Fractalkine stimulates angiogenesis by activating the Raf-1/MEK/ERK- and PI3K/Akt/eNOS-dependent signal pathways

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PI3K/Akt/eNOS-dependent signal pathways

Fractalkine (FKN) has been implicated in modulation of angiogenesis and vascular inflammation, but the underlying mechanism has not been elucidated. We have investigated the molecular mechanism by which FKN regulates angiogenesis. We found that recombinant FKN increases in vitro proliferation, migration, and tube formation of human umbilical vein endothelial cells and stimulates in vivo angiogenesis. FKN-induced angiogenesis was accompanied by phosphorylation of ERK, Akt, and endothelial nitric oxide (NO) synthase (eNOS), as well as an increase in NO production. These biochemical events and angiogenesis were completely inhibited by the G protein-coupled receptor inhibitor pertussis toxin. Inhibitors of Raf-1, MEK, phosphatidylinositol 3-kinase (PI3K), and eNOS or transfection with dominant-negative forms of ERK and Akt significantly suppressed the angiogenic activity of FKN. However, inhibitors of Raf-1 and MEK or a dominant-negative ERK mutant blocked FKN-induced ERK, but not Akt and eNOS, phosphorylation. The PI3K inhibitor and a dominant-negative mutant of Akt suppressed Akt and eNOS phosphorylation and NO production. Our results demonstrated that FKN stimulated angiogenesis by activating the Raf-1/MEK/ERK and PI3K/Akt/eNOS/NO signal pathways via the G protein-coupled receptor CX3CR1, indicating that two pathways are required for full angiogenic activity of FKN. This study suggests that FKN may play an important role in the pathophysiological process of inflammatory angiogenesis.

chemokine; vascular inflammation; vascular endothelial growth factor; endothelial cells

ANGIOGENESIS, the formation of new blood vessels, is essential for the physiological processes of embryogenesis, tissue growth, and tumorigenesis (12). Angiogenesis has also been found to be central to the progression of various chronic inflammatory conditions, including diabetic retinopathy, wound healing, and rheumatoid arthritis (40). These diseases are characterized by chronic inflammation associated with a marked increase in vascular remodeling. The angiogenic process is controlled by a wide variety of positive and negative regulators, which are composed of growth factors, cytokines, adhesion molecules, and chemokines (1).

Members of the chemokine family have been initially recognized for their ability to recruit leukocytes to sites of injury. More recent studies have shown that this family participates in the regulation of a number of pathophysiological conditions, including leukocyte homeostasis, development, cancer, and response to infection (13, 29). Chemokines are subdivided into four subfamilies, C, CC, CXC, and CX3C, on the basis of the number of cysteines and the spacing of the first two cysteines in a conserved cysteine structural motif. Although chemokines are generally thought to function as leukocyte attractants, members of the CXC chemokine family (CXCL1 to CXCL8, except CXCL4) and the CC chemokine family [monocyte chemoattractant protein (MCP)-1, viral macrophage inflammatory protein (vMIP) I, and vMIP II] have been shown to possess angiogenic activity (14, 42, 45). However, some of the CXC family chemokines (CXCL4, CXCL9, and CXCL10) exert angiostatic activity (36).

Fractalkine (FKN, CX3CL1), a member of the CX3C chemokine family, plays a crucial role in the initiation and progression of inflammation. FKN binds to the specific CX3C chemokine receptor CX3CR1, inducing numerous monocyte-mediated proinflammatory signals and monocyte chemotaxis. FKN is a unique chemokine, which contains three amino acids between the first two cysteine residues and exists in soluble and membrane-anchored forms. The membrane-anchored form of FKN consists of a chemokine head tethered to the cell surface by a mucinlike stalk followed by a single transmembrane-spanning domain (4, 37). FKN is expressed on activated endothelial cells, dendritic cells, and intestinal epithelial cells (2, 28, 34). However, CX3CR1, the unique receptor for FKN, is expressed on subsets of peripheral CD4+ and CD8+ T cells, natural killer cells, and monocytes (47), as well as microvascular endothelial cells (44). Recent studies have suggested that the interaction of FKN and CX3CR1 contributes to the pathogenesis of atherosclerosis (9, 11) and kidney diseases (39) through the firm adhesion of leukocytes to endothelial cells (43). FKN has been also shown to participate in the pathogenesis of rheumatoid arthritis, probably by increasing the angiogenic process (5, 44) through endothelial cell activation (33). However, its in vivo angiogenic activity and molecular mechanism have not been clearly elucidated. In this study, we have investigated the in vivo effect of FKN on angiogenesis and its underlying molecular mechanism in cultured human endothelial cells. Our results clearly show that FKN plays a significant role in facilitating inflammatory angiogenesis by activating the G protein-coupled receptor-mediated Raf-1/MEK/ERK signaling pathways.
MEK/ERK and phosphatidylinositol 3-kinase (PI3K)/Akt/endothelial nitric oxide (NO) synthase (eNOS) signal pathways.

