The Nonreceptor Protein-tyrosine Kinase c-Fes Is Involved in Fibroblast Growth Factor-2-induced Chemotaxis of Murine Brain Capillary Endothelial Cells*

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Fibroblast growth factor-2 (FGF-2)-induced migration of endothelial cells is involved in angiogenesis in vivo. However, signal transduction pathways leading to FGF-2-induced chemotaxis of endothelial cells are largely unknown. Previous studies have shown that the cytoplasmic protein-tyrosine kinase c-Fes is expressed in vascular endothelial cells and may influence angiogenesis in vivo. To investigate the contribution of c-Fes to FGF-2 signaling, we expressed wild-type or kinase-inactive human c-Fes in the murine brain capillary endothelial cell line, IBE (Immortomouse brain endothelial cells). Wild-type c-Fes was tyrosine-phosphorylated upon FGF-2-stimulation in transfected cells, whereas kinase-inactive c-Fes was not. Overexpression of wild-type c-Fes promoted FGF-2-independent tube formation of IBE cells. Tube formation was not observed with endothelial cells expressing kinase-inactive c-Fes, indicating a requirement for c-Fes kinase activity in this biological response. Expression of kinase-defective c-Fes suppressed endothelial cell migration following FGF-2 treatment, suggesting that activation of endogenous c-Fes may be required for the chemotactic response. Expression of either wild-type c-Fes or the kinase-inactive mutant did not affect the tyrosine phosphorylation of Frs2, Shc, or phospholipase C-γ, nor did it influence the kinetics of mitogen-activated protein kinase activation. These results implicate c-Fes in FGF-2-induced chemotaxis of endothelial cells through signaling pathways not linked to mitogenesis.

Angiogenesis is involved in many physiological and pathological processes, such as ovulation, embryogenesis, malignant tumor growth, retinopathies, and rheumatoid arthritis (1–3). In angiogenesis, activated endothelial cells produce proteases that digest the basement membrane of blood vessels, allowing them to migrate into interstitial tissue, proliferate, and finally form lumen-containing, tube-like structures (1, 4). These biological responses are tightly regulated by fibroblast growth factors (FGFs)1 and vascular endothelial growth factors, which mediate their effects by binding to specific receptor tyrosine kinases (5–7). These receptor tyrosine kinases are linked to a number of downstream effector molecules including components of the ubiquitous Ras/mitogen-activated protein kinase (Ras/MAPK) pathway and phospholipase C-γ.

The FGF receptor (FGFR) subfamily of receptor tyrosine kinases consists of four structurally related members, designated FGFR-1 through FGFR-4 (8, 9). All members of the family are composed of an extracellular domain with two or three immunoglobulin-like loops, a single transmembrane domain, and an intracellular kinase domain, which is divided by an insert region. Seven tyrosine residues in the cytoplasmic region of FGFR-1 have been identified as autophosphorylation sites (10). Of these, Tyr-766 in the C-terminal tail of FGFR-1 binds to the Src homology 2 domain of PLC-γ, leading to its tyrosine phosphorylation and activation (11, 12). Substitution of Tyr-766 with Phe resulted in slower internalization of FGFR-1, suggesting a function in receptor turnover (13).

FGFR-1 is also linked to several small G-protein/kinase cascades. Activation of the Ras pathway has been implicated in both proliferation and tube formation of cultured endothelial cells (14). The adaptor proteins Shc and FRS2 are tyrosine-phosphorylated by FGF-2 treatment, leading to recruitment of the Grb2/Sos guanine nucleotide exchange complex for Ras (14–16). Activated Ras binds to Raf-1, leading to activation of the extracellular signal regulated kinases Erk-1 and Erk-2 through the intermediate kinase mitogen-activated protein kinase/Erk kinase-1. The farnesyl transferase inhibitor, manumycin, the prenylated protein methyltransferase inhibitor, FTS, both attenuate tube formation, presumably by interfering with Ras function (14). The mitogen-activated protein kinase/Erk kinase inhibitor PD98059 suppressed proliferation of endothelial cells without affecting tube formation (14), suggesting that the differentiation signal diverges above the level of mitogen-activated protein kinase/Erk kinase activation. In addition to Shc, FRS2, and Grb2/Sos, the adaptor protein Crk also binds to the activated FGFR-1 through phosphotyrosine 463 in the juxtamembrane region. Crk in turn recruits C3G, a guanine nucleotide exchanger factor for Rap1 (17). Substitution of Tyr-463 with Phe disrupts FGF-2-induced DNA synthesis (17), implicating the Crk-C3G interaction in the mitogenic response.

