Inhibition of endothelial cell chemotaxis toward FGF-2 by gefitinib associates with downregulation of Fes activity

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Abstract. Gefitinib inhibits epidermal growth factor-independent angiogenesis, but the molecular mechanism underlying this inhibition has yet to be defined. Here we show that gefitinib dose-dependently inhibited chemotaxis of endothelial cells toward fibroblast growth factor-2 (FGF-2), but not toward vascular endothelial growth factor-A (VEGF-A). Gefitinib inhibited lamellipodium formation by endothelial cells induced by FGF-2, but not by VEGF-A. Gefitinib at 10 μM did not inhibit autophosphorylation of FGF receptor 1 or VEGF receptor 2. A non-receptor protein tyrosine kinase, Fes, has two coiled-coil domains (CCDs) in its N-terminal region. Fes is activated by trans-autophosphorylation through CCD functions. An inactivating mutation in the second CCD abolished FGF-2 activation of Fes, indicating involvement of this CCD in FGF-2-induced Fes activation. Gefitinib-treatment decreased both CCD-independent and FGF-2- or VEGF-A-promoted Fes activity with a maximal decrease at 1 μM. The same results were observed in cells stably expressing kinase-inactive Fes; a dominant negative effect was observed in cells treated with FGF-2, but not with VEGF-A. Taken together, these results indicate that FGF-2 activates Fes via the second CCD, leading to lamellipodium formation and chemotaxis by endothelial cells, and gefitinib may act through Fes as an inhibitor of FGF-2-driven angiogenesis.

Introduction

A prerequisite for tumor progression and metastasis is angiogenesis, a new vascular sprout from pre-existing blood vessels, along with vascular co-option and vasculogenesis promoted by circulating or bone marrow-derived endothelial progenitor/precursor cells (1-4). Key players in angiogenesis are vascular endothelial cells, and their proliferation, migration, survival, and differentiation are essential for this process. In these biological responses, vascular endothelial growth factor (VEGF)-A plays central roles (5,6), and targeting VEGF-A signaling has been extensively applied for the treatment of advanced human cancers. However, many clinical trials have shown that targeting VEGF-A signaling was effective only for a limited period followed by resistance to this therapeutic strategy (7,8). One of the mechanisms underlying this resistance is a switch from VEGF-A-dependent angiogenesis to angiogenesis dependent on other proangiogenic factors, such as fibroblast growth factor (FGF)-2 (9). In recent years, the significance of FGF-2 in tumor angiogenesis has been widely recognized (10-14). Thus, for the eradication of VEGF-A-independent tumors, targeting FGF-2 signaling may become the potential second-line therapeutic strategy for anti-angiogenesis. Because stromal-derived factor (SDF)-1α and angiopoietin 2 (Ang2) may also substitute for VEGF-A-dependent angiogenesis during therapy (15-17), inhibition of intracellular signaling pathways commonly acting downstream of receptors for FGF-2, SDF-1α, and Ang2 would be a favorable strategy as a potential anti-angiogenic therapy.

Fes (also known as Fps) and Fer define a unique subfamily of non-receptor protein tyrosine kinases and Fes is exclusively expressed in the monocytic lineage of hematopoietic cells, vascular endothelial cells, some neuronal cell types and epithelial cells (reviewed in refs. 18-20). Fes has a unique N-terminal region, containing two coiled-coil domains (CCDs). One of the CCD functions is inter- or intra-molecular association; however, the role of CCDs in signal transduction pathways upstream or downstream of Fes is largely unknown. Fes is activated by autophosphorylation at tyrosine 713 through functional N-terminal CCDs (21,22). Using mutant Fes harboring point mutations that disrupt the coiled-coil function has revealed involvement of the first CCD in tight suppression of basal kinase activity and of the second CCD in a positive regulatory role in Rat 1 fibroblasts and TF-1 myeloid leukemia cells (23). We have previously shown that kinase activity of Fes was required for chemotaxis toward

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FGF-2 by a cultured murine brain capillary endothelial cell line, denoted IBE cells (24). IBE cells expressed endogenous Fes, and FGF-2 activated wild-type (wt) Fes in IBE cells. Stable expression of kinase-inactive Fes inhibited this FGF-2-induced Fes activation, suggesting that expression of kinase-inactive Fes exerts a dominant-negative effect on endogenous Fes. Fes kinase activity was also required for Ang2-directed chemotaxis and SDF-1α-induced morphological differentiation of IBE cells (25-27), suggesting that Fes may be involved in angiogenesis driven by Ang2 or SDF-1α.