MATERIALS AND METHODS

Materials. Recombinant human FKN (chemokine domain) was purchased from R & D systems (Minneapolis, MN); basic fibroblast growth factor and vascular endothelial growth factor (VEGF) from Upstate Biotechnology (Lake Placid, NY); BAY 43-9006 from Calbiochem (San Diego, CA); wortmannin, PD-98059, and growth factor and vascular endothelial growth factor (VEGF) from R&D systems (Minneapolis, MN); antibodies against phosphorylated (phospho)-ERK, phospho-Akt, Akt, phospho-eNOS, eNOS, and ERK from New England Biolabs (Beverly, MA); and 4-amin-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) diacetate from Molecular Probes (Eugene, OR). All other reagents were purchased from Sigma (St. Louis, MO) unless indicated otherwise.

Cell culture and transfection. Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cord veins by collagenase treatment, as previously described (20), and used in passages 2–6. The cells were grown in M199 supplemented with 20% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 3 ng/ml basic fibroblast growth factor, and 5 U/ml heparin at 37°C in humidified 5% CO2–95% air. The hemagglutinin-tagged kinase-dead form of Akt (K179M, DN-Akt) (25) and Hissx6-tagged dominant-negative (DN) ERK (K71R, DN-ERK) (22) were subcloned into pcDNA3 vectors. HUVECs were transiently transfected for 4 h in 60-mm dishes with 2 µg of the empty vector (pcDNA3) or vector containing the gene for DN-AKT or DN-ERK using Lipofectamine (Invitrogen), according to the manufacturer’s instructions. The cells were supplemented with M199 with 20% serum and cultured for 36 h and then subjected to Western blotting for confirmation of protein expression. When HUVECs were transfected with pEGFP-C1 vector using Lipofectamine, the transfected cells were conveniently monitored by fluorescence microscopy for green fluorescent protein expression, revealing typical transfection efficiency of ~35% (data not shown).

Proliferation assay. HUVEC proliferation was determined by DNA synthesis. Briefly, HUVECs were seeded at a density of 2.5 × 10⁴ cells/well in gelatin-coated 24-well plates. The cells were incubated in growth medium and allowed to attach for 18 h. The cells were washed twice with M199 containing 1% FBS and 0.1% FBS. The cells were stimulated with FKN or VEGF for 24 h before addition of 1 µCi/ml [3H]thymidine for 30 min. After the cells were washed twice with ice-cold H2O, [3H] radioactivity was solubilized in 0.2 N NaOH containing 0.1% SDS, and DNA synthesis was determined by a liquid scintillation counter.

Cell migration assay. Migration assays were performed as previously described (25). Briefly, the chemotactic motility of HUVECs was assayed using Transwell plates with 6.5-µm-diameter polycarbonate filters (8-µm pore size). The lower surface of the filter was coated with 10 µg of gelatin. Fresh M199 containing 1% FBS and FKN or VEGF was placed in the lower wells. HUVECs were trypsinized and suspended at a final concentration of 1 × 10⁶ cells/ml in M199 containing 1% FBS. Each inhibitor was incubated with the cells for 30 min at room temperature before seeding. One hundred microliters of the cell suspension were loaded into each of the upper wells. The chamber was incubated at 37°C for 4 h. After incubation, the cells were fixed and stained with hematoxylin and eosin. The upper surface of the filter was wiped with a cotton swab to remove nonmigrating cells, and chemotaxis was observed using an inverted phase contrast microscope. Images were captured with a video graphic system. Chemotaxis was quantified by counting the cells that migrated to the lower side of the filter at low-power (×100) fields.

