MEM containing 1% FCS and 0.1% DMSO, PD98059 or SB203580 for 60 min; then human recombinant bFGF was added to a final concentration of 1 n*M*. After a 6-day incubation, tube-like structures formed in the gel were measured in terms of total tube length/field (×200) with NIH image software.

DNA synthesis DNA synthesis was analyzed with cell proliferation ELISA/5-bromo-2′-deoxyuridine (BrdU)-chemiluminescence (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's protocol. Briefly, MSS31 cells were seeded into 96-well microtiter plates, cultured in α-MEM containing 10% FCS for 24 h, washed with phosphate-buffered saline (PBS) and serum-free medium, and cultured with α-MEM containing 0.1% FCS for a further 24 h. After preincubation with 0.1% dimethyl sulfoxide (DMSO), PD98059 or SB203580, the medium was replaced with that containing bFGF for stimulation and BrdU was added simultaneously. After a 24-h incubation, the cells were fixed. Peroxidase (POD)-conjugated anti-BrdU antibody was then added and incorporated BrdU was measured by chemiluminescence.

Cell migration Cell migration was examined by wound migration assay as previously described.[11] Briefly, confluent cultures were preincubated with 0.1% DMSO,

PD98059 or SB203580 for 60 min and wounded with a razor blade. The cellular debris was subsequently removed by washing with PBS. The monolayers were incubated in α-MEM containing 0.1% FCS and 0.1% DMSO, PD98059 or SB203580, with or without 1 n*M* bFGF. After a 24-h incubation, the number of cells that had migrated across the edge of the wound was counted under ×100 magnification.

Fluorescence staining of F-actin and vinculin Cells were fixed with 3.7% formaldehyde in PBS for 30 min at room temperature and then treated with 0.1% Nonidet P-40 in PBS for 10 min. Nonspecific staining was blocked by incubating cells with 5% skim milk in PBS and normal goat serum. After the cells had been incubated with monoclonal anti-vinculin antibody (Seikagaku Co., Tokyo) for 60 min at room temperature, fluorescein-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) were applied to them, and incubation was carried out for 30 min at 37°C. Cells were mounted and microscopic observation was done with an LSM410 Laser Scan Microscope (Carl Zeiss, Jena, Germany).

Northern blot analysis Confluent monolayers of MSS31 cells in 60-mm plastic dishes were preincubated for 16 h in α-MEM containing 1% FCS, and then human recombinant bFGF was added. After incubation for 2 h, total RNA was extracted from the monolayers with ISOGEN (Nippon Gene Co., Ltd., Tokyo) used according to the manufacturer's protocol. Northern blotting was performed as described.[19] In brief, equal amounts of total RNA were loaded onto 1% agarose gel containing 2.2 *M* formaldehyde, electrophoresed, and transferred to a Hybond N^+ filter (Amersham Life Sciences Inc.). The filter was hybridized with ^{32}P-labeled probe in hybridization solution overnight at 42°C. The filter was washed in 2× SSC/0.1% SDS at room temperature and then in 0.2× SSC/0.1% SDS at 65°C. Autoradiography was carried out with an imaging plate and analyzed with a BAS2000 Image Analyzer (Fuji, Tokyo). Murine *ets-1* cDNA template (737 bps) was prepared by reverse-transcriptional PCR using the following primer pairs: sense, 5′-CCCTGGGTAAAGAATGCTTTCTCG-3′; antisense, 5′-GGACTGACAAGACTTATCAGTGAG-3′.

Calculations and statistical analysis The statistical significance of differences in the results was evaluated by use of unpaired ANOVA, and *P* values were calculated by Fisher's PLSD.

RESULTS

bFGF activates ERK1/2, JNK1, and p38 MAP kinase in MSS31 cells The activation of three MAP kinases, ERK1/2, JNK1, and p38 MAP kinase, in MSS31 after treatment with bFGF was analyzed. The in-gel kinase assay with MBP as a substrate showed activation of ERK1 (p44 MAP kinase) and ERK2 (p42 MAP kinase) with peaks of 35.0 and 10.3 fold activation, respectively, at 10 min, which levels gradually declined to 18.3 and 4.6 fold activation at 60 min (Fig. 1A). JNK1 and p38 MAP kinase activations were measured by the immune-complex kinase assay with GST-c-Jun (1-79) and GST-ATF-2 (1-96), respectively, as substrates. bFGF induced the activation of JNK1 and p38 MAP kinase with peaks of 4.1 and 2.2 fold activation, respectively, at 10 min (Fig. 1, B and C). The activation of p38 MAP kinase was transient, as the activity returned to the basal level by 30 min (Fig. 1C).