The c-Fes protein-tyrosine kinase is structurally distinct from FGFRs and has been implicated in mitogenesis, angiogenesis, and chemotaxis in multiple cell types (18–20). This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture, Japan, and by National Institutes of Health Grant CA58667 (to T. E. S.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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c-Fes in Migration of Endothelial Cells

from c-Src, c-Abl, and other nonreceptor tyrosine kinases (reviewed in Ref. 18). c-Fes is expressed predominantly in hematopoietic cells, vascular endothelial cells, and some epithelial and neuronal cells (19). Several lines of evidence suggest that c-Fes may play a direct role in myeloid differentiation. Expression of c-Fes in the myeloid leukemia cell line K-562, an immature blast cell line devoid of c-Fes expression, results in growth suppression and terminal differentiation (20, 21). This result suggests that c-Fes expression is sufficient for differentiation in some cellular contexts. On the other hand, suppression of c-Fes expression with antisense oligonucleotides interferes with myeloid differentiation in HL-60 promyelocytes, and in some cases, it results in apoptosis instead (22, 23). Thus, c-Fes may be required for differentiation to occur. c-Fes may regulate differentiation in extrahematopoietic sites as well. Expression of an activated form of c-Fes bearing the N-terminal myristylation signal sequence from v-Src produced a marked increase in vascularity and hemangioma formation in transgenic mice (24). This result indicates that c-Fes is involved in vascular development in vivo. However, the precise roles of c-Fes in the regulation of cellular responses by endothelial cells are unknown.

In this report, we examined the effect of c-Fes expression on FGF-2-induced proliferation, chemotaxis, and tube formation of IBE cells, a murine brain capillary endothelial cell line (25). Transfected c-Fes was autophosphorylated upon FGF-2 stimulation, suggesting that FGF-2 may regulate c-Fes kinase activity in vivo. Expression of a kinase-inactive c-Fes mutant interfered with FGF-2-induced chemotaxis of IBE cells, suggesting that endogenous c-Fes activation may be important for this response. In addition, tube formation by IBE cells was stimulated by overexpression of wild-type c-Fes in the absence of FGF-2 treatment. These results indicate that c-Fes is both necessary and sufficient for FGF-2-induced cellular responses by endothelial cells.

EXPERIMENTAL PROCEDURES

Antibodies—Anti-c-Fes polyclonal antibody, anti-PLC-γ mixed monoclonal antibodies, and anti-phospho-tyrosine monoclonal antibody (4G10) were obtained from Upstate Biotechnologies, Inc. (Lake Placid, NY). Anti-FLAG monoclonal antibody (M2) was purchased from Sigma. Anti-phosphorylated MAPK polyclonal antibodies were obtained from New England BioLabs (Beverly, MA), and anti-MAPK polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell Culture—Murine brain capillary endothelial cells (IBE cells) isolated from H-2Kb-tsA58 transgenic mice (Immortomice) were grown from H-2Kb-tsA58 transgenic mice (Immortomice) in Ham's F-12 medium supplemented with 20% heat-inactivated fetal bovine serum (Life Technologies, Inc.). 75 μg/ml endothelial cell growth supplement (Sigma), 5 μg/ml bovine pancreas insulin (Sigma), and 10 ng/ml human recombinant epidermal growth factor (Roche Molecular Biochemicals) at 33 °C as described previously (25).