Recent findings have indicated that FGF/FGF receptor (FGFR) signaling seems to contribute significantly to the progression of non-small cell lung cancer (NSCLC) (28-30). Because gefitinib efficiently inhibit the progression of NSCLC tumors in the clinical setting, it may inhibit an as-yet unidentified kinase acting downstream of FGFR in NSCLC cells. Certain lung cancer cells express Fes (31); however, whether gefitinib affects Fes tyrosine kinase activity remains unknown. Gefitinib is known to inhibit tumor angiogenesis (32,33). NSCLC cells produce FGF (28-30), and gefitinib-mediated inhibition of NSCLC tumor growth *in vivo* may arise because of a blockade of FGF-activated Fes in tumor cells and in angiogenesis. To test this hypothesis, we examined the effect of gefitinib on FGF-2-mediated cellular responses and Fes activity in endothelial cells, and found that gefitinib decreased the Fes activity and chemotaxis toward FGF-2.

Materials and methods

Materials. Human recombinant FGF-2 and epidermal growth factor (EGF) were obtained from Roche Diagnostics (Tokyo, Japan). Human recombinant VEGF-A_{165} was purchased from PeproTech Inc., (Rocky Hill, NJ). Anti-Fes polyclonal antiserum (denoted Fps-QE) was kindly provided by Dr Peter A. Greer (Queen's Cancer Research Institute (Ontario, Canada). Anti-pY713 Fes antibody was described elsewhere (34). Anti-FLAG monoclonal antibody (M2) was purchased from Sigma Chemical Co. (St. Louis, MO). Anti-EGF receptor (EGFR) blocking antibody (mouse monoclonal, clone LA1) was purchased from Upstate Cell Signaling Solutions (Lake Placid, NY). Other antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Gefitinib was kindly supplied by AstraZeneca (Cheshire, UK). It was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 30 mM as a stock solution and stored at -80˚C. The stock solution was further diluted with DMSO and dissolved in culture medium just before use. The final concentration of DMSO was 0.1% in all cases. FuGENE 6 transfection reagent was obtained from Roche Diagnostics GmbH (Mannheim, Germany).

Cell culture. Porcine aortic endothelial (PAE) cells and cells stably expressing VEGFR2 (denoted KDR/PAE cells) were transfected with plasmid encoding either WT or kinase-inactive (K590E mutant; KE) Fes and several stable cell lines were obtained (27). Among these, in the present study, we used two stable PAE cell lines (WT-6 PAE cells and KE-12 PAE cells), and two stable KDR/PAE cell lines (WT-29 KDR/PAE cells or KE-4 KDR/PAE cells). PAE cells endogenously express FGFR1 and VEGFR1, whereas KDR/PAE cells additionally express human VEGFR2 (27). PAE cells over-expressing WT FGFR1 (35) was kindly provided by Dr Carl-Henrik Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). They were cultured in Ham's F-12 medium containing 10% fetal bovine serum (FBS) and 1 μg/ml of hygromycin B as a selection antibiotics. Human umbilical vein endothelial cells (HUVECs) and their culture medium were obtained from Cambrex, Walkersville, MD, and the cells were cultured in endothelial cell basal medium-2 (EBM-2) supplemented with 2% FBS, 10 ng/ml VEGF-A, 20 ng/ml FGF-2, 10 ng/ml EGF, 10 ng/ml insulin-like growth factor-I, 50 μg/ml ascorbic acid, 100 ng/ml heparin, and 10 pM dexamethasone.

Stable expression of WT and mutant Fes protein in PAE cells. The cDNAs encoding the WT human Fes with a C-terminally-FLAG-tag and those encoding mutations disrupting CCD functions were the kind gifts of Dr Thomas E. Smithgall (University of Pittsburgh School of Medicine, Pittsburgh, PA). Disruption of function of the first CCD was achieved with the L145P mutation, while disruption of the second CCD function was achieved with the L344P mutation. We also included a double mutant (L145P + L344P; 2LP) (23). The cDNAs were transfected into PAE cells using FuGENE 6. After 48 h, resistant clones were selected for culturing in the presence of 200 μg/ml of G418 and the pool of resistant cells was examined for immunoprecipitation followed by immunoblotting as described below.

Chemotaxis assay. The chemotaxis assay to assess migration for PAE cells has been described elsewhere (27). Briefly, PAE cells suspended in Ham's F-12 medium containing 2% FBS were seeded onto the upper surface of type I collagen-coated membranes of Transwell inserts (diameter, 6.7 mm; pore size, 8 μm). In the lower wells, Ham's F-12 medium containing 2% FBS with or without the indicated conditions was added and incubated for 4 h. Cells that migrated to the lower membrane surface of the Transwell inserts were counted microscopically. FGF-2 at 20 ng/ml and VEGF-A at 50 ng/ml gave the maximal stimulation of chemotaxis by PAE cells. For chemotaxis of HUVECs, membranes of the Transwell inserts were coated with type I collagen and cells were suspended in EBM-2 containing 0.5% FBS. In the lower wells, EBM-2 containing 0.5% FBS with or without the indicated factors was added. FGF-2 at 20 ng/ml reproducibly gave the maximal stimulation of chemotaxis by HUVECs.