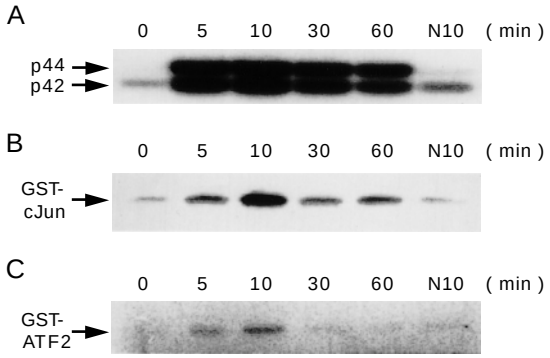

Fig. 1. Activation of MAP kinase homologs in response to bFGF. Confluent monolayers of MSS31 in plastic dishes were preincubated in α-MEM containing 0.1% BSA for 16 h and were then stimulated with bFGF (1 n*M*). After the indicated periods, the cells were lysed and the activities of MAK kinase homologs were determined as described in "Materials and Methods." (A) ERK1/2 activity was assayed by an in-gel kinase assay with MBP as a substrate. (B) JNK1 activity was assayed by an immune-complex kinase assay with GST-c-Jun (1-79) as a substrate. (C) p38 MAP kinase activity was assayed by an immune-complex kinase assay with GST-ATF-2 (1-96) as a substrate. The samples "N10" were harvested, in the same way, at 10 min after the addition of the vehicle without bFGF.

PD98059 and SB203580 inhibit ERK1/2 and p38 MAP kinase of MSS31 cells in a specific manner To clarify the roles of MAP kinases in the signal transduction of bFGF effects in ECs, we employed two specific inhibitors. PD98059 is a specific inhibitor of MEK1,[20] an upstream element of ERK1/2, and SB203580 is a specific inhibitor of p38 MAP kinase.[21] The specificity of these two inhibitors in our system was shown as follows: PD98059 at the concentration of 50 μM reduced the activation of ERK1/2 by 56%, whereas it did not affect the activation of p38 MAP kinase in ECs after the treatment with bFGF (Fig. 2A). SB203580 at the concentration of 10 μM reduced the bFGF-mediated activation of p38 MAP kinase by about 67%, as determined by the activation of MAPKAP kinase-

2, a downstream element of p38 MAP kinase, whereas it slightly augmented the activation of ERK1/2 (Fig. 2B). Neither of the inhibitors showed any apparent cytotoxic effect in terms of the cellular morphology.

PD98059 and SB203580 inhibit bFGF-stimulated tube formation by MSS31 cells To investigate whether ERK1/2 and/or p38 MAP kinase activities are required for bFGF-mediated angiogenesis, we analyzed tube formation by ECs in type 1 collagen gel. When MSS31 cells were cultured on type 1 collagen gel, they invaded the gel and formed tube-like structures in it. This tube formation by MSS31 cells was enhanced by the treatment with bFGF. In the presence of PD98059 or SB203580, tube formation was inhibited in a dose-dependent manner. PD98059 at the concentration of 50 μM completely inhibited tube formation, whereas inhibition by 10 μM SB203580 was partial but significant (Fig. 3). These results indicate that the activities of ERK1/2 and p38 MAP kinase are indispensable for angiogenesis.

PD98059 and SB203580 inhibit bFGF-stimulated DNA synthesis of MSS31 cells Angiogenesis is a complex phenomenon that relies on several independent properties of ECs, including cell proliferation, cell migration, and gene induction.

We next examined the effect of these inhibitors on DNA synthesis of MSS31 cells stimulated with bFGF. DNA synthesis was measured by BrdU incorporation as described in "Materials and Methods." PD98059 as well as SB203580 inhibited bFGF-stimulated DNA synthesis in a dose-dependent manner; and 50 μM PD98059 or 10 μM SB203580 reduced it to near the basal level (Fig. 4). Thus, both ERK1/2 and p38 MAP kinase activities are required for bFGF-stimulated DNA synthesis.

PD98059 and SB203580 inhibit bFGF-stimulated migration of MSS31 cells Cell migration was analyzed by the wound migration assay. bFGF stimulated the migration of MSS31 cells. PD98059 as well as SB203580 inhibited bFGF-stimulated cell migration in a dose-dependent manner (Fig. 5). Thus, both ERK1/2 and p38 MAP kinase activities are required for bFGF-stimulated cell migration.

