

Fractalkine: A Novel Angiogenic Chemokine in Rheumatoid Arthritis

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Angiogenesis is an important aspect of the vasculo-proliferation found in the rheumatoid arthritic (RA) pannus. We have previously implicated members of the CXC chemokine family as potent angiogenic mediators in RA. We investigated the possibility that the sole member of the CX₃C chemokine family, fractalkine (fkn), induces angiogenesis and that fkn might mediate angiogenesis in RA. Recombinant human fkn significantly induced migration of human dermal microvascular endothelial cells (HMVECs), a facet of the angiogenic response, in the pmol/L range in a concentration-dependent manner ($P < 0.05$). Fkn also induced the formation of significantly more endothelial tubes on Matrigel than did a negative control ($P < 0.05$). Fkn significantly induced 2.3-fold more blood vessel growth than control in the *in vivo* Matrigel plug assays ($P < 0.05$). We identified HMVEC expression of the fkn receptor, CX₃CR1. Next, we determined if RA synovial fluid (SF)-induced angiogenesis was fkn-dependent. SFs from six RA patients immunodepleted of soluble fkn induced 56% less migration of HMVECs than did sham-depleted RA SFs ($P < 0.05$). *In vivo*, immunodepletion of fkn from six RA SFs significantly inhibited their angiogenic activity in Matrigel plug assays ($P < 0.05$). Immunodepletion of fkn from five RA synovial tissue homogenates inhibited their ability to induce angiogenesis in *in vivo* Matrigel plug assays ($P < 0.05$). These results establish a new function for fkn as an angiogenic mediator and suggest that it may mediate angiogenesis in RA. (*Am J Pathol* 2001, 159:1521–1530)

Angiogenesis, the growth and proliferation of new blood vessels, is an important aspect of the vasculoproliferation found in tumor growth, wound repair, and inflammatory states such as rheumatoid arthritis (RA).^{1–3} A number of mediators orchestrate the angiogenic process. These include members of the adhesion molecule superfamily, such as vascular cell adhesion molecule-1 (VCAM-1) or E-selectin^{4,5} and chemokines such as interleukin (IL)-8.⁶

The chemokines are mainly homologous 8- to 10-kd proteins that are subdivided into four families (C, CC, CXC, and CX₃C) on the basis of the relative position of the cysteine residues in the mature protein.^{7,8} Although the chemokines are generally thought to function as leukocyte attractants, we have previously identified the CXC chemokine, IL-8, as a mediator of angiogenesis.⁶ Further studies have shown that the CXC chemokines containing the ELR motif, consisting of glutamic acid-leucine-arginine preceding the CXC sequence are not only chemotactic for neutrophils but are angiogenic.⁹ The CC chemokine monocyte chemoattractant protein-1 (MCP-1) has recently been identified as an inducer of endothelial cell (EC) chemotaxis *in vitro*¹⁰ and as a mediator of inflammatory angiogenesis *in vivo*.¹¹ Viral CC chemokine-like proteins vMIP-I and vMIP-II, but not their cellular counterpart macrophage inflammatory protein-1 α (MIP-1 α), have also been shown to induce angiogenesis *in vivo*.¹²

We have previously described fractalkine (fkn), the sole member of the CX₃C chemokine family, as a mediator of inflammation in RA.¹³ As many inflammatory mediators are also angiogenic mediators in RA synovial tissues (STs) and synovial fluids (SFs) we investigated the angiogenic properties of fkn. In this report we define fkn as a potent angiogenic mediator in RA. Fkn was named for its fractal geometry and is distinct from other chemokines in that it contains the CX₃C motif with three amino acids between the two terminal cysteines.¹⁴ Fkn also is unique in that it is a transmembrane protein displaying its chemokine domain perched on a long (241 amino acid) negatively charged mucin-like stalk that extends away from the cell surface. In addition, fkn is much larger than any other chemokine consisting of 373 amino acids and can be cleaved via a syndecan-like cleavage motif proximal to the membrane resulting in a soluble 95-kd glycoprotein.^{14,15}

Soluble fkn is a monomer that like other chemokines functions as a chemoattractant for natural killer cells, T lymphocytes, and monocytes.^{14,16–19} Unlike other chemokines, membrane-bound fkn can directly mediate firm