Immunoprecipitation and immunoblotting. Immunoprecipitation and immunoblotting were performed as described previously (24). In short, cells grown subconfluently were serum-starved with culture medium overnight, then stimulated or left unstimulated with 100 ng/ml FGF-2 for indicated periods. Cells were washed once with Tris-buffered saline (TBS), pH 7.5, containing 100 μM orthovanadate on ice and lysed in lysis buffer (50 mM Tris-HCl, pH 7.5 containing 0.15 M NaCl, 1% Nonidet P-40, 10 mM NaF, 1 mM EDTA, 2 mM Pefabloc® SC, 0.02% sodium dodecyl sulfate (SDS), 1 μM leupeptin, 100 μM orthovanadate, and 100 U/ml aprotinin). After centrifugation to remove insoluble materials, the cell lysates were immunoprecipitated with the indicated antibodies, followed by the adsorption to Protein A- or

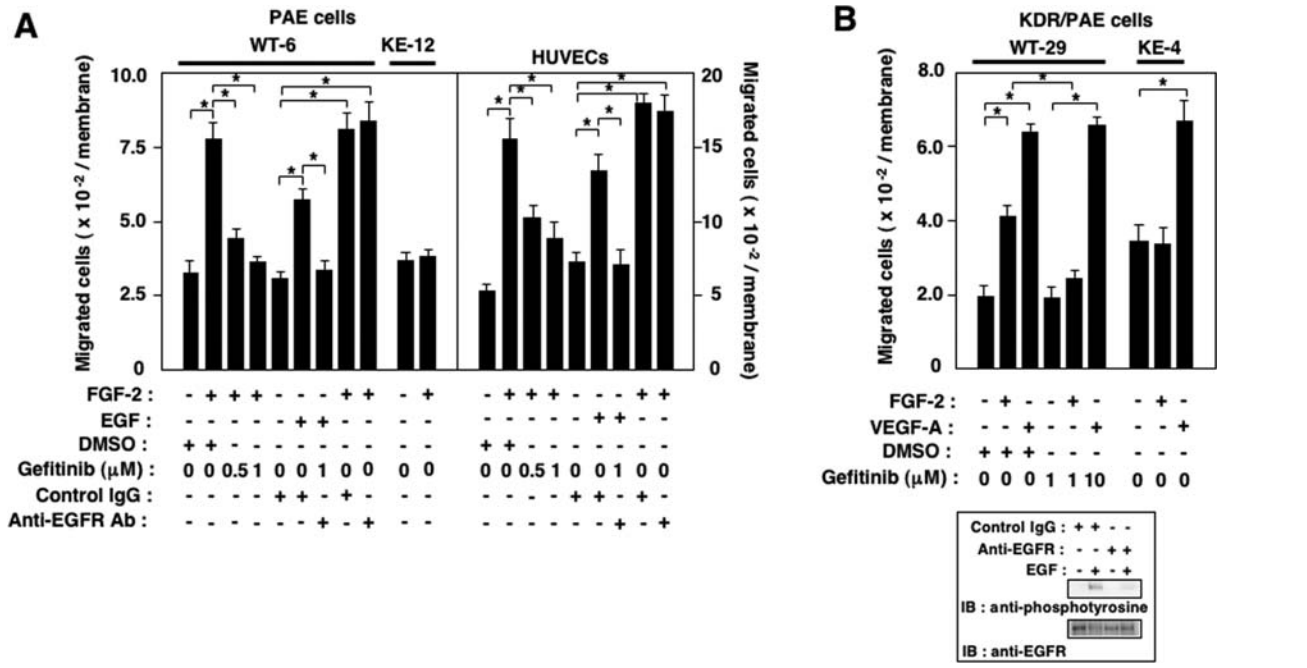

Figure 1. (A) Gefitinib inhibits chemotaxis toward FGF-2 of a PAE cell line expressing WT Fes (WT-6 cells) and HUVECs toward FGF-2, and a PAE cell line expressing kinase-inactive Fes (KE-12 cells) exhibited a dominant-negative effect on chemotaxis toward FGF-2. Culture media containing FGF-2 at 20 ng/ml or EGF at 100 ng/ml with either DMSO (0.1%), gefitinib, control IgG (4 μg/ml), or anti-EGFR blocking antibody (4 μg/ml) were placed in the lower wells of Transwell culture plates and PAE cells or HUVECs that migrated onto the lower surface of the Transwell inserts were counted. Reproducible results were obtained from three independent experiments. (B) Gefitinib fails to inhibit chemotaxis of a KDR/PAE cell line expressing WT Fes (WT-29 cells) toward VEGF-A, and a KDR/PAE cell line expressing kinase-inactive Fes (KE-4 cells) exhibited no dominant-negative effect on chemotaxis toward VEGF-A. Culture media and counting were as described for (A) except that media contained FGF-2 at 20 ng/ml or VEGF-A at 50 ng/ml with either DMSO (0.1%) or gefitinib at indicated concentrations. Reproducible results were obtained from three independent experiments. Inserted immonoblot shows the effect of blocking antibody against EGFR on EGF-induced phosphorylation of EGFR expressed in LLC-PK$_1$ cells. Cells were treated with control IgG or anti-EGF antibody for 60 min, and then stimulated or left unstimulated with 100 ng/ml EGF for 7 min. EGFR was immunoprecipitated followed by immunoblotting. Reproducible results were obtained from two independent experiments.