Tube formation assay. The formation of tubelike structures by HUVECs on growth factor-reduced Matrigel was performed as previously described (25). Twenty-four-well culture plates were coated with Matrigel according to the manufacturer’s instructions. After HUVECs were incubated in M199 containing 1% FBS for 6 h and plated onto the layer of Matrigel at a density of 2.0 × 10⁵ cells/well, FKN or VEGF with or without inhibitors was added. Matrigel cultures were incubated at 37°C for 20 h. Tube formation was observed using an inverted phase contrast microscope. Images were captured with a video graphic system. The degree of tube formation was quantified by measurement of the length of tubes in five randomly chosen low-power (×100) fields from each well using ImagePro Plus (version 4.5, Media Cybernetics, San Diego, CA).

Western blotting. HUVECs were cultured in M199 containing 1% FBS for 6 h and then stimulated with FKN or VEGF with inhibitors. The cells were scraped off the plates and lysed in RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 0.1% SDS). Cell lysates (50 µg) were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were incubated with antibodies against target proteins for 2 h. After they were washed twice, the membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody, and signal intensities of target proteins were detected by the enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ), as previously described (26).

RT-PCR. Total RNA was prepared from HUVECs using TRIzol reagent. Five micrograms of total RNA were converted to cDNA by incubation with 200 U of reverse transcriptase and 500 ng of oligo(dT) primer in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, and 1 mM DNTPs at 42°C for 1 h. The levels of VEGF were determined from cDNA by PCR. The sequence of primers and the conditions of PCR were the same as those previously described (22).

Aortic ring sprouting assay. Aortas harvested from 6-wk-old Sprague-Dawley rats (Orient, Sungnam, Korea) were transversely cut into 1-mm-thick sections. The aortic rings were placed in 96-well plates coated with 50 µl of Matrigel and sealed in place with an overlay of 20 µl of Matrigel. FKN (20 ng/ml) or VEGF (20 ng/ml) was added to the wells at a final volume of 100 µl in human endothelial serum-free medium (Invitrogen). On day 5, microvessel outgrowth was photographed under a phase contrast microscope. The assay was scored from 0 (least positive) to 5 (most positive) in a double-blinded manner.

Choriovitallantoic membrane assay. To investigate the in vivo angiogenic activity of FKN, the modified choriovitallantoic membrane (CAM) assay was carried out as described previously (18). Briefly, FKN (20 ng) or VEGF (20 ng) in type I collagen was applied to Thermofax disks and polymerized at room temperature. The disks were loaded onto the CAM of 10-day-old embryos. After 70 ± 4 h of incubation at 37°C, the area around the loaded disk was photographed with a digital camera, and the number of newly formed vessels was counted by two observers in a double-blinded manner. Assays for each test sample were carried out using 18–20 eggs.

Intravital fluorescence microscopy. Male BALB/c mice (6–8 wk old) were obtained from Daehan Biolink and maintained at the specific pathogen-free housing facility at the School of Medicine, University of Kangwon National University (Chunchon, Korea). All procedures performed on these animals were in accordance with the guidelines of the University Animal Care and Use Committee. The mice were anesthetized by inhalation of 1.5% isoflurane and O₂-N₂O using a vaporizer (Surgivet, Waukesha, WI), and abdominal wall windows were implanted. A titanium circular mount was attached to the skin and the abdominal wall. Growth factor-reduced Matrigel containing VEGF (100 ng/mouse) or FKN (100 ng/mouse) was applied to the space between the windows, and a circular glass coverslip was placed on top and fixed by a snap ring. After 4 days, the...
animals were anesthetized and injected intravenously with 50 µl of 25 mg/ml fluorescein isothiocyanate-labeled dextran (250,000 mol wt) via tail vein. The mice were then placed on a Zeiss Axiovert 200M microscope. The epi-illumination microscopy setup included a 100-W mercury lamp and filter set for blue light (440- to 475-nm excitation wavelength, 530- to 550-nm emission wavelength). Fluorescence images were recorded in five random locations of each window by an electron-multiplying charge-coupled device camera (Photon Max 512, Princeton Instruments, Trenton, NJ) and digitized for subsequent offline analysis using the MetaMorph program (Universal Imaging, Downingtown, PA). The assay was scored from 0 (least positive) to 5 (most positive) in a double-blinded manner.