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cell adhesion, and initiate leukocyte capture.^{14,17,20} Specifically, membrane-bound fkn has been shown to be involved in adhesion of monocytes, T lymphocytes, and natural killer cells to ECs *in vitro*.^{14,20,21} Fkn expression on human ECs is induced by the inflammatory cytokines IL-1 or tumor necrosis factor- α .¹⁴ The mouse homologue of fkn, neurotactin, is up-regulated on ECs in inflamed brain in allergic encephalomyelitis.²² Thus, in inflammation fkn can function both as a chemoattractant for leukocytes and as an EC adhesion molecule.

We show fkn to induce both EC migration and tube formation *in vitro*, to induce angiogenesis *in vivo* and to function as an angiogenic mediator present in RA SF and ST. Thus, in addition to being an adhesion molecule and chemoattractant for leukocytes, fkn functions as an angiogenic mediator.

Materials and Methods

Reagents

Human recombinant fkn, IL-8, epithelial neutrophil-activating protein-78 (ENA-78), basic fibroblast growth factor (bFGF), bovine acidic fibroblast growth factor (aFGF), goat polyclonal antibody (pAb) anti-human fkn, and control goat IgG were purchased from R&D Systems (Minneapolis, MN). Rabbit purified pAb anti-CX₃CR1 was purchased from Imgenex (San Diego, CA). Dimethyl sulfoxide and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma (St. Louis, MO). Matrigel was purchased from Becton Dickinson (Bedford, MA). Enhanced chemiluminescence Western blotting detection reagents and goat horseradish peroxidase-conjugated antibody were obtained from Amersham Life Sciences (Arlington Heights, IL).

Cells

Human dermal microvascular endothelial cells (HMVECs) were purchased from BioWhittaker (San Diego, CA) and grown in EC growth medium (BioWhittaker) with 10% fetal bovine serum (FBS). Cell assays were performed in endothelial basal medium (BioWhittaker) supplemented with the appropriate amount of FBS for the assay and 0.1% gentamicin. THP-1 cells were purchased from the American Type Culture Collection (Manassas, VA) and grown in RPMI with 10% FBS.

EC Chemotaxis

HMVECs were cultured in endothelial cell growth medium containing 10% FBS. Chemotaxis was performed in 48-well blind-well chemotaxis chambers using gelatin-coated polycarbonate membranes with an 8- μ m pore size (Neuroprobe, Cabin John, MD).^{4,23} HMVECs (2.5×10^4 cells in 25 μ l of endothelial basal medium containing 0.1% FBS) were added to the bottom wells. The chambers were inverted and incubated for 2 hours at 37°C allowing HMVEC attachment to the membrane. Fkn (10^{-12} to 10^2 nmol/L), phosphate-buffered saline (PBS),

or positive control bFGF (60 nmol/L) were added to the top wells and the chambers incubated for 2 hours at 37°C. The membranes were removed, fixed in methanol, and stained with Diff-Quik (Baxter Scientific, Deerfield, IL). The number of cells that had migrated through the pores in the filter was counted per three high-power fields and each test group was assayed in quadruplicate. Checkerboard analyses were performed in a similar manner to chemotaxis assays except that the concentrations of fkn were varied in the upper and lower chambers.

Immunodepletion of Fkn in HMVEC Chemotaxis Assays

Fkn (10^1 nmol/L and 10^{-3} nmol/L) or PMA (60 nmol/L) were incubated with 10 to 25 μ g/ml of either pAb anti-fkn or control goat IgG for 1 hour at 37°C. On completion of this neutralization period, the fkn/Ab and PMA/Ab combinations were assayed in the HMVEC chemotaxis assay as described above.

Immunodepletion of Fkn in RA SFs for HMVEC Chemotaxis Assays

SFs were isolated from six patients with RA during therapeutic arthrocentesis with Institutional Review Board approval. RA SF was diluted 1 to 50 with PBS and preincubated with 25 μ g/ml of pAb anti-fkn or goat IgG control for 1 hour at 37°C. On completion of this neutralization period, the RA SF/Ab combination was assayed in the HMVEC chemotaxis assay as described above.