Transfection of FLAG-tagged c-Fes Constructs into IBE Cells—Lipo- somes, denoted TFL-3, -5, -6, and -8, were kindly provided by Dr. Hiroshi Kikuchi (Daiichi Pharmaceuticals, Tokyo, Japan). Empty pcDNAs (Invitrogen, San Diego, CA) or expression plasmids containing C-terminally FLAG-tagged wild-type c-Fes or kinase-inactive (K590E) c-Fes were used (26). Dried liposomes were resuspended in sterile Milli-Q water containing purified plasmid DNA (1 μg of DNA/10 μl cationic lipid) and left for 15 min at room temperature. IBE cells (2 × 10^6 cells/10-cm dish) were incubated with Ham's F-12 medium containing 1.5 μg/ml plasmid DNA for 7 h, and then the medium was changed to a fresh growth medium. On the third day, 0.4 mg/ml G418 (Life Technologies, Inc.) was added, and the culture was continued for 10 days. After selection, G418-resistant clones were expanded for analysis of expression of the c-Fes-FLAG constructs by immunoblotting.

Cell Proliferation Assay—The cell proliferation assay was performed as described previously, with some modifications (27). Briefly, transfected IBE cells were plated in human plasma fibronectin-coated 24-well culture plates at a density of 1.5 × 10^4 cells/cm² (3 × 10^5 cells/well) in Ham's F-12 medium containing 10% fetal bovine serum and cultured at 33 °C. The next day, the medium was changed to Ham's F-12 medium containing 0.25% fatty acid-free bovine serum albumin (BSA) with or without G418, and the culture was continued for 3 days. Cells were detached with trypsin, and the cells were counted using a hemocytometer. The number of cells in the untreated samples was set to 100%.

Chemotaxis Assay—Chemotaxis assay was performed using Transwell membrane filters (Corning Costar Japan, Tokyo, Japan). Membrane filters were coated with 8 μg/ml fibronectin. Ham's F-12 medium containing 0.25% BSA with or without the indicated concentrations of FGF-2 was added into lower wells of 24-well plates. Transfected IBE cells were harvested with trypsin, incubated with soybean trypsin inhibitor (Sigma), and washed. Cells were resuspended in Ham's F-12 medium containing 0.25% BSA at a density of 3 × 10^5 cells/ml. The membrane was then replaced with fresh Ham's F-12 medium containing 0.25% BSA with or without indicated concentrations of FGF-2 and cultured for 24 h. The culture medium was collected and centrifuged to remove cell debris, and aliquots of the medium were electrophoresed in SDS-polyacrylamide gels (10% polyacrylamide) under nonreducing conditions. Gels were washed extensively with phosphate-buffered saline containing 2.5% Triton X-100 and put horizontally on top of a 1.1% agarose gel containing 1.8% nonfat milk (as a source of casein) and 0.12 units/ml plasminogen (Calbiochem, La Jolla, CA) and incubated at 37 °C for 18 h. PA activity was visualized as translucent areas in the white agarose gel. The gels were photographed, and quantitation was performed using the NIH Image program, version 1.52. The intensity of the band in lanes of untreated cells was set to 100%.

Zymographic Assay for the Determination of Plasminogen Activator (PA) Activity—Zymographic assay for the determination of secreted PA activity was described previously (25). In brief, IBE cells were plated in fibronectin-coated 24-well plates and cultured in growth medium for 24 h at 33 °C. Once confluent, the cells were washed with Ham's F-12 medium three times and cultured in Ham's F-12 medium containing 0.25% BSA. Cells were plated on the upper wells (100 μl/well), and incubated for 4 h at 33 °C. Cells were washed, fixed in 100% methanol for 10 min at room temperature, washed again, and stained with Glemsa solution. Cells on the upper surface of the membranes were removed with cotton swabs, and the cells attached to the lower surface of the membranes were counted microscopically. The number of cells counted in untreated samples was set to 100%.