Protein G-agarose beads. After the washing, proteins were eluted from beads by heating in SDS-sample buffer and then separated by SDS-polyacrylamide gel electrophoresis. After electrophoretic transfer onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA), the blots were probed with indicated antibodies. Antibody incubation was followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG, and detection was through an enhanced chemiluminescence reaction (Thermo Scientific, Rockford, IL). Between two probings, stripping of the membrane was performed 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. In indicated blots, total cell lysates were prepared by the incubation of cells with boiled SDS sample buffer and proteins from an equal number of cells were electrophoresed and analyzed.

Visualization of polymerized actin. Actin reorganization was examined as described previously (36). In brief, cells were grown on cover slips and incubated with medium containing 0.5% FBS for 16 h. Cells were either stimulated with 50 ng/ml FGF-2 or VEGF-A or left unstimulated for indicated periods, and then washed with TBS. Cells were fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature, followed by incubation with 0.05% Triton X-100 in TBS for 3 min. Cells were washed with TBS and incubated with TRITC-conjugated phalloidin (Sigma Chemical Co.) for 30 min. After washing and mounting, photographs were taken under fluorescence microscopy. To quantify the number of lamellipodium >100 cells in each group were examined.

Statistical analysis. Values are presented as mean ± SD. Differences between two groups were examined using the Mann-Whitney's U test. Differences were considered significant when P-value was <0.05.

Results

Gefitinib inhibits chemotaxis of endothelial cells toward FGF-2, but not toward VEGF-A. We have previously established the stable cell lines expressing either WT (WT-6 PAE cells and WT-29 KDR/PAE cells, respectively) or kinase-inactive Fes (KE-12 PAE cells and KE-4 KDR/PAE cells, respectively) (27). FGF-2 activated WT Fes via endogenous FGFR1 in both PAE and KDR/PAE cells, whereas VEGF-A activated WT Fes only in KDR/PAE cells (27). To examine the anti-angiogenic activity of gefitinib *in vitro*, we tested the effect of gefitinib on chemotaxis of these cells and HUVECs as primary endothelial cells. As shown in Fig. 1A, gefitinib inhibited the chemotaxis of WT-6 PAE cells toward EGF. Interestingly, gefitinib strongly inhibited the chemotaxis of these cells toward FGF-2. Blocking antibody against EGFR, which inhibits the tyrosine phosphorylation of porcine EGFR