Formation of EC Tubes on Matrigel in Vitro

Matrigel was thawed on ice to prevent premature polymerization; 125 μ l were plated into individual wells of eight-well chamber slides (Falcon, Bedford, MA) and allowed to polymerize at 37°C for 30 to 60 minutes. HMVECs were removed from culture by trypsinization and resuspended at 4×10^4 cells/ml in Medium 199 (Life Technologies, Inc., Grand Island, NY) containing 2% FBS and 200 μ g/ml EC growth supplement.²⁴ Four hundred μ l of cell suspension containing fkn, 50 nmol/L PMA, or vehicle control (PBS for fkn, PBS and dimethyl sulfoxide for PMA) were plated in each well and plates incubated for 16 to 18 hours at 37°C in a 5% CO₂ humidified atmosphere.²⁵ Culture medium was aspirated off and cells were fixed with Diff-Quik Fixative and stained with Diff-Quik Solution II. Each chamber was photographed using a Polaroid Microcam camera at a final magnification of $\times 22$. The number of tube branches was quantitated by a blinded observer.²⁶ Each concentration of control or test substance was assayed in triplicate.

HMVEC Proliferation Assay

HMVEC proliferation was quantified using a CellTiter 96 Aq_{ueous} assay (Promega, Madison, WI).^{4,23} HMVECs in endothelial basal medium, 2% FBS, and 0.1% gentamicin

were plated in 96-well plates (2500 cells/well) for 4 hours, allowing cells to adhere to the plates. The test substances, diluted in medium, were added to the appropriate wells and incubated according to the manufacturer's suggested conditions of 37°C and 5% CO₂ for 72 hours. After the incubation, viable cells were detected by their reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) into a formazan. The number of living cells in culture is directly proportional to the quantity of formazan product as measured at a wavelength of 490 nm. These absorbance values were compared to a positive control, bFGF, and a negative control, medium alone.

Reverse Transcriptase-Polymerase Chain Reaction (PCR) Amplification of HMVEC CX₃CR1

HMVECs were cultured in endothelial cell growth medium (BioWhittaker) containing 10% FBS. Total RNA (1 µg) was prepared from HMVECs and first-strand cDNAs were synthesized using an oligo dT primer and AMV RT (Promega, Madison, WI). Subsequent amplification of CX₃CR1 from HMVEC cDNA was performed using specific 5' and 3' primers: forward primer 5'CTCTATGACTCTTTCCCAGTTGT3'; reverse primer 5'AGACACAAGGCTTTGGGATTC3'.²⁷ PCR cycling conditions were 95°C for 5 minutes followed by 30 cycles of 95°C for 1 minute, 52°C for 1 minute, and 72°C for 1 minute, and ended by 10 minutes at 72°C. Amplification products were characterized by size fractionation on 1% agarose gels.

Western Blot Analysis

HMVECs were cultured in endothelial cell growth medium (BioWhittaker) containing 10% FBS. THP-1 cells were cultured in RPMI containing 10% FBS. Cells were lysed in extraction buffer containing 10 mmol/L Tris, pH 7.4, 100 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L ethyleneglycoltetraacetic acid, 1 mmol/L NaF, 20 mmol/L NaP₂O₄, 2 mmol/L Na₃VO₄, 1% Triton X-100, 10% glycerol, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, 1 mmol/L phenylmethyl sulfonyl fluoride, and protease inhibitors (1 tablet/10 ml, Proteinase inhibitor cocktail tablets; Boehringer Mannheim, Mannheim, Germany). Cell lysates were mixed 1:1 with Laemmli's sample buffer and boiled for 5 minutes. Equal amounts of sample were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Separated proteins were electrophoretically transferred from the gel onto nitrocellulose membranes using a Tris-glycine buffer. To block nonspecific binding, membranes were incubated with 5% milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour at room temperature. The blots were incubated with anti-human CX₃CR1 Ab (Imgenex) diluted 1:500 in TBST and 5% milk at 4°C overnight. After washing with TBST, the blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (diluted 1:10,000) for 45 minutes at room temperature.

An enhanced chemiluminescence detection system (ECL+, Amersham) was used to detect the CX₃CR1 band.