NO measurement. The NO level in HUVECs was measured in situ by using DAF-FM diacetate (Molecular Probes). Briefly, 7 h after FKN treatment, the cells were washed twice with serum-free medium and then incubated with 5 µM DAF-FM diacetate for 1 h at 37°C. After the excess probe was removed, the cells were incubated for an additional 20 min to allow for complete deesterification of the intracellular DAF-FM diacetate to the nonpermeable and nonfluorescent DAF-FM, which is converted to the highly fluorescent triazol form in the presence of NO and oxygen. The fluorescence images were captured from ≥10 randomly selected cells per dish using a confocal laser microscope. The relative levels of intracellular NO were determined from the fluorescence intensity of DAF-FM.

Statistical analysis. Values are means ± SD. Statistical comparisons between groups were performed using one-way ANOVA followed by Student’s t-test. P < 0.05 was considered statistically significant.

Fig. 1. Fractalkine (FKN) induces human umbilical vein endothelial cell (HUVEC) proliferation, migration, and tube formation. A: [3H]thymidine incorporation assay of proliferation in HUVECs stimulated with FKN or vascular endothelial growth factor (VEGF) for 24 h and then treated with 1 µCi/ml [3H]thymidine for 6 h. B: [3H]thymidine incorporation assay of proliferation in HUVECs stimulated with 10 ng/ml FKN or 10 ng/ml VEGF. Ctl, control. C: quantification of chemotatic migration in HUVECs treated with FKN or VEGF in Transwell plates for 4 h. Cells that migrated to the bottom of the filter were counted using optical microscopy at ×100 magnification. D: tube formation in HUVECs cultured on a layer of Matrigel with or without FKN or 10 ng/ml VEGF for 20 h observed using an inverted phase contrast microscope with a video graphic system. Area covered by the tube network was quantitated using Image-Pro Plus software. Values are means ± SD (n = 3). *P < 0.01 vs. control (Ctl or 0).

RESULTS

FKN stimulates ex vivo and in vivo angiogenesis. Angiogenesis is a complex biological process that requires the precise coordination of multiple and various steps, such as endothelial cell proliferation, migration, and tube formation. As shown in previous studies (5, 44), FKN treatment significantly increased endothelial cell proliferation in a dose- and time-dependent manner compared with the well-known angiogenic factor VEGF (Fig. 1, A and B). When endothelial cells were counted after FKN treatment, cell proliferation was also significantly increased in a concentration-dependent manner (data not shown). Using Transwell plates, we next examined whether FKN would induce endothelial cell migration. Treatment of HUVECs with FKN for 4 h significantly increased migration in a concentration-dependent manner up to 20 ng/ml, which is comparable to that of VEGF treatment (Fig. 1C). Furthermore, FKN treatment resulted in a dose-dependent increase in intracellular NO. The angiogenic effects of FKN did not further increase at >20 ng/ml, whereas the angiogenic effects of VEGF reached maximum at 40 ng/ml (Fig. 1, A, C, and D). Treatment with 10 ng/ml FKN resulted in almost maximum in vitro proliferation, migration, and tube formation, with 2.3-, 2.2-, and 3.0-fold increases compared with control, and these angiogenic activities induced by 10 ng/ml of FKN were comparable to those of VEGF (10 ng/ml).
We further confirmed the ex vivo and in vivo angiogenic activity of FKN. Treatment with FKN resulted in an about threefold increase in vessel sprouting at the cut edge of rat aortic rings compared with control (Fig. 2A). Addition of FKN stimulated a significant number of newly formed blood vessels compared with control, as measured by the chick CAM assay (Fig. 2B). Angiogenic activity of FKN was also observed in a mouse model by intravital microscopy (Fig. 2C). All these angiogenic activities observed with FKN were comparable with those observed with VEGF at the same concentration. These results indicate that FKN is a potent angiogenic factor ex vivo and in vivo.