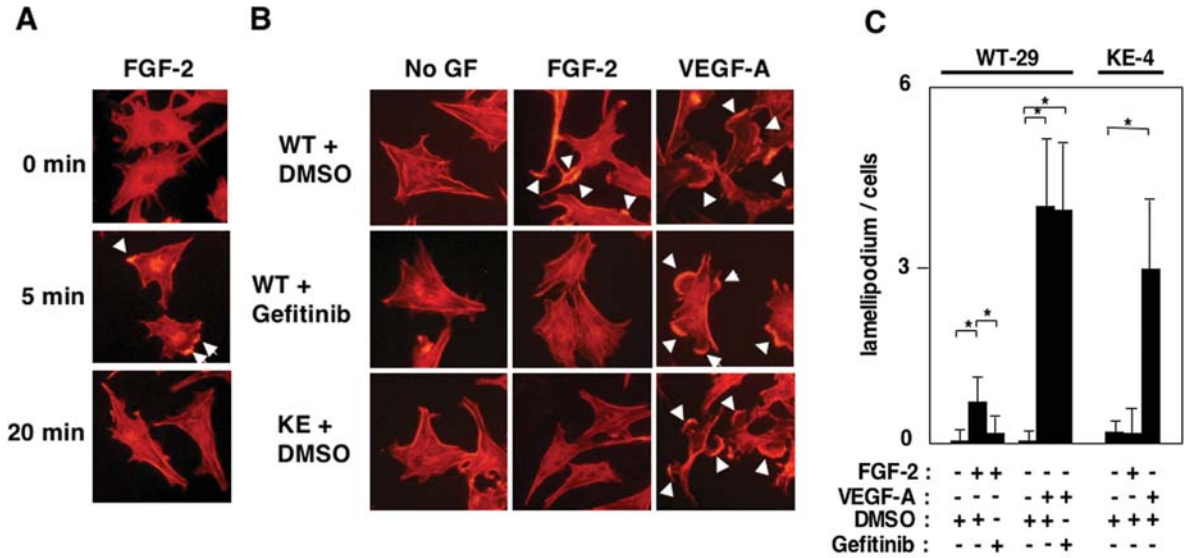

Figure 2. (A) Kinetics of FGF-2-induced actin reorganization. WT-29 KDR/PAE cells were cultured on coverslips, serum-starved for 2 h, and treated with FGF-2 (50 ng/ml) or left unstimulated for indicated periods. Cells were fixed and polymerized actin bundles were visualized with TRITC-conjugated phalloidin. White arrowhead indicates lamellipodia. Reproducible results were obtained from three independent experiments. (B) FGF-2-induced lamellipodium formation in WT-29 KDR/PAE cells (WT) is inhibited by 1 μM of gefitinib or is not observed in KE-4 KDR/PAE cells (KE). Cells were cultured in the presence of either 0.1% DMSO or 1 μM of gefitinib for 2 h and were stimulated or not for 5 min with FGF-2 (50 ng/ml) or VEGF-A (50 ng/ml). Cells were fixed and polymerized actin bundles were visualized with TRITC-conjugated phalloidin. White arrowhead indicates lamellipodia. Reproducible results were obtained from three independent experiments. (C) Quantification of lamellipodium formation. Number of lamellipodium in each cell was counted. In each treatment, more than 100 cells were examined.

expressed in LLC-PK1 porcine renal tubular epithelial cells (Fig. 1B insert), did not inhibit the chemotaxis toward FGF-2. This result suggested that gefitinib inhibited chemotaxis toward FGF-2 independently of signaling via EGFR. Stable expression of kinase-inactive Fes (KE-12 PAE cells) exerted a dominant-negative effect on FGF-2-directed chemotaxis, as has been observed in IBE cells (24). Gefitinib also inhibited the chemotaxis of HUVECs toward FGF-2 independently of signaling via EGFR as well. We next examined the effect of gefitinib on FGF-2- or VEGF-A-induced chemotaxis. As shown in Fig. 1B, gefitinib inhibited the chemotaxis of WT-29 KDR/PAE cells toward FGF-2, but failed to inhibit the chemotaxis toward VEGF-A even at 10 μM. Stable expression of kinase-inactive Fes (KE-4 KDR/PAE cells) also failed to exert a dominant-negative effect on chemotaxis toward VEGF-A. These results suggest that gefitinib inhibited chemotaxis toward FGF-2, but not toward VEGF-A, similar to the results obtained using cells expressing kinase-inactive Fes.

Gefitinib inhibits FGF-2-promoted lamellipodium formation in PAE cells. Actin reorganization is one of the critical cellular responses of cytokine-initiated cell motility. Especially, stress fiber fomation, fillopodium or lamellipodium formation are main features of actin reorganization observed during motility of a variety of cells. We first examined the kinetics of actin reorganization in FGF-2-treated WT-29 KDR/PAE cells. As shown in Fig. 2A, FGF-2 induced lamellipodium formation at 5 min and smaller lamellipodia were observed at 20 min. Stress fiber formation or fillopodium formation were not remarkable during FGF-2-treatment in these cells. We then examined the effect of gefitinib on lamellipodium formation by either FGF-2 or VEGF-A at 5 min. As shown in Fig. 2B and C, FGF-2 induced lamellipodium formation in WT-29 cells, as did VEGF-A. Gefitinib only inhibited lamellipodium formation induced by FGF-2. In KE-4 KDR/PAE cells, FGF-2 failed to induce lamellipodium formation; however, VEGF-A did induce it in these cells. These cellular responses were closely associated with chemotaxis of PAE or KDR/PAE cells expressing kinase-inactive Fes.

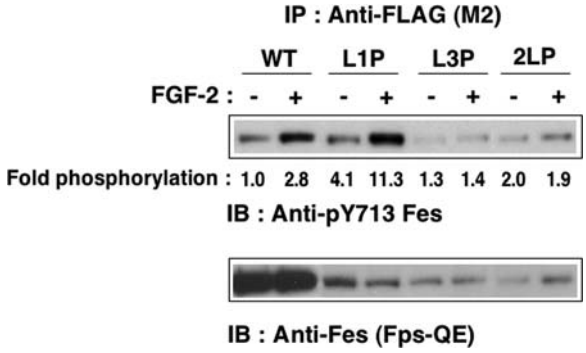

Figure 3. Mutations in the CCDs affect basal or FGF-2-induced kinase activity of Fes in PAE cells. PAE cells stably expressing FLAG-tagged WT, a first CCD mutant (L145P; denoted L1P), a second CCD mutant (L344P; denoted L3P), or the double CCD mutant (denoted 2LP) Fes were serum-starved for 2 h and then stimulated with FGF-2 (100 ng/ml) for 15 min. Fes was immunoprecipitated with anti-FLAG antibody (M2), and separated by SDS-PAGE, followed by transfer onto PVDF membranes; autophosphorylation of Fes was examined by immunoblotting with the use of anti-phospho-Y713 Fes antibody (pY^{713} Fes). Fold phosphorylation was calculated by the ratio of an intensity of pY713 Fes/total Fes and normalized to the ratios observed with untreated WT Fes-expressing cells. Reproducible results were obtained from three independent experiments.