SB203580 inhibits bFGF-stimulated actin reorganization and vinculin assembly of MSS31 cells Since coordinated regulation of actin reorganization and focal adhesion are required for cell migration,[22] we examined whether bFGF and MAP kinases are involved in these two phenomena. MSS31 cells were exposed to bFGF for 24 h. Thereafter, F-actin was stained with rhodamine-conjugated phalloidin, and vinculin with anti-vinculin antibody, as

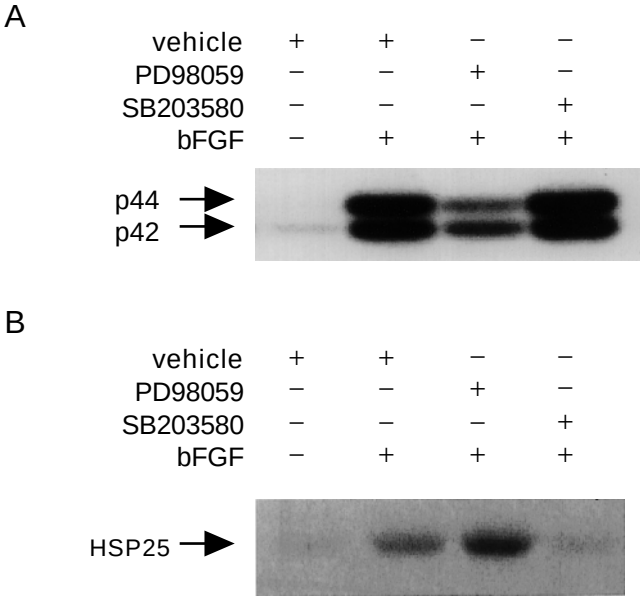

Fig. 2. Specific inhibition of ERK1/2 and p38 MAP kinase by PD98059 and SB203580. Confluent cultures of MSS31 cells in plastic dishes were preincubated in α-MEM containing 0.1% bovine serum albumin (BSA) for 16 h, and then for 60 min in the presence of vehicle alone (0.1% DMSO), PD98059 (50 μM), or SB203580 (10 μM). Ten minutes after addition of bFGF (1 nM), the cells were lysed, ERK1/2 activity was determined by the in-gel kinase assay (A), and MAPKAP kinase-2 activity was determined by the immune-complex kinase assay with HSP-25 as a substrate (B).

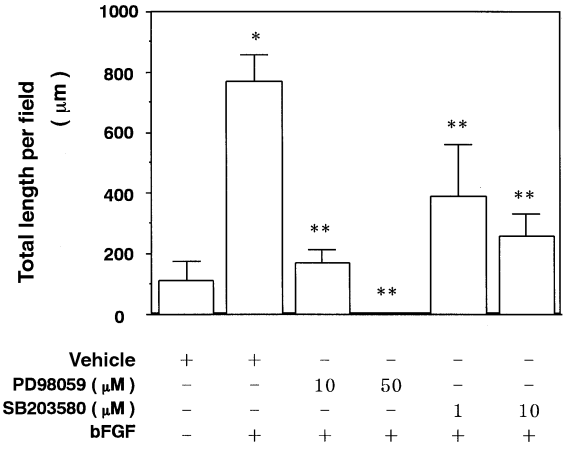

Fig. 3. Effect of PD98059 and SB203580 on tube formation by MSS31 in type 1 collagen gel. MSS31 cells were grown on the surface of type 1 collagen gel. After they had reached confluence, the medium was changed to α-MEM containing 1% FCS. The cells were then incubated with vehicle alone (0.1% DMSO), PD98059 or SB203580 for 60 min, after which bFGF (1 nM) was added. After 6 days of incubation in α-MEM containing 0.1% FCS and 0.1% DMSO, PD98059 or SB203580, the total length of the tube-like structures formed in the gel was measured from 4 fields. Values represent means and SEMs. $*$ $P<0.01$, vs. vehicle control, and $**$ $P<0.01$, vs. bFGF-treated cultures.