Tube Formation Assay—Tube formation assay was performed as described elsewhere (25). Briefly, IBE cells were cultured between two layers of type I collagen gels without serum or growth supplements in the presence or absence of 10 ng/ml FGF-2 at 33 °C for 18 h. Cultures were photographed under the phase-contrast microscope.

Immunoprecipitation and Immunoblotting—IBE cells grown to confluence in fibronectin-coated dishes were cultured in growth supplement-starved with Ham's F12 containing 20 units/ml aprotinin and 0.25% BSA overnight, followed by treatment in the presence or absence of 100 ng/ml FGF-2 for 8 min at 33 °C. Cells were washed once with Tris-buffered saline, pH 7.5, containing 100 μM orthovanadate on ice and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, containing 0.15 mM NaCl, 1% Triton X-100, 10 mM NaF, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.02% SDS, 100 μg/ml aprotinin, and 100 units/ml aprotinin). Clarified cell lysates were incubated with either anti FLAG M2 antibody, anti-PLC-γ antibody, or anti-Shc antibody followed by the adsorption to protein A-agarose beads. Alternatively, p130Cas-agarose beads (Upstate Biotechnologies, Inc.) were used to precipitate FRS-2. After washing, proteins were eluted from beads by heating in SDS-sample buffer and then separated by SDS-polyacrylamide gel electrophoresis. Proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA) and probed with the indicated antibodies. Antibody incubation was followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG, and detection was through enhanced chemiluminescence reaction (ECL, Amersham Pharmacia Biotech). Membranes were stripped of antibodies by soaking in 62.5 mM Tris-HCl, pH 6.8, containing 2% SDS and 0.7% 2-mercaptoethanol at 50 °C for 30 min. For some blots, total cell lysates were prepared by heating the cells in SDS sample buffer, and 50 μg of the resulting proteins extracts were electrophoresed and analyzed.

RESULTS

FGF-2 Induces Tyrosine Phosphorylation of c-Fes—The model system for all studies was the IBE capillary endothelial cell line, which was established from the brains of transgenic mice expressing a temperature-sensitive mutant of SV40 large T antigen. At the permissive temperature (33 °C), IBE cells proliferate, migrate, secret urokinase-type plasminogen activator (u-PA), and

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and form tube-like structures in response to FGF-2 treatment. IBE cells express mainly FGFR-1 with small amounts of FGFR-2 and -4, and the signals for FGF-2-induced biological responses require the intracellular region of the FGFR-1 (25). Recent studies have established marked c-Fes expression in vascular endothelial cells (19). To examine the expression of endogenous c-Fes in IBE cells, total cell lysates were prepared from human umbilical cord vein endothelial cells (HUVEC) which have been shown previously to express high levels of c-Fes (24). As shown in Fig. 1A, endogenous c-Fes expression was readily detectable in both cells. To examine the role of c-Fes in FGF-2-induced cellular responses of IBE cells, IBE cells were transfected with expression plasmids containing either WT or K590E c-Fes cDNAs, and G418-resistant clones were isolated. c-Fes proteins were immunoprecipitated (IP) from clarified lysates of 2 × 10⁶ cells using the M2 anti-FLAG monoclonal antibody. Immunoprecipitated proteins were resolved in SDS-polyacrylamide gel electrophoresis, and c-Fes proteins were visualized by immunoblotting (IB) with the anti-FLAG antibody. C, c-Fes is tyrosine-phosphorylated by FGF-2 treatment in IBE cells. IBE cells expressing the WT (clone 6-1) and K590E (clone 5-8) forms of c-Fes were incubated with Ham’s F-12 medium containing 0.25% BSA overnight. Cells (1 × 10⁷ cells each) were then incubated in the presence or absence of 100 ng/ml FGF-2 for 8 min, rinsed, and lysed. Fes proteins were immunoprecipitated with the anti-FLAG antibody, resolved by SDS-polyacrylamide gel electrophoresis, and immunoblotted with the anti-phosphotyrosine monoclonal antibody 4G10 (top). After stripping, the membranes were reprobed with anti-FLAG antibody (bottom).
This result suggests that signals for u-PA secretion in response to FGF-2 treatment do not involve c-Fes. We next assessed the role of c-Fes in the chemotactic response of endothelial cells to FGF-2. As shown in Fig. 5, FGF-2 stimulated marked chemotaxis of mock-transfected IBE cells in a dose-dependent manner, consistent with previous results (25). Expression of wild-type c-Fes did not affect the chemotactic response toward FGF-2. In contrast, expression of the kinase-inactive mutant of c-Fes completely inhibited the chemotactic response of IBE cells to FGF-2. Previous studies have shown that this kinase-inactive mutant of c-Fes suppresses wild-type c-Fes autophosphorylation in vitro (30), suggesting that it may be capable of suppressing endogenous c-Fes activation by FGF-2 in IBE cells. These results implicate c-Fes in FGF-2-induced chemotaxis.