G protein-coupled receptor-mediated phosphorylation of ERK, Akt, and eNOS are involved in FKN-induced angiogenesis. Because phosphorylation of ERK, Akt, and eNOS is an important signaling event for angiogenesis (10, 32), we determined whether FKN regulates phosphorylation of ERK, Akt, and eNOS. Western blot analyses revealed that treatment of HUVECs with FKN increased phosphorylation of ERK, Akt, and eNOS in a time- and dose-dependent manner (Fig. 3, A and B). ERK phosphorylation was apparent 15 min after FKN treatment, which preceded Akt and eNOS phosphorylation at 45 min. It has been suggested that FKN executes its biological activities by activating the G protein-coupled receptor CX3CR1 (8). Therefore, we investigated the functional role of the G protein-coupled receptor on these FKN-dependent phosphorylation events. The G protein-coupled receptor inhibitor pertussis toxin (PTX) efficiently blocked FKN-induced phosphorylation of these proteins (Fig. 3C), suggesting that FKN stimulates angiogenesis through a receptor linked to PTX-sensitive Gi/Go proteins. Treatment of the cells with the Raf-1 kinase inhibitor BAY 43-9006 or the MEK1/2 inhibitor PD-98059 blocked ERK, but not Akt and eNOS, phosphorylation (Fig. 3C). However, the PI3K inhibitor wortmannin suppressed Akt and eNOS phosphorylation without affecting ERK phosphorylation. These results suggest that FKN stimulates the Raf-1/MEK/ERK and PI3K/Akt/eNOS pathways by activating G protein-coupled receptors. We next examined the functional

![Fig. 2. FKN stimulates ex vivo and in vivo angiogenesis.](image)
involvement of these two signal pathways in FKN-induced angiogenesis. Treatment of HUVECs with PTX completely blocked FKN-induced HUVEC proliferation, migration, and tube formation, whereas treatment with BAY 43-9006, PD-98059, and wortmannin partially inhibited FKN-induced proliferation, migration, and tube formation of HUVECs (Fig. 4, A–C). On the basis of these findings, we hypothesized that activation of ERK and Akt plays a critical role in FKN-induced angiogenesis. To determine the involvement of ERK and Akt in FKN-induced angiogenesis in endothelial cells, we transiently transfected HUVECs with DN-ERK and DN-Akt and examined their effects on FKN-induced signaling events and angiogenesis (Fig. 5). HUVECs expressing DN-ERK slightly reduced basal ERK phosphorylation and significantly blocked FKN-mediated ERK phosphorylation without changing Akt and eNOS phosphorylation. Conversely, transfection with DN-Akt suppressed basal and FKN-stimulated Akt and eNOS phosphorylation without altering ERK activation (Fig. 5A). However, both transfectants significantly inhibited FKN-induced proliferation, migration, and tube formation compared with mock-transfected cells and also partially reduced the basal angiogenic activity compared with mock-transfected and non-transfected control cells (Fig. 5, B–D). These results suggest that FKN activates two distinct signal pathways downstream of G protein-coupled receptor activation, Raf-1/MEK/ERK and PI3K/Akt/eNOS, which are involved in stimulating angiogenesis.

**FKN increases PI3K/Akt-dependent NO generation.** The chemokine for endothelial progenitor cells CXCL12 and VEGF activates Akt-dependent eNOS activation and leads to an increase in NO production, which potentiates angiogenesis (16, 30). We next examined the effect of eNOS inhibition on FKN-mediated angiogenesis and the intracellular signal pathway. Treatment of HUVECs with the NOS inhibitor NMA significantly inhibited HUVEC proliferation, migration, and tube formation induced by FKN (Fig. 6, A–C), without affecting the increases in FKN-induced phosphorylation of ERK, Akt, and eNOS (Fig. 6D). We next determined NO production in HUVECs stimulated with FKN in the presence or absence of several inhibitors, as measured by the intensity of fluorescent DAF-FM formed in the presence of NO. Stimulation with FKN resulted in a significant increase in NO production compared with control cells, and this increase was inhibited in the presence of NMA (Fig. 6, E and F). In addition, NO production by FKN was also significantly inhibited by treatment with PTX and wortmannin, which inhibited FKN-induced Akt and eNOS phosphorylation and angiogenesis. However, NO production was not inhibited by treatment with PD-98059 but was observed to suppress angiogenesis without affecting Akt and eNOS phosphorylation (Figs. 3 and 4). These results indicate that eNOS phosphorylation-dependent NO production through the PI3K/Akt pathway is an important signal cascade in FKN-induced angiogenesis.