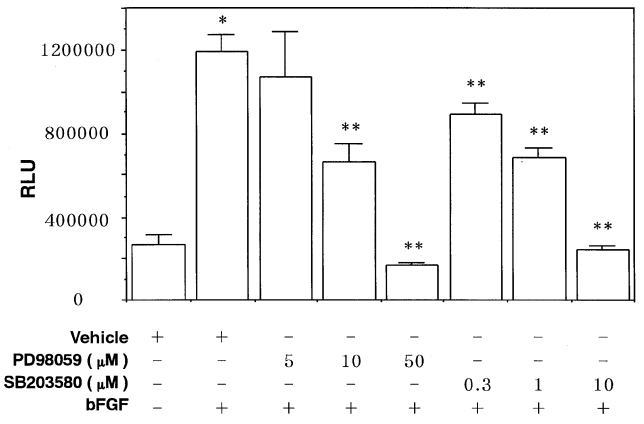

Fig. 4. Effect of PD98059 and SB203580 on proliferation of MSS31. MSS31 cells were preincubated in α-MEM containing 0.1% FCS for 16 h and were then treated with 0.1% DMSO, PD98059 or SB203580 for a further 60 min. Next, the medium was replaced with that containing bFGF (1 n*M*) for stimulation and BrdU was added simultaneously. After a 24-h incubation in the presence of vehicle alone (0.1% DMSO), PD98059 or SB203580, incorporated BrdU was measured by chemiluminescence. Values were expressed as means and SDs of 5 samples. * *P*<0.01, vs. vehicle control, and ** *P*<0.01, vs. bFGF-treated cultures.

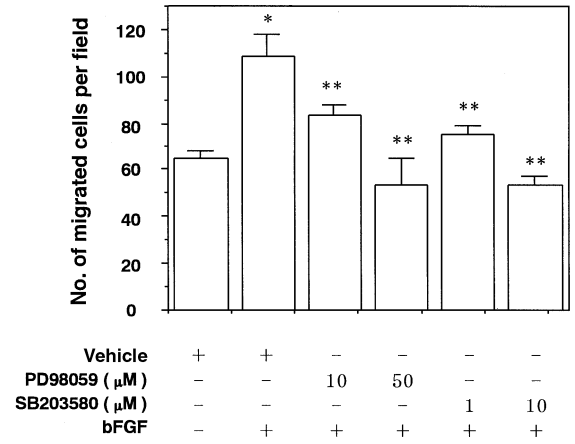

Fig. 5. Effect of PD98059 and SB203580 on migration of MSS31. Confluent monolayers of MSS31 cells were treated with vehicle alone (0.1% DMSO), PD98059 or SB203580 for 60 min and wounded with a razor blade. After a 24-h incubation in α-MEM containing 0.1% FCS and 0.1% DMSO, PD98059 or SB203580, with or without bFGF (1 n*M*), the number of cells that had migrated across the edge of the wound was counted under ×100 magnification. Values were expressed as means and SDs of 4 fields. * *P*<0.01, vs. vehicle control, and ** *P*<0.01, vs. bFGF-treated cultures.