Matrigel Plug Assay for Angiogenesis in Vivo

Female 8- to 12-week-old C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were each injected subcutaneously near their abdominal midline using a 30-gauge needle with 0.5 ml of Matrigel combined with either PBS, fkn (100 nmol/L), IL-8 (100 nmol/L), ENA-78 (100 nmol/L), or positive control bovine aFGF (63 pmol/L).^{28,29} Seven to 10 days later, the mice were sacrificed and the Matrigel plugs were removed, weighed, and processed for histology or hemoglobin concentration determination. For histological analysis plugs were formalin-fixed, paraffin-embedded, cut into 4-µm sections, and Masson trichrome-stained. For hemoglobin determination, which correlates with the number of blood vessels, plugs were homogenized in 1 ml of distilled water. Hemoglobin concentration was determined either by the Drabkin method using a Drabkin's reagent kit (Sigma) or using 3,3',5,5'-tetramethylbenzidine liquid substrate system (Sigma).

Immunodepletion of Fkn in RA SFs for Matrigel Plug Angiogenesis Assays

SFs were isolated from six patients with RA during therapeutic arthrocentesis with Institutional Review Board approval. RA SFs were pooled and diluted 1 to 10 with PBS and preincubated with 25 µg/ml of pAb anti-fkn or goat IgG control for 1 hour at 37°C. On completion of this neutralization period, the RA SF/Ab combination was diluted again 1 to 10 with Matrigel and assayed in the *in vivo* Matrigel plug angiogenesis assay as described above.

Immunodepletion of Fkn in RA STs for Matrigel Plug Angiogenesis Assays

STs were obtained from five patients undergoing total joint replacement who met the American College of Rheumatology criteria for RA.³⁰⁻³² RA STs were homogenized in 1 ml of an anti-protease buffer as described.³³ Samples were sonicated, centrifuged at 900 × *g* for 15 minutes and filtered through a 1.2-µm pore size sterile Acrodisk (Gelman Sciences, Ann Arbor, MI), and frozen at -80°C until thawed for assay. ST homogenates were thawed, normalized, pooled, and preincubated with 25 µg/ml of pAb anti-fkn or goat IgG control for 1 hour at 37°C. On completion of this neutralization period, the RA ST homogenates/Ab combination was diluted 1 to 25 with Matrigel and assayed in the *in vivo* Matrigel plug angiogenesis assay as described above.

Statistical Analysis

Data were analyzed using Student's *t*-test. *P* values <0.05 were considered significant.

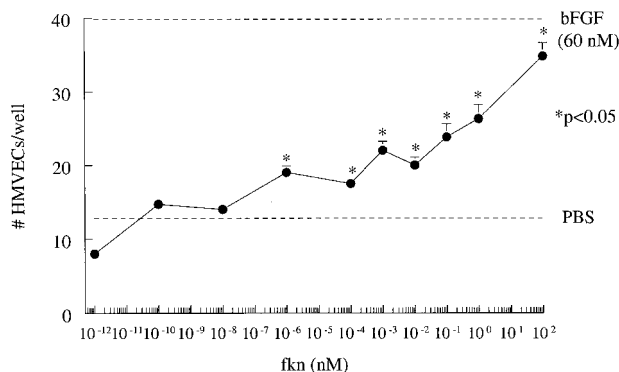


Figure 1. Fkn induces HMVEC migration. Results represent the mean number of cells/well \pm SE of one representative assay of four. *, $P < 0.05$, significantly different from PBS control.

Results

Fkn Induces HMVEC Migration (Chemotaxis and Chemokinesis) *In Vitro*

Fkn was assayed for its ability to induce HMVEC chemotaxis *in vitro*. Results of a representative experiment of four are shown in Figure 1. Fkn induced chemotaxis in a concentration-dependent manner in the pmol/L and nmol/L concentration range. Fkn (10^{-1} pmol/L to 10^2 nmol/L) significantly increased EC chemotaxis over negative control PBS ($P < 0.05$). Checkerboard analysis was performed to determine whether fkn was chemotactic and/or chemokinetic for ECs. Representative results of four checkerboard assays showing fkn as both chemotactic and chemokinetic for HMVECs are shown in Table 1.