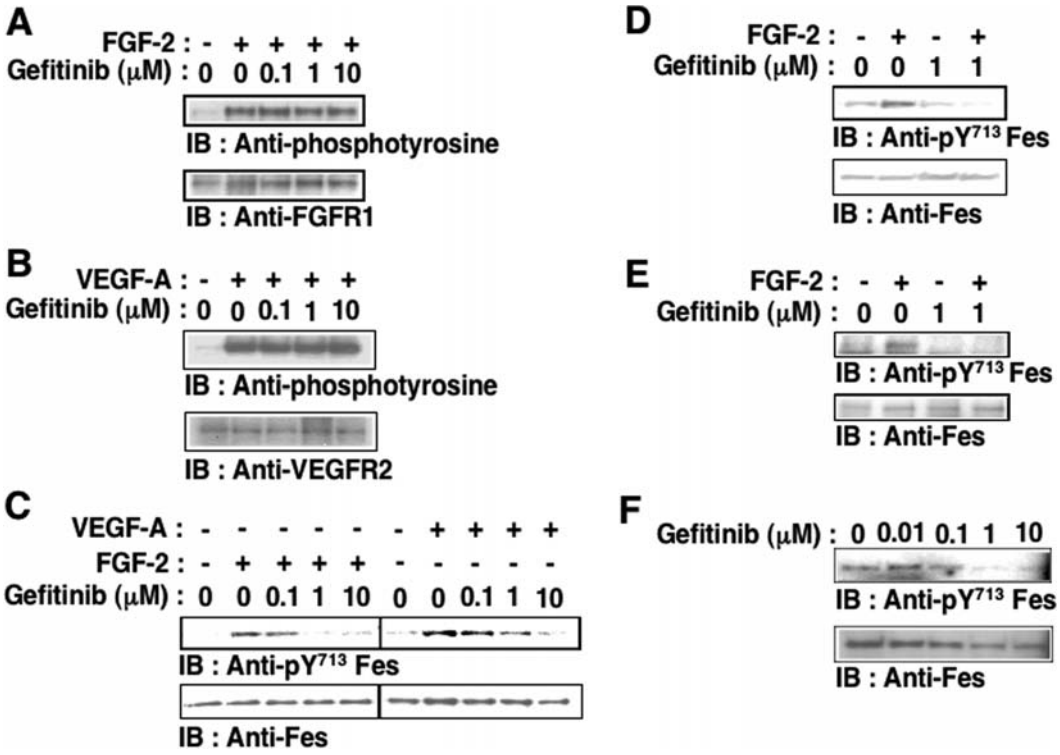

Figure 4. (A) Gefitinib does not inhibit the autophosphorylation of FGFR1. PAE cells overexpressing FGFR1 were cultured in 6-cm dishes, serum-starved overnight, and further incubated with DMSO or gefitinib at indicated concentrations for 2 h. Cells were stimulated or left unstimulated with FGF-2 at 100 ng/ml for 8 min. FGFR1 was immunoprecipitated, followed by the separation with SDS-PAGE. Autophosphorylation of FGFR1 was examined by immunoblotting. Reproducible results were obtained from two independent experiments. (B) Gefitinib does not inhibit the autophosphorylation of VEGFR2. WT-29 KDR/PAE cells were cultured in 6-cm dishes, serum-starved overnight, and further incubated with DMSO or gefitinib at indicated concentrations for 2 h. Cells were stimulated or left unstimulated with VEGF-A at 100 ng/ml for 8 min. VEGFR2 was immunoprecipitated, followed by the separation with SDS-PAGE. Autophosphorylation of VEGFR2 was examined by immunoblotting. Reproducible results were obtained from two independent experiments. (C) Gefitinib decreases FGF-2- and VEGF-A-promoted Fes activity in KDR/PAE cells. KDR/PAE cells expressing WT Fes were serum-starved for 2 h and then stimulated with FGF-2 (100 ng/ml) or VEGF-A (100 ng/ml) for 15 min. FLAG-tagged Fes was immunoprecipitated, followed by the separation with SDS-PAGE. Autophosphorylation of Fes was examined by immunoblotting with the use of anti-pY^{713} Fes antibody. Reproducible results were obtained from three independent experiments. (D) Gefitinib decreases FGF-2-promoted Fes activity in PAE cells. PAE cells expressing WT Fes were processed as in (C) except for being stimulated only with FGF-2 (100 ng/ml) for 15 min. Reproducible results were obtained from two independent experiments. (E) Gefitinib decreases FGF-2-promoted Fes activity in HUVECs. HUVECs were processed as in (D). Reproducible results were obtained from two independent experiments. (F) Gefitinib decreases the kinase activity of Fes harboring the loss-of-coiled-coil mutations. PAE cells expressing Fes with double CCD mutations (L145P and L344P, denoted 2LP cells) were serum-starved for 2 h and then processed for blotting as in (D). Reproducible results were obtained from three independent experiments.