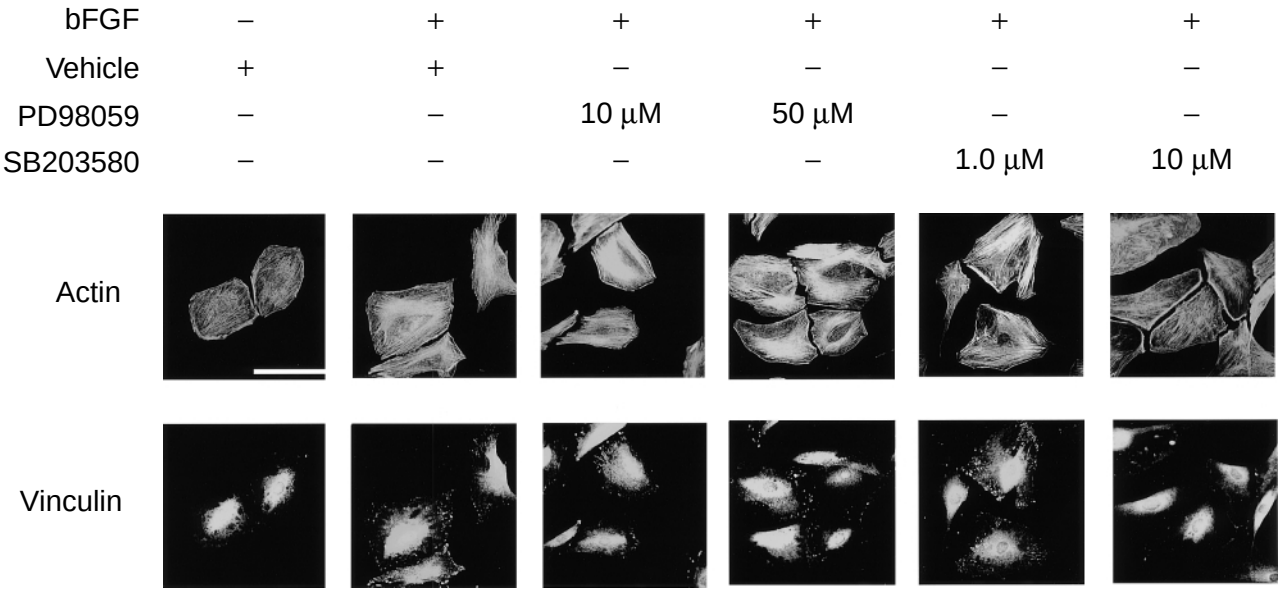

Fig. 6. Effect of PD98059 and SB203580 on actin reorganization and assembly in focal adhesion complex of MSS31. MSS31 cells were preincubated in α-MEM containing 0.1% FCS for 16 h and were then treated with 0.1% DMSO, PD98059 or SB203580 for a further 60 min. Next the medium was replaced with that containing bFGF (1 n*M*) for stimulation. After a 24-h incubation in the presence of 0.1% DMSO, PD98059 or SB203580 with or without bFGF, the cells were fixed and stained with rhodamine-conjugated phalloidin and anti-vinculin antibody. A bar represents 100 μm.

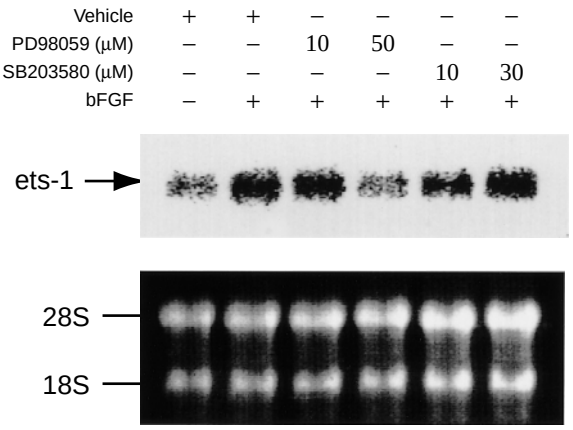

Fig. 7. Effect of PD98059 and SB203580 on the induction of *ets-1* mRNA. Confluent cultures of MSS31 cells in plastic dishes were preincubated in α-MEM containing 0.1% BSA for 16 h and then for 60 min in the presence of 0.1% DMSO, PD98059 or SB203580 for 60 min. After a 2-h incubation in α-MEM containing 0.1% FCS and 0.1% DMSO, PD98059 or SB203580, with or without 2 n*M* bFGF, total RNA was extracted and northern blotting was performed. An autoradiogram of *ets-1* mRNA is shown in the upper panel, and ethidium bromide-stained ribosomal RNA, in the lower panel.

described in "Materials and Methods." bFGF induced actin reorganization, resulting in stress fiber formation. Additionally, vinculin was visualized in both ends of stress fibers in bFGF-treated cells. Though PD98059 at a concentration of 50 μM did not inhibit stress fiber formation or vinculin assembly, SB203580 inhibited these processes at a concentration of 10 μM (Fig. 6).

bFGF-mediated induction of *ets-1* mRNA in MSS31 cells is mediated via the activation of ERK1/2 We have previously shown that ETS-1, the prototype of ets family transcription factors, is induced in ECs by angiogenic growth factors, including bFGF.[11] bFGF induced the expression of *ets-1* mRNA in MSS31 cells. Though 50 μM PD98059 inhibited *ets-1* mRNA induction, SB203580 showed no inhibition, even at 30 μM (Fig. 7). Thus, the induction of *ets-1* mRNA by bFGF in MSS31 cells was preferentially mediated via the ERK1/2 pathway.

DISCUSSION

We have examined the transduction of bFGF-mediated signals in ECs by using a murine EC line, MSS31, as a model. We found that bFGF activated three MAP kinase homologs, ERK1/2, JNK1, and p38 MAP kinase. Among them, ERK1/2 and p38 MAP kinase were found to be indispensable for bFGF-mediated angiogenesis *in vitro*, i.e., tube formation in type I collagen gel. They appeared to regulate proliferation and migration of, and gene induction in, ECs in a concomitant but coordinated fashion.

bFGF-stimulated DNA synthesis of ECs was inhibited by both PD98059 and SB203580, suggesting both ERK1/2 and p38 MAP kinase activities to be required for bFGF-stimulated DNA synthesis. It is generally accepted that the activation of ERK1/2 is required for DNA synthesis in various cell types,[23] and this effect of ERK1/2 is thought to be mediated via the induction of cyclin D1.[24] However, evidence that p38 MAP kinase regulates DNA synthesis is rather sparse. However, recent reports suggest that p38 MAP kinase is involved in the DNA synthesis of certain cell types.[25, 26] p38 MAP kinase is known to phosphorylate a number of transcription factors, resulting in the induction of a number of genes. For example, Elk-1 and ATF-2 are directly phosphorylated by p38 MAP kinase.[27] cAMP response element binding protein (CREB), on the other hand, is phosphorylated via MAPKAP kinase-2/3, an immediate downstream kinase of p38 MAP kinase.[28] However, it remains to be elucidated which molecule participates in the p38 MAP kinase-mediated DNA synthesis in ECs.