Fkn-Induced Chemotactic Activity for HMVECs Is Decreased by Immunodepletion of Fkn

We next determined whether the chemokine domain of fkn was responsible for the EC chemotactic ability of fkn. Fkn was incubated with 25 μ g/ml of an antibody specific for the CX₃C chemokine domain of fkn and then assayed for HMVEC chemotaxis ability. Figure 2A shows that at concentrations from 1 pmol/L to 10 nmol/L of fkn, the anti-CX₃C domain antibody completely inhibited fkn-induced HMVEC migration ($P < 0.05$). This inhibition of migration by anti-CX₃C domain antibody was specific for fkn-induced HMVEC migration as bFGF-induced migration was not affected by incubation with this antibody (Figure 2B).

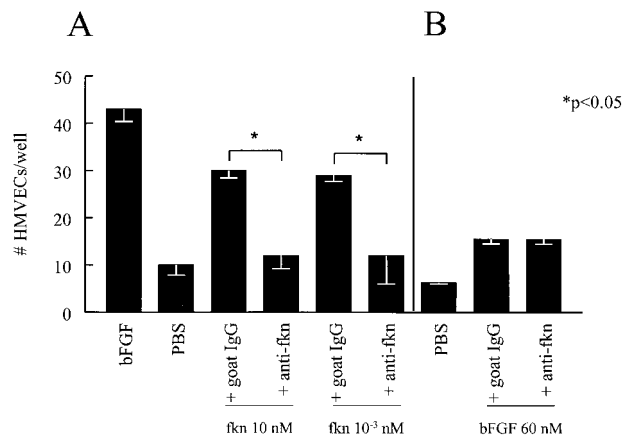


Figure 2. Anti-fkn inhibits fkn-induced, but not bFGF-induced HMVEC migration. **A:** Anti-fkn inhibited fkn-induced HMVEC migration. **B:** Anti-fkn did not inhibit bFGF-induced HMVEC migration. Results represent the mean number of cells/well \pm SE of one of three similar assays. *, $P < 0.05$, significantly different from goat IgG control.

Fkn Does Not Induce HMVEC Proliferation *In Vitro*

We next assessed the ability of fkn to act as a mitogen for ECs *in vitro*. When assayed in concentrations of 10^{-10} to 10^2 nmol/L, fkn did not induce a mitogenic response, in contrast to 60 nmol/L of bFGF that induced potent EC proliferation. We have shown previously that angiogenic soluble adhesion molecules such as soluble VCAM-1 (sVCAM-1) or sE-selectin did not induce EC mitogenesis *in vitro* although they were potentially angiogenic *in vivo*.⁴ Results of a representative experiment of four experiments is shown in Figure 3.

Fkn-Induced HMVEC Tube Formation on Matrigel *In Vitro*

Tube formation, one facet of the angiogenic response, can be assayed for *in vitro* by testing the ability of HMVECs plated on Matrigel to form tubes. We investigated the ability of fkn to induce tube formation on Matrigel in eight-well chamber slides. The results of a representative experiment of four experiments is shown in Figure 4. Figure 4A shows a photomicrograph of tube formation induced by fkn. In contrast, PBS did not induce EC tube formation. To quantify tube formation in the Matrigel matrices, a blinded observer counted EC tubes in each experimental well. Figure 4B shows EC tube counts for

Table 1. Checkerboard Analysis of Fkn-Mediated HMVEC Migration

Lower chamber fkn*	Upper chamber fkn*			
	0 nmol/L	10^{-5} nmol/L	10^{-3} nmol/L	10^{-1} nmol/L
0 nmol/L	8.5 \pm 0.5	16.3 \pm 0.3	16.0 \pm 1.8	22.0 \pm 0.9
10^{-5} nmol/L	17.8 \pm 2.6	15.0 \pm 0.7	14.3 \pm 1.4	18.3 \pm 1.0
10^{-3} nmol/L	16.8 \pm 2.8	15.5 \pm 0.7	13.3 \pm 0.8	22.0 \pm 1.6
10^{-1} nmol/L	18.0 \pm 0.7	18.5 \pm 1.9	13.0 \pm 1.6	18.0 \pm 0.5

*fkn (10^{-5} to 10^{-1} nmol/L) was assayed for EC migration. The results represent 12 high-power fields ($\times 400$) per sample and are expressed as the number of cells \pm SE per replicate well. This results represents one of four experiments. Positive control migration in response to bFGF (60 nmol/L) was a mean of 26 cells/well. Negative control migration in response to PBS was nine cells/well.

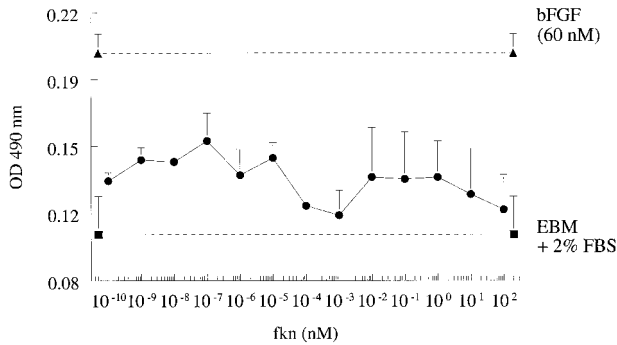


Figure 3. Fkn does not induce HMVEC proliferation. Results represent the mean absorbance of quadruplicate wells \pm SE of one representative assay of four assays. No values were significantly different ($P < 0.05$) from media alone.

fkn-induced tube formation along with tube counts induced by positive control PMA and the vehicle controls dimethyl sulfoxide and PBS. Fkn induced significantly more EC tube formation than negative control PBS (152 ± 11.7 versus 90 ± 10.7 tubes/well; $P < 0.05$, $n = 4$). We have also used this technique to test the ability of the

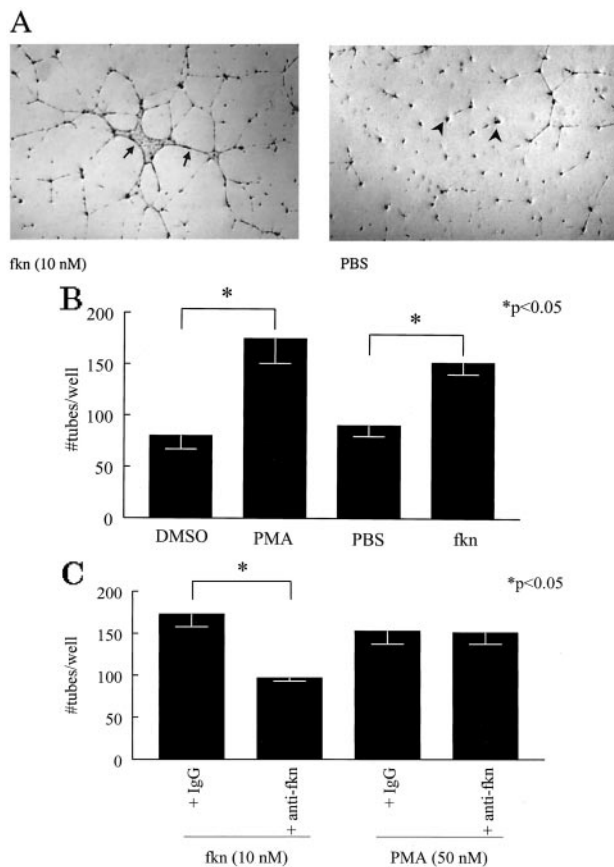


Figure 4. Fkn induces EC tube formation *in vitro*. **A:** Representative assay showing fractalkine-induced HMVEC tube formation and PBS control (original magnification, $\times 22$). Individual tubes are shown in fractalkine-treated well (**arrows**) and non-tube-forming ECs are identified in a PBS-treated well (**arrowheads**). **B:** Fkn and PMA both induce HMVEC tube formation relative to their negative controls. **C:** Anti-fkn inhibits fkn-induced, but not PMA-induced HMVEC tube formation. Values represent the mean number of HMVEC tube branches/well \pm SE for three or four assays. *, $P < 0.05$, significantly different from vehicle control.

angiogenic chemokines, IL-8 and ENA-78, to induce EC tube formation. Both IL-8 and ENA-78 induced EC tube formation greater than PBS controls (data not shown). Next, to help discern whether fkn-induced tube formation was because of the chemokine domain or to the mucin stalk, we incubated fkn with an anti-CX₃C domain antibody. Anti-fkn significantly inhibited fkn-induced tube formation over isotype control antibody ($P < 0.05$, $n = 3$) (Figure 4C). This inhibition of tube formation by anti-fkn was specific to fkn-induced formation as PMA-induced tube formation was not affected by incubation with the antibody (Figure 4C).