A point mutation in the second CCD abolishes the FGF-2-mediated increase in Fes kinase activity in PAE cells. The mechanisms of how FGF-2 activates Fes are unknown. To examine the role of CCDs in the activation of Fes, we transfected the plasmid encoding FLAG-tagged WT and coiled-coil mutant Fes into PAE cells, obtaining pools of G418-resistant stable cell lines. Because cell density affects intracellular signaling pathways, we always used subconfluent cultures of transfected cells in the experiments described below. We examined the activation of Fes by immunoblotting, probing with anti-pY713 Fes antibody, which recognizes the autophosphorylated Fes responsible for the kinase activation. As shown in Fig. 3, the basal kinase activity of L145P mutant Fes was ~4 times higher than that of WT Fes. FGF-2 increased the autophosphorylated WT and L145P Fes in PAE cells. By contrast, Fes harboring point mutation in the second CCD (L344P and L145P/L344P double mutations) was defective for FGF-2-induced activation. Thus, it seems likely that mutation of the first CCD is responsible for the release of basal kinase activity, and the intact second CCD was required for FGF-2-induced activation of Fes in PAE cells.

Gefitinib dcreases the autophosphorylation of Fes in endothelial cells. A previous report has demonstrated that gefitinib inhibited VEGFR2 kinase activity, or FGF-2- and VEGF-A-induced proliferation of HUVECs at the micromolar level (37). Expression of endogenous FGFR1 in PAE cells is extremely low and thus, it is difficult to examine the autophosphorylation of endogenous FGFR1. Therefore, to determine whether gefitinib inhibits FGFR1 tyrosine kinase activity, we studied the autophosphorylation of overexpressed FGFR1 in PAE cells. As shown in Fig. 4A, FGF-2-treatment promoted autophosphorylation of FGFR1, and gefitinib even at 10 μM did not inhibit the phosphorylation. We also examined the effect of gefitinib on the autophosphorylation of VEGFR2. VEGF-A potently promoted autophosphorylation of VEGFR2 in WT-29 cells and gefitinib at 10 μM showed no significant effect (Fig. 4B). These results suggest that

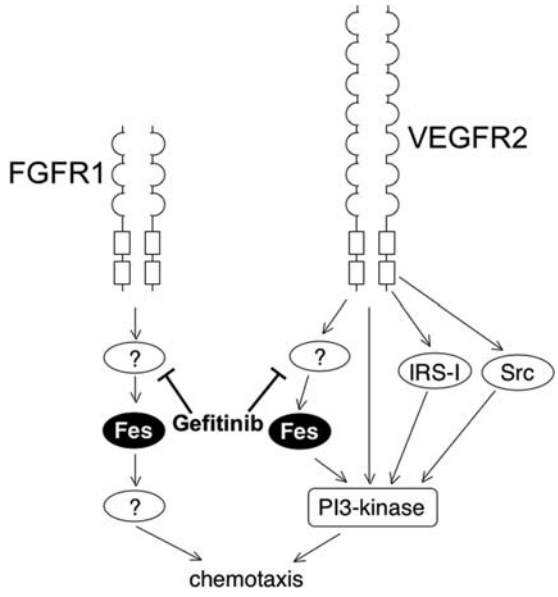

Figure 5. Proposed signal transduction pathway leading to chemotaxis toward FGF-2 or VEGF-A by PAE cells. Gefitinib inhibits certain target signaling molecule(s) upstream of Fes, resulting in the decrease in Fes kinase activity. FGF-2-activated Fes regulates chemotaxis toward FGF-2. Chemotaxis toward VEGF-A is inhibited by a PI3-kinase inhibitor, LY294002 (27). VEGF-A activates PI3-kinase through VEGFR-2 by inducing the binding of PI3-kinase to VEGFR2, insulin receptor substrate-I (IRS-I), Fes, and Src (27). Inhibition of Fes alone with gefitinib fails to inhibit VEGF-A-activated PI3-kinase; thus, chemotaxis toward VEGF-A is insensitive to gefitinib-treatment.

gefitinib does not seem to inhibit FGFR1 or VEGFR2 in these cultured endothelial cells even at 10 μM.

We next examined the effect of gefitinib on FGF-2- or VEGF-A-induced autophosphorylation of Fes in WT-29 KDR/PAE cells. As shown in Fig. 4C, gefitinib at 1 μM maximally decreased both FGF-2- and VEGF-A-treated autophosphorylation of Fes, and gefitinib at 10 μM did not further decrease. We also examined the effect of gefitinib on FGF-2-induced autophosphorylation of Fes in PAE cells and HUVECs. As shown in Fig. 4D and E, gefitinib at 1 μM strongly decreased the FGF-2-treated autophosphorylation of Fes in these cells. FGF-2-induced autophosphorylation of Fes is regulated by CCDs. To examine whether gefitinib decreases autophosphorylation of Fes independent of CCD function, we studied the Fes activity in gefinitib-treated or untreated 2LP cells. As shown in Fig. 4F, gefitinib at 1 μM resulted in a maximal decrease in the autophosphorylation of Fes.