In a final series of experiments, FGF-2-induced tube forma-
Data were obtained from two independent experiments. Open bars, Data are shown as the means that had migrated to the lower surface of the membranes were counted. Surface of the membranes were wiped away with cotton swabs, and cells washed, and stained with Giemsa solution. Cells present on the upper without FGF-2. After incubation for 4 h, cells were fixed with methanol, wells prefilled with Ham’s F-12 medium containing 0.25% BSA with or without FGF-2. After incubation for 4 h, cells were fixed with methanol, washed, and stained with Giemsa solution. Cells present on the upper surface of the membranes were wiped away with cotton swabs, and cells that had migrated to the lower surface of the membranes were counted. Data are shown as the means ± S.D. for quadruplicate wells. Comparable data were obtained from two independent experiments. Open bars, no FGF-2; hatched bars, 5 ng/ml FGF-2; solid bars, 50 ng/ml FGF-2.

**DISCUSSION**

In this report, we show for the first time that the nonreceptor protein-tyrosine kinase encoded by the c-fes proto-oncogene plays a role in FGF-2-induced angiogenic responses in cultured vascular endothelial cells. Overexpression of wild-type c-Fes in IBE capillary endothelial cells was sufficient to induce tube formation in the absence of FGF-2. On the other hand, expression of a kinase-defective mutant of c-Fes completely blocked FGF-2-induced chemotaxis, suggesting that activation of endogenous c-Fes may be required for this FGF-2 response. In contrast, neither wild-type nor kinase-defective c-Fes markedly influenced the proliferative response of IBE cells or expression of u-PA. These results suggest that c-Fes may be responsible for generating signals for cellular movement and differentiation and are consistent with previous data showing that expression of a membrane-targeted form of c-Fes results in hypervascularization in transgenic mice (24).

At the molecular level, we observed that FGF-2 was able to stimulate tyrosine phosphorylation of c-Fes in the transfected IBE cells. One mechanism to explain these data involves recruitment of c-Fes to the activated receptor and stimulation of c-Fes autophosphorylation. Other studies have shown that autophosphorylation of c-Fes on Tyr-713 within its kinase domain activation loop is essential for full kinase activity and substrate phosphorylation (31). Interestingly, we also observed that Src kinases strongly phosphorylate a kinase-defective form of c-Fes in SF-9 cells (Fig. 2). This result suggests that activation of c-Fes may occur downstream of FGF-2-induced Src family kinase activation. Other work from our laboratory has shown that the Src family kinase inhibitor PP1, as well as overexpression of kinase-inactive c-Src, inhibited chemotaxis toward FGF-2 by IBE cells. Together with the strong phosphorylation