bFGF-stimulated migration of ECs was inhibited by both PD98059 and SB203580, suggesting that both ERK1/2 and p38 MAP kinase activities are required for bFGF-stimulated cell migration. Cell migration is a complex phenomenon that requires cytoskeleton-regulated cell motility, as well as cell adhesion.[22] bFGF elicited actin reorganization and the assembly of vinculin in focal adhesion plaques. SB203580 but not PD98059 inhibited these processes, indicating that p38 MAP kinase preferentially regulates these processes. Growth factors such as VEGF and oxidative stress are reported to induce actin reorganization in ECs via the activation of p38 MAP kinase.[16, 29] One of the downstream elements that p38 MAP kinase phophorylates is MAPKAP kinase 2/3, which is a serine/threonine kinase that has been suggested to play a role in actin reorganization.[30]

As PD98059 did not affect actin reorganization or vinculin assembly in bFGF-stimulated ECs, ERK1/2 was suggested to regulate bFGF-stimulated cell migration via a distinct mechanism. The fact that PD98059 but not SB203580 inhibited the induction of ETS-1 in ECs might be responsible for the inhibitory effect of PD98059 on cell migration. Recent work in our laboratory revealed that ETS-1 converts EC to an invasive phenotype by inducing the expression of proteases including u-PA, MMP-1, MMP-3, and MMP-9, as well as integrin β3 subunit as target genes in ECs.[11, 31] u-PA, MMP-1, MMP-3, and MMP-9 are responsible for degradation of extracellular matrices, whereas u-PA[32, 33] and integrin β3[34, 35] are responsible for cell migration. Therefore, the inhibitory effect of PD98059 on cell migration may derive at least in part from the impaired expression of ETS-1 target genes.

The 5′ ETS-1 promoter contains Sp1, Ap1, Ap2, and ETS binding sites,[36] and ETS-1 autoinduces the expression of ETS-1 via binding to ETS binding site.[37] Moreover, the Pointed domain within ETS-1, which is homologous to the *Drosophila* ETS family transcription factor Pointed, contains a target amino acid sequence for MAP kinases including ERK1/2, and once this sequence is phosphorylated, transactivation activity of ETS-1 is markedly enhanced.[38] These findings explain how ERK1/2 enhances the expression of ETS-1 in ECs. We previously reported that ETS-1 was induced in ECs after denuding injury and that this induction of ETS-1 was mediated via p38 MAP kinase, as SB203580 inhibited the induction of ETS-1.[19] The discrepancy of growth factor-mediated and denudation-mediated induction of ETS-1 in ECs raises the possibility that distinct sets of p38 MAP zkinase homologs are activated by growth factors and denuding injury. p38 MAP kinase contains 4 isoforms, namely p38α, p38β, p38γ and p38δ,[39–41] and the functions of these p38 MAP kinase homologs are not identical. For example, p38α induces apoptosis, whereas p38β induces hypertrophy of cardiomyocytes.[42] Further study is under way to determine what homologs of p38 MAP kinase are activated by various stimuli.

ACKNOWLEDGMENTS

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Japanese Ministry of Education, Science, Sports and Culture.

(Received March 1, 1999/Revised April 9, 1999/Accepted April 13, 1999)