Discussion

Gefitinib has been appreciated as a highly specific inhibitor for Erb B family receptor tyrosine kinases, especially for Erb B1, an EGFR tyrosine kinase (38), and is widely used for the treatment of patients with advanced NSCLC. Gefitinib also can inhibit angiogenesis (32,33). Recent studies have indicated that the progression of NSCLC utilizes FGF/FGFR signaling (28-30). Therefore, gefitinib may inhibit angiogenesis promoted by NSCLC cell-derived FGF during treatment. The present study provides evidence for the first time that gefitinib can inhibit chemotaxis toward FGF-2 by endothelial cells, the critical cellular response for angiogenesis.

In endothelial cells in this study, Fes kinase activity was downregulated by treatment with gefitinib (Fig. 4C-F). However, in other work, gefitinib failed to inhibit Fes activity directly as assessed by *in vitro* tubulin phosphorylation assay (unpublished observation by Hellwig and Smithgall), suggesting that gefitinib indirectly inhibited Fes activity in endothelial cells. Inhibition of Fes does not seem to involve EGFR-mediated signaling, because blocking antibody against EGFR showed no effect on chemotaxis toward FGF-2 in either PAE cells or HUVECs (Fig. 1A). Screening with an immobilized compound from gefitinib identified that GAK and RICK as possible target kinases for gefitinib (39). According to the competition of binding between T7 bacteriophage-tagged kinases and immobilized gefitinib, GAK was again identified as a potential target for gefitinib (40,41). A Src homology 2 (SH2) domain is required for full kinase activation (42). Association of an SH2 domain with autophosphorylated Fes kinase itself stabilizes the activated conformation of the kinase domain and binding of particular ligands to the SH2 domain further stabilizes the active conformation of the catalytic domain (43). Therefore, an as yet undefined protein tyrosine kinase, such as GAK, or association of the Fes SH2 domain with tyrosine-phosphorylated ligands may be involved in the regulation of Fes kinase activity, and gefitinib may modulate these signaling events.

In the present study, we showed that FGF-2-induced activation of Fes seems to require the second CCD function. FGF-2-induced activation was not observed in Fes with the mutant second CCD, while FGF-2 did activate Fes with the intact second CCD even in the presence of the mutant first CCD (Fig. 3). We performed transfection experiments three times independently, and the expression level of WT Fes protein was always considerably higher than mutant Fes proteins in all transfection experiments (data not shown). The reason for the higher expression of WT Fes is not yet clear. One possibility is the decreased stability of the mutant Fes. Alternatively, high basal kinase activity of mutant Fes may be toxic for endothelial cells, because kinase activity of WT Fes (endogenous Fes) is tightly downregulated in endothelial cells.

Activation of class I phosphoinositide 3 (PI3)-kinase involves multiple mechanisms, including the binding of PI3-kinase to tyrosine-phosphorylated proteins, which bring PI3-kinase close to the plasma membrane (44). In VEGF-A-treated KDR/PAE cells, PI3-kinase was activated through binding to active Fes (27). VEGF-A-directed chemotaxis and VEGF-A-promoted morphological differentiation of KDR/PAE cells are dependent on PI3-kinase activation, because PI3-kinase inhibitor, LY294002, inhibited these cellular responses (27). Expression of kinase-inactive Fes did not inhibit VEGF-A-activated PI3-kinase; VEGF-A activates PI3-kinase in KDR/PAE cells through multiple pathways involving the binding of PI3-kinase to VEGF receptor (VEGFR) 2, insulin receptor substrate-I, and Src, all of which can substitute for the expression of kinase-inactive Fes (27). Gefitinib-treatment did not decrease the phosphorylation of Ser473 of Akt1 promoted by VEGF-A in KDR/PAE cells (unpublished data). Thus, it seems likely that inhibition of Fes by gefitinib did not affect chemotaxis of KDR/PAE cells toward VEGF-A

because of intact PI3-kinase activation (Fig. 5). By contrast, Fes does not activate PI3-kinase in FGF-2-treated endothelial cells (24) and LY294002 did not inhibit the chemotaxis of PAE cells toward FGF-2 (unpublished data). It seems that FGF-2-activated Fes uses different downstream signaling molecule(s) other than PI3-kinase. The effecter molecules of Fes in FGF-2-treated endothelial cells have not yet been identified.

A previous report has shown that gefitinib inhibited FGF-2- or VEGF-A-induced proliferation of HUVECs at 1-3 μM and that the IC_{50} value of gefitinib on VEGFR2 kinase activity was 3.7-10 μM (37). In the present study, gefitinib at 10 μM failed to inhibit the autophosphorylation of FGFR1 and VEGFR2. Nevertheless, gefitinib at 1 μM gave the maximal inhibition of Fes activity (Fig. 4C-F). Notably, oral administration of gefitinib in cancer patients achieves a concentration of 1 μM in serum (45). Thus, gefitinib may inhibit angiogenesis via Fes during the treatment of NSCLC in humans.

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