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**FIG. 5.** Kinase-defective c-Fes blocks FGF-2-induced chemotaxis of IBE cells. Mock, K590E (KE) (clones 5-8 and 5-15), or WT (clones 6-1 and 6-8) c-Fes-transfected cells were plated on the fibronectin-coated upper well membranes of Transwell plate inserts in Ham’s F-12 medium containing 0.25% BSA. The inserts were then placed into wells prefilled with Ham’s F-12 medium containing 0.25% BSA with or without FGF-2. After incubation for 4 h, cells were fixed with methanol, washed, and stained with Giemsa solution. Cells present on the upper surface of the membranes were wiped away with cotton swabs, and cells that had migrated to the lower surface of the membranes were counted. Data are shown as the means ± S.D. for quadruplicate wells. Comparable data were obtained from two independent experiments. Open bars, no FGF-2; hatched bars, 5 ng/ml FGF-2; solid bars, 50 ng/ml FGF-2.

**FIG. 6.** Overexpression of wild-type c-Fes results in FGF-2-independent tube formation by IBE cells. Mock, K590E (clones 5-8 and 5-15), or WT (clones 6-1 and 6-8) c-Fes-transfected cells were cultured between two layers of type I collagen gels without serum and growth supplements in the presence or absence of 5 ng/ml FGF-2 at 33 °C for 18 h. Phase-contrast photomicrographs of the result are shown.

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2 T. Shono, H. Kanetake, and S. Kanda, submitted for publication.
of c-Fes by Src family kinases reported here, these results point to c-Fes as a downstream substrate and effector for Src family kinases in some FGF-2-initiated angiogenic signals.

We also investigated whether signaling pathways known to be activated by FGF-2 are affected by overexpression of wild-type or kinase-inactive c-Fes in IBE cells. One major substrate for FGFR-1 is PLC-γ, which binds through its Src homology 2 domain to the activated receptor via Tyr(P)-766. Although substitution of FGFR-1 Tyr-766 with Phe blocks recruitment and activation of PLC-γ, this receptor mutant still retains the ability to transduce signals for chemotaxis (32). In this study, overexpression of both wild-type and kinase-defective c-Fes had no effect on tyrosine phosphorylation of PLC-γ in response to FGF-2 treatment. This result also supports the idea that PLC-γ is not involved in FGF-2-induced cellular migration. Other studies have shown that both wortmannin and LY294002, which are potent inhibitors of PI3K, inhibit chemotaxis of porcine aortic endothelial cells (32). However, we observed that LY294002 inhibited PDGF-BB-induced chemotaxis but not FGF-2-induced chemotaxis in both control and c-Fes-expressing IBE cells (data not shown). Although c-Fes has been shown to activate PI3K in other systems (33), this result suggests that activation of PI3K is not required for FGF-2-induced chemotaxis of IBE cells. We also looked at signaling molecules that have been implicated in c-Fes signaling in fibroblast transformation systems, including the Ras/MAPK pathway and Stat3 activation (34, 35). These pathways were unaffected under conditions in which wild-type or kinase-defective c-Fes produced biological effects on FGF-2-dependent responses in IBE cells. Taken together, these data suggest that c-Fes signals for angiogenesis in vascular endothelial cells are distinct from the mitogenic pathways known to be stimulated in fibroblasts transformed by activated forms of c-Fes (e.g., PI3K pathway, Stat3 activation, and small G protein/MAPK pathways). Similar findings have been made in macrophage cell lines overexpressing c-Fes. In these studies, phosphorylation of p130 Cas and other proteins related to cellular adhesion and cell-cell contact have been reported in the absence of phosphorylation of signaling molecules related to proliferation (36, 37).

In conclusion, this study provides evidence that c-Fes is a novel downstream signaling molecule in FGF-2-regulated angiogenesis, affecting both chemotaxis and tube formation. Future studies will address the specific downstream signaling molecules activated by c-Fes in endothelial cells; identification of such molecules will provide a better understanding of the mechanisms underlying FGF-2-induced angiogenic responses.

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