HMVECs Express CX₃CR1 in Vitro

To better understand how fkn may interact with ECs to induce migration (chemotaxis and chemokinesis) and tube formation, we tested whether the only known fkn receptor, CX₃CR1, was expressed by ECs. Previous reports have identified endothelial expression of fkn but did not examine expression of its receptor CX₃CR1. Reverse transcriptase-PCR was performed on HMVEC cDNAs along with cDNAs from THP-1 cells, a myeloid cell line previously reported to express high amounts of CX₃CR1 mRNA.³⁴ PCR products were synthesized using specific human CX₃CR1 primers that amplify a 320-bp fragment. As shown in Figure 5A, a 320-bp PCR product was amplified from both HMVEC cDNAs as well as the positive control, THP-1 cDNAs, indicating EC expression of CX₃CR1. Next, Western blot analysis was performed to demonstrate endothelial CX₃CR1 protein expression. Cell lysates were prepared from both HMVECs and THP-1 cells and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Western blotting, performed with a IgG-purified pAb specific for human CX₃CR1, revealed a band of the correct size (50 kd) in both the EC and positive control, THP-1 cell, lanes (Figure 5B).³⁵

Fkn-Induced Angiogenesis in Matrigel Plugs in Vivo

To determine whether fkn functions as an angiogenic mediator *in vivo*, we used the mouse Matrigel plug assay. Matrigel plugs containing negative control PBS, fkn, angiogenic chemokines IL-8 and ENA-78, or positive control aFGF were implanted subcutaneously into the abdomen of mice. A representative photomicrograph of a plug fixed and Masson trichrome-stained is shown in Figure 6A. In fkn-containing plugs marked new blood vessel growth can be seen. In contrast, minimal blood vessel growth was induced by negative control PBS. Figure 6B shows the hemoglobin content normalized to the weight of the Matrigel plugs. The hemoglobin content correlates with the number of blood vessels in the plugs. By this method, fkn induced significantly more blood vessels in the Matrigel plugs than did negative control PBS (0.77 ± 0.15 versus 0.33 ± 0.08 g/dl of hemoglobin/mg of plug weight, respectively; $n = 18$, $P < 0.05$). To compare the relative angiogenic potency of fkn to other known angio-