REFERENCES

1) Fidler, I. J. and Ellis, L. M. The implications of angiogenesis for the biology and the therapy of cancer metastasis. *Cell*, **79**, 185–188 (1994).
2) Folkman, J. Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat. Med.*, **1**, 27–31 (1995).
3) Holmgren, L., O'Reilly, M. S. and Folkman, J. Dormancy of micrometastases: balanced proliferation and apoptosis in the presence of angiogenesis suppression. *Nat. Med.*, **1**, 149–153 (1995).
4) Folkman, J. and Shing, Y. Angiogenesis. *J. Biol. Chem.*, **267**, 10931–10934 (1992).
5) Zagzag, D. Angiogenic growth factors in neural embryogenesis and neoplasia. *Am. J. Pathol.*, **146**, 293–309 (1995).
6) Ferrara, N. and Davis-Smith, T. The biology of vascular endothelial growth factor. *Endocr. Rev.*, **18**, 4–25 (1997).
7) Mustonen, T. and Alitalo, K. Endothelial receptor tyrosine kinases involved in angiogenesis. *J. Cell Biol.*, **129**, 895–898 (1995).
8) Rifkin, D. B. and Moscatelli, D. Recent development in the cell biology of basic fibroblast growth factor. *J. Cell Biol.*, **109**, 1–6 (1989).
9) Hori, A., Sasada, R., Matsunami, E., Naito, K., Sakura, Y., Fujita, T. and Kozai, Y. Suppression of solid tumor growth by immunoneutralizing monoclonal antibody against human basic fibroblast growth factor. *Cancer Res.*, **51**, 6180–6184 (1991).
10) Brem, S., Tsanaclis, A. M., Gately, S., Gross, J. L. and Herblin, W. F. Immunolocalization of basic fibroblast growth factor to the microvasculature of human brain tumors. *Cancer*, **70**, 2673–2680 (1992).
11) Iwasaka, C., Tanaka, K., Abe, M. and Sato, Y. Ets-1 regulates angiogenesis by inducing the expression of urokinase-type plasminogen activator and matrix metaloproteinase-1 and the migration of vascular endothelial cells. *J. Cell. Physiol.*, **169**, 522–531 (1996).
12) Waskiewicz, A. J. and Cooper, J. A. Mitogen and stress response pathways: MAP kinase cascades and phosphatase regulation in mammals and yeast. *Curr. Opin. Cell Biol.*, **7**, 798–805 (1995).
13) Force, T., Pombo, C. M., Avruch, J. A., Bonventre, J. V. and Kyriakis, J. M. Stress-activated protein kinases in cardiovascular disease. *Circ. Res.*, **78**, 947–953 (1996).
14) Pages, G., Lenormand, P., L'Allemain, G., Chambard, J.-C., Meloche, S. and Pouyssegur, J. Mitogen-activating protein kinases p42mapk and p44mapk are required for fibroblast proliferation. *Proc. Natl. Acad. Sci. USA*, **90**, 8319–8323 (1993).
15) Gardner, A. M. and Johnson, G. L. Fibroblast growth factor-2 suppression of tumor necrosis factor α-mediated apoptosis requires Ras and the activation of mitogen-activated kinase. *J. Biol. Chem.*, **271**, 14560–14566 (1996).
16) Rousseau, S., Houle, F., Landry, J. and Huot, J. p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. *Oncogene*, **15**, 2169–2177 (1997).
17) Hibi, M., Lin, A., Smeal, T., Minden, T. and Karin, M. Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev.*, **7**, 2135–2148(1993).
18) Yanai, N., Satoh, T. and Obinata, M. Endothelial cells create a hematopoietic inductive microenvironment preferential to erythropoiesis in the mouse spleen. *Cell Struct. Funct.*, **16**, 87–93 (1991).
19) Tanaka, K., Oda, N., Iwasaka, C., Abe, M. and Sato, Y. Induction of Ets-1 in endothelial cells during reendothelialization after denuding injury. *J. Cell. Physiol.*, **176**, 235–244 (1998).
20) Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiei, A. R. PD098059 is a specific inhibitor of the activation of mitogen activated protein kinase kinase *in vitro* and *in vivo*. *J. Biol. Chem.*, **270**, 27489–27494 (1995).