**FKN does not induce VEGF expression but shares the same downstream signal pathway with VEGF.** The angiogenic activity of some chemokines may be the result of direct action on endothelial cells or induction of other genes, including VEGF, which is involved in angiogenesis (41). VEGF stimulates endothelial cell proliferation and migration by activating phosphorylation-dependent activation of ERK and Akt. A number of angiogenic inducers, including TNF-α, transforming growth factor-β, interleukin-1β, prostaglandins, and endothelins, have been shown to induce the expression of VEGF in various cell types (7). To test whether FKN-induced angiogenesis is mediated by the expression of VEGF, we examined the effect of VEGF-neutralizing antibody on FKN (10 ng/ml)-induced in-
increases in ERK and Akt phosphorylation. VEGF-neutralizing antibody reduced VEGF (10 ng/ml)-mediated phosphorylation of ERK, Akt, and eNOS but did not affect FKN-mediated phosphorylation (Fig. 7A). In addition, FKN (20 ng/ml) + VEGF (40 ng/ml), which resulted in maximum angiogenic activity, partially increased the phosphorylated activation of ERK, Akt, and eNOS, suggesting that both angiogenic factors utilize the same or an overlapping signaling mechanism. Furthermore, this neutralizing antibody significantly reduced VEGF-induced HUVEC proliferation but did not alter the proliferative effect of FKN (Fig. 7B). We also observed that FKN-induced HUVEC migration and tube formation were not affected by the VEGF-neutralizing antibody (data not shown). We next examined whether FKN regulated VEGF expression as assessed by RT-PCR analysis. As previously reported, TNF-α markedly increased the VEGF transcript in HUVECs after 8 h of treatment, whereas FKN did not increase the levels of VEGF mRNA (Fig. 7C). These results indicate that FKN may act as a direct angiogenic modulator in endothelial cells without inducing VEGF expression. We further examined the effect of the G protein-coupled receptor inhibitor PTX, the MEK inhibitor PD-98059, and the PI3K inhibitor wortmannin on FKN- and VEGF-mediated angiogenesis. Treatment of HUVECs with PTX completely inhibited FKN-mediated, but not VEGF-induced, cell proliferation (Fig. 7D). Similar inhibitory effects of PTX were also observed in the migration and tube formation of HUVECs stimulated by FKN or VEGF (data not shown). FKN (10 ng/ml) + VEGF (20 ng/ml) significantly increased cell migration in HUVECs, which was significantly blocked by treatment with PTX, PD-98059, or wortmannin. In addition, this combination effect was almost completely inhibited by PD-98059 + wortmannin. However, saturating or maximum concentrations FKN (20 ng/ml) + VEGF (40 ng/ml) revealed a slight, but not synergistic, increase in endothelial cell proliferation (Fig. 7E). This proliferation was partially blocked by PTX, PD-98059, or wortmannin but was almost completely inhibited by PD-98059 + wortmannin (data not shown). These results suggest that the angiogenic processes of FKN and VEGF act independently of one another but utilize MEK/ERK and PI3K/Akt activation as common mediators in their angiogenic signal pathways.

**DISCUSSION**

This study was undertaken to examine the potential effect of FKN on angiogenesis in vitro and in vivo and to investigate its
underlying molecular mechanism. Our results show that FKN significantly stimulates the angiogenic process in vitro and in vivo. The FKN-induced angiogenic process was accompanied by G protein-coupled receptor-mediated phosphorylation of ERK, Akt, and eNOS, as well as an increase in intracellular NO production. Inhibition of Raf-1, MEK, PI3K, and eNOS partially suppressed the angiogenic activity of FKN. However, the inhibitors of Raf-1 and MEK blocked FKN-induced ERK, but not Akt and eNOS, phosphorylation. The PI3K inhibitor suppressed only Akt and eNOS phosphorylation and NO production. Moreover, overexpression of DN-ERK and DN-Akt significantly, but not completely, inhibited the angiogenic activity of FKN. Our results indicate that the Raf-1/MEK/ERK and PI3K/Akt/eNOS/NO signal pathways via G protein-coupled receptor play critical roles in FKN-induced angiogenesis.

Chemokines are divided into four families: CXC, CC, C, and CX3C. The CXC chemokine family is based on the presence or absence of the amino acid sequence Glu-Leu-Arg (the ELR motif). This motif has tremendous physiological implication in terms of effect on angiogenesis, because ELR chemokines (CXCL1 to CXCL8, except CXCL4) have been shown to possess angiogenic activity, whereas most of the non-ELR chemokines (CXCL4, CXCL9, and CXCL10) exert antiangiostatic activity (36, 42). In addition, the CC chemokines (MCP-1, vMIP I, and vMIP II) have been shown to be strong inducers of angiogenesis (14, 36, 45). FKN, a novel transmembrane molecule with a CX3C-motif chemokine domain atop a mucin stalk, is a unique class of chemokine that may constitute part of the molecular control of leukocyte traffic at the endothelium (4, 19). FKN has been shown to play an important role in inflammatory vascular diseases, including atherosclerosis and rheumatoid arthritis (27, 38). We have demonstrated that FKN functions as a stimulator of endothelial cell proliferation, migration, and tube formation in vitro, as well as blood vessel formation in vivo. The angiogenic property of FKN may play a pathological role in the recruitment of blood inflammatory cells and the supply of nutrients and oxygen to the proliferating pannus tissue (38). These results indicate that FKN, a member of the CX3C chemokine family, is an inflammatory chemokine with angiogenic activity. In addition, because FKN does not contain an ELR motif, the mechanism by which FKN induces endothelial cell proliferation, migration, and tube formation seems different from that of other angiogenic chemokines.

FKN stimulated HUVEC proliferation, migration, and tube-like structure formation in vitro in a concentration-dependent manner from 1 to 10 ng/ml, where it exhibited activity similar to that of VEGF, a well-known angiogenic factor, at 10 ng/ml (Fig. 1). FKN also exerted angiogenic activity ex vivo and in vivo similar to that of VEGF at the same concentration (Fig. 2). However, treatment of HUVECs with a higher concentration...
(20 ng/ml) of FKN showed no further significant increase in angiogenic activity, whereas VEGF elevated endothelial cell proliferation and migration in a dose-dependent manner and reached a maximum at 40 ng/ml (Fig. 1), suggesting that FKN and VEGF receptors are likely saturated by their ligands at 20 and 40 ng/ml, respectively. Although FKN exists in soluble and membrane-anchored forms, it is predominantly found in the membrane-bound form. The expression level of FKN is very low in normal cells but increases markedly after stimulation of cells by TNF-α/H9251 and interleukin-1β/H9252 (4, 37). The level of FKN is very low or undetectable in physiological fluid but significantly increases under pathological conditions (35). The dose of FKN used in this study (20 ng/ml) is much lower than the local FKN concentration (0.6–1.0 μg/ml) detected in the seminal plasma of healthy and infertile men (46). Although 20 ng/ml of VEGF is higher than the physiological level, higher levels of VEGF (14–146 ng/ml) have been detected in the plasma of human patients with metastatic melanoma (31). Thus the concentrations of FKN and VEGF used in this study are comparable with pathophysiological levels.

Molecular weights of FKN (monomer) and VEGF (dimer) used in this study were 8,500 and 44,000, respectively, indicating that molar concentrations of these angiogenic inducers (10 ng/ml) used in this in vitro study (Fig. 1) were 1.17 and 0.23 nM, resulting in the angiogenic activity of FKN that is fivefold lower than that of VEGF at the same molar concentration. Therefore, this difference in angiogenic activity can be explained by different biological potencies and/or different receptor levels. Moreover, the well-known angiogenic chemokine interleukin-8 has been shown to induce doubling in HUVEC chemotaxis at 1.25 nM and induce angiogenesis at 10 nM (24). Other angiogenic chemokines, CXCL1 and CXCL5, induced angiogenesis in a rat cornea neovascularization assay at 10 nM (42). Thus, although FKN is slightly weaker than VEGF, our results suggest that FKN is likely to be a strong angiogenic factor in the nanomolar range, similar to other angiogenic chemokines.
The action mode of angiogenic chemokines may be the result of its direct action on endothelial cells or through the induction of other genes involved in angiogenesis (36). A number of angiogenic chemokines, including MCP-1 and CXCL12, have been shown to induce the expression of VEGF in various cell types, which is, in part, responsible for their role in angiogenesis (17, 36). MCP-1-induced angiogenesis was completely blocked by the specific VEGF inhibitor Flt2-11, suggesting that VEGF mainly orchestrates MCP-induced angiogenesis (17). We tested the possibility that the effect of FKN on angiogenesis is mediated through the expression of VEGF. Our results showed that FKN did not increase VEGF mRNA expression and that VEGF-neutralizing antibody did not affect FKN-induced endothelial cell proliferation and phosphorylation of ERK, Akt, and eNOS. In addition, inhibition of FKN signaling by PTX did not affect VEGF-induced angiogenic responses in HUVECs. These results indicate that FKN directly stimulates angiogenesis without increasing VEGF expression.

Expression of CX3CR1, a specific seven-transmembrane-domain G protein-coupled receptor for FKN, has been found in different cell types, such as myeloid cells (44), microvascular endothelial cells (44), and human peripheral blood mononuclear cells (3). We observed that CX3CR1 mRNA was highly expressed in HUVECs and slightly increased by FKN treatment (data not shown). As reported in G protein-coupled receptor activation (6, 21, 32), FKN also activated downstream angiogenic signal mediators, such as ERK, Akt, and eNOS, in HUVECs (Fig. 3). Using its specific inhibitor PTX, we have also provided direct evidence that the G protein-coupled receptor CX3CR1 is essentially involved in FKN-mediated activation of ERK, Akt, and eNOS and angiogenesis. In addition, the inhibitors of Raf and MEK completely suppressed FKN-induced ERK activation without altering Akt and eNOS phosphorylation and significantly, but not completely, suppressed FKN-induced angiogenesis in HUVECs. Inhibition of PI3K, however, effectively suppressed phosphorylation of Akt and eNOS without affecting ERK phosphorylation and partially inhibited the angiogenic activity of FKN. Inhibition of PI3K, however, effectively suppressed phosphorylation of Akt and eNOS without affecting ERK phosphorylation and partially inhibited the angiogenic activity of FKN. Inhibition of ERK and Akt by transfection with their biologically inactive genes also significantly inhibited the angiogenic activity of FKN. These results indicate that Raf and PI3K are independently stimulated by activation of CX3CR1 with FKN. In addition, treatment with Raf-1 and PI3K inhibitors completely suppressed FKN-induced endothelial cell proliferation and tube formation (data not shown). These results suggest that FKN requires Raf/MEK/ERK and PI3K/Akt/eNOS-dependent pathways for its angiogenic activity (Fig. 7).

Many studies have shown that angiogenic factors, including VEGF, increase Akt activation, leading to eNOS activation,
and the consequent endothelial NO production is required for VEGF-induced angiogenesis (15, 23). We have shown that the NOS inhibitor NMA suppressed FKN-induced NO production and angiogenesis without inhibiting ERK, Akt, and eNOS phosphorylation. A PI3K inhibitor upstream of Akt completely suppressed NO production and partially reduced angiogenesis in HUVECs treated with FKN. However, inhibition of MEK did not affect NO production but significantly suppressed the angiogenic process in HUVECs stimulated with FKN. These results indicate that increased NO production via activation of the PI3K/Akt/eNOS-dependent pathway may vitally contribute to the angiogenic processes triggered by FKN. Although upstream signaling events induced by FKN are different from those induced by VEGF, activation of MEK/ERK and PI3K/Akt is a common intracellular event in both angiogenic pathways (Fig. 7). In addition, our findings suggest that coactivation of the Raf-1/MEK/ERK and PI3K/Akt/eNOS/NO pathways may result in full angiogenic activity exerted by FKN.

In conclusion, the present study has characterized the signal pathway of CX3CR1 activated by FKN in HUVECs, which is likely to provide further information on the relation between signaling events and the angiogenic action linked to activation of this receptor. Moreover, FKN activated the Raf-1/MEK/ERK and PI3K/Akt/eNOS/NO pathways and stimulated in vitro and in vivo angiogenesis. Although further studies are needed, blocking the biological activity of FKN, its receptor, or associated downstream signals may suppress the pathogenesis of angiogenesis-associated inflammatory diseases, including wound healing, rheumatoid arthritis, and tumor growth and metastasis.

GRANTS
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