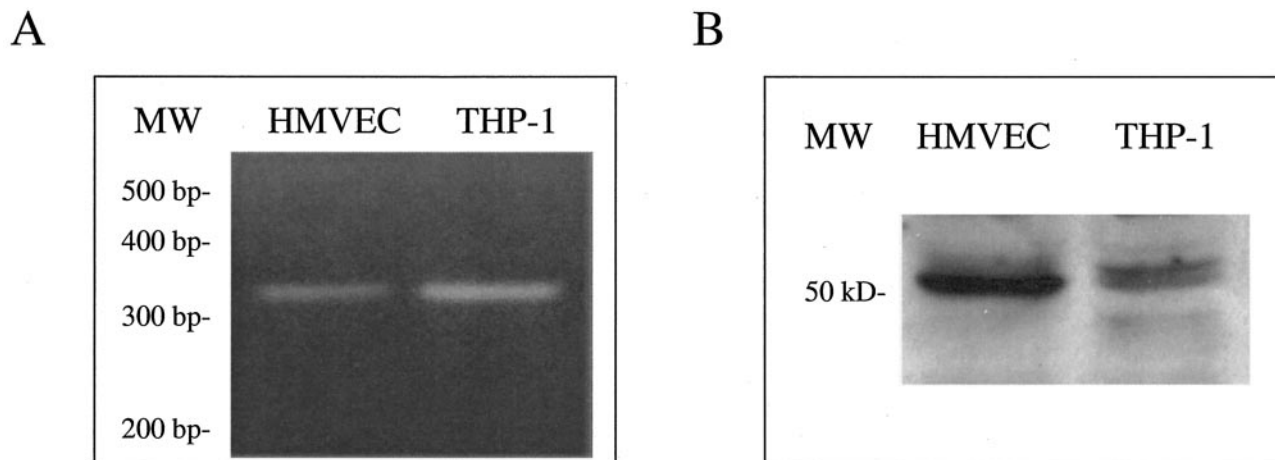


Figure 5. HMVECs express CX₃CR1. **A:** Agarose gel showing 320-bp CX₃CR1 reverse transcriptase-PCR products from HMVECs and THP-1 cells. **B:** Western blot showing 50-kd CX₃CR1 band in both HMVECs and THP-1 cells. MW, molecular weight markers.

genic chemokines, IL-8 and ENA-78 were also tested in the Matrigel plug model. The relative angiogenic potencies for fkn, IL-8, and ENA-78 as a percentage of the angiogenic potency of the positive control, aFGF, are shown in Figure 6C. Fkn exhibited 78% of the angiogenic potency of aFGF, whereas IL-8 and ENA-78 exhibited 65% and 44%, respectively ($n = 7$ to 9).

RA SF Chemotactic Activity for HMVECs Is Decreased by Immunodepletion of Fkn

To determine whether fkn has biological relevance in a disease characterized by angiogenesis, SFs from six patients with RA were immunodepleted of fkn and assayed for their HMVEC chemotactic activity. Results of immunodepletion experiments are shown in Table 2. Although RA SF was potently chemotactic for HMVECs, immunodepletion of fkn resulted in significantly decreased ($56.1 \pm 2.4\%$, mean \pm SE) chemotactic ability for HMVECs relative to immunodepletion with isotype control antibody ($P < 0.05$).

RA SF Angiogenic Activity Is Decreased by Immunodepletion of Fkn

To determine whether fkn is responsible for a portion of the angiogenic properties of RA SF, SFs from six RA patients were pooled, immunodepleted of fkn, and assayed for angiogenic activity *in vivo*. Fkn-immunodepleted SFs were diluted in Matrigel and injected subcutaneously into mice. Results of these immunodepletion experiments are shown in Figure 7. The angiogenesis induced by the pooled SFs was significantly decreased by immunodepleting fkn compared to sham immunodepletion (0.028 ± 0.02 versus 1.38 ± 0.57 g/dl of hemoglobin/mg of plug weight, $n = 12$, respectively, $P < 0.05$).

RA ST Angiogenic Activity Is Decreased by Immunodepletion of Fkn

To determine whether fkn is responsible for a portion of the angiogenic properties of RA ST homogenates, ST homogenates from five RA patients were pooled, immunodepleted of fkn, and assayed for *in vivo* angiogenic activity. Representative photomicrographs of these Matrigel plugs fixed and Masson trichrome-stained are shown in Figure 8A. Matrigel plugs containing pooled RA ST homogenates have significant new blood vessel growth, whereas Matrigel plugs containing RA ST homogenate immunodepleted with anti-fkn have minimal blood vessel growth. Figure 8B shows the hemoglobin content normalized to the weight of the Matrigel plugs. The angiogenesis induced by the pooled ST homogenates was significantly decreased by immunodepleting fkn compared to sham immunodepletion (0.09 ± 0.08 versus 0.66 ± 0.12 g/dl of hemoglobin/mg of plug weight, $n = 12$, respectively, $P < 0.05$).

Discussion

Chemokines are divided into four families CXC, CC, C, and CX₃C. Members of the CXC chemokine family containing the amino acid sequence Glu-Leu-Arg (the ELR motif) and the CC chemokines MCP-1, vMIP-I, and vMIP-II have been shown to be angiogenic.⁹⁻¹² Here we show fkn inducing HMVEC migration in a concentration-dependent manner from as low as 10^{-6} nmol/L upwards to 10^2 nmol/L where it had similar activity to the potent EC chemoattractant bFGF (60 nmol/L) (Figure 1). In Table 1, a checkerboard assay shows fkn to induce EC migration both when it is added directly to the cells and when it is added to the chamber on the other side of the membrane, indicating that in addition to inducing directional migration (chemotaxis), fkn stimulates ECs to randomly migrate (chemokinesis). Fkn (100 nmol/L) induced ECs to