21) Cuenda, A., Rouse, J., Doza, Y. N., Meiser, R., Cohen, P., Gallagher, T. F., Young, P. R. and Lee, J. C. SB203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.*, **364**, 229–233 (1995).
22) Lauffenburger, D. A. and Horwitz, A. F. Cell migration: a physically integrated molecular process. *Cell*, **84**, 359–369 (1996).
23) Cook, S. J. and McCormick, F. Kinetic and biochemical correlation between sustained p44ERK1 (44kDa extracellular signal-regulated kinase 1) activation and lysophosphatidic acid-stimulated DNA synthesis in Rat-1 cells. *Biochem. J.*, **320**, 237–245 (1996).
24) Weber, J. D., Raben, D. M., Phillips, P. J. and Baldassare, J. J. Sustained activation of extracellular signal-regulated kinase 1 (ERK1) is required for the continued expression of cyclin D1 in G1 phase. *Biochem. J.*, **326**, 61–68 (1997).
25) Crawley, J. B., Rawlinson, L., Lali, F. V., Page, T. H., Saklatvala, J. and Foxwell, B. M. J. T cell proliferation in response to interleukins 2 and 7 requires p38 MAP kinase activation. *J. Biol. Chem.*, **272**, 15023–15027 (1997).
26) Craxton, A., Shu, G., Graves, J. D., Saklatvala, J., Krebs, E. G. and Clark, E. A. p38 MAPK is required for CD40-induced gene expression and proliferation in B lymphocyte. *J. Immunol.*, **161**, 3225–3236 (1998).
27) Raingeaud, J., Whitmarsh, A. J., Barrett, T., Derijard, B. and Davis, R. J. MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. *Mol. Cell. Biol.*, **16**, 1247–1255 (1996).
28) Tan, Y., Rouse, J., Zhang, S. C., Cohen, P. and Comb, M. J. FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2. *EMBO J.*, **15**, 4629–4642 (1996).
29) Huot, J., Houle, F., Marceau, F. and Landry, J. Oxidative stress-induced actin reorganization mediated by the p38 mitogen-activated protein kinase/heat shock protein 27 pathway in vascular endothelial cells. *Circ. Res.*, **80**, 383–392 (1997).
30) Guay, J., Lambert, H., Gingras-Breton, G., Lavoie, J. N., Huot, J. and Landry, J. Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. *J. Cell Sci.*, **110**, 357–368 (1997).
31) Oda, N., Abe, M. and Sato, Y. ETS-1 converts endothelial cells to the angiogenic phenotype by inducing the expression of matrix metalloproteinases and integrin β3. *J. Cell. Physiol.*, **178**, 121–132 (1999).
32) Fibbi, G., Ziche, M., Morbidelli, L., Magnelli, L. and Rosso, M. D. Interaction of urokinase with specific receptors stimulates mobilization of bovine adrenal capillary endothelial cells. *Exp. Cell Res.*, **179**, 385–395 (1988).
33) Odekon, L. E., Sato, Y. and Rifkin, D. B. Urokinase-type plasminogen activator mediates basic fibroblast growth factor-induced bovine endothelial cell migration independent of its proteolytic activity. *J. Cell. Physiol.*, **150**, 258–263 (1992).
34) Leavesley, D. I., Schwartz, M. A., Rosenfeld, M. and Cheresh, A. Integrin β1- and β3-mediated endothelial cell migration is triggered through distinct signaling mechanisms. *J. Cell Biol.*, **121**, 163–170 (1993).
35) Liaw, L., Lindner, V., Schwartz, S. M., Chambers, A. F. and Giachelli, C. M. Osteopontin and αvβ3 integrin are coordinately expressed in regenerating endothelium *in vivo* and stimulate Arg-Gly-Asp dependent endothelial migration *in vitro*. *Circ. Res.*, **77**, 665–672 (1995).
36) Jorcyk, C. L., Watson, D. K., Mavrothalassitis, G. J. and Papas, T. S. The human ETS1 gene: genomic structure, promoter characterization and alternative splicing. *Oncogene*, **6**, 523–532 (1991).
37) Majerus, M. A., Bibollet-Ruche, F., Telliez, J. B., Wasylyk, B. and Bailleul, B. Serum, AP-1 and Ets-1 stimulate the human ets-1 promoter. *Nucleic Acids Res.*, **20**, 2699–2703 (1992).
38) Slupsky, C. M., Gentile, L. N., Donaldson, L. W., Mackereth, C. D., Seidel, J. J., Graves, B. J. and McIntosh, L. P. Structure of the Ets-1 pointed domain and mitogen-activated proten kinase phosphorylation site. *Proc. Natl. Acad. Sci. USA*, **95**, 12129–12134 (1998).
39) Jiang, Y., Chen, C., Li, Z., Guo, W., Gegner, J. A., Lin, S. and Han, J. Characterization of the structure and function of a new mitogen-activated protein kinase (p38β). *J. Biol. Chem.*, **271**, 17920–17926 (1996).
40) Li, Z., Jiang, Y., Ulevitch, R. J. and Han, J. The primary structure of p38γ a new member of p38 group of MAP kinases. *Biochem. Biophys. Res. Commun.*, **228**, 334–340 (1996).
41) Wang, X. S., Diener, K., Manthey, C. L., Wang, S., Rosenzweig, B., Bray, J., Delaney, J., Cole, C. N., Chan-Hui, P. Y., Mantlo, N., Lichenstein, H. S., Zukowski, M. and Yao, Z. Molecular cloning and characterization of a novel p38 mitogen-activated protein kinase. *J. Biol. Chem.*, **272**, 23668–23674 (1997).
42) Wang, Y., Huang, S., Sah, V. P., Ross, J., Jr., Brown, J. H., Han, J. and Chien, K. R. Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. *J. Biol. Chem.*, **273**, 2161–2168 (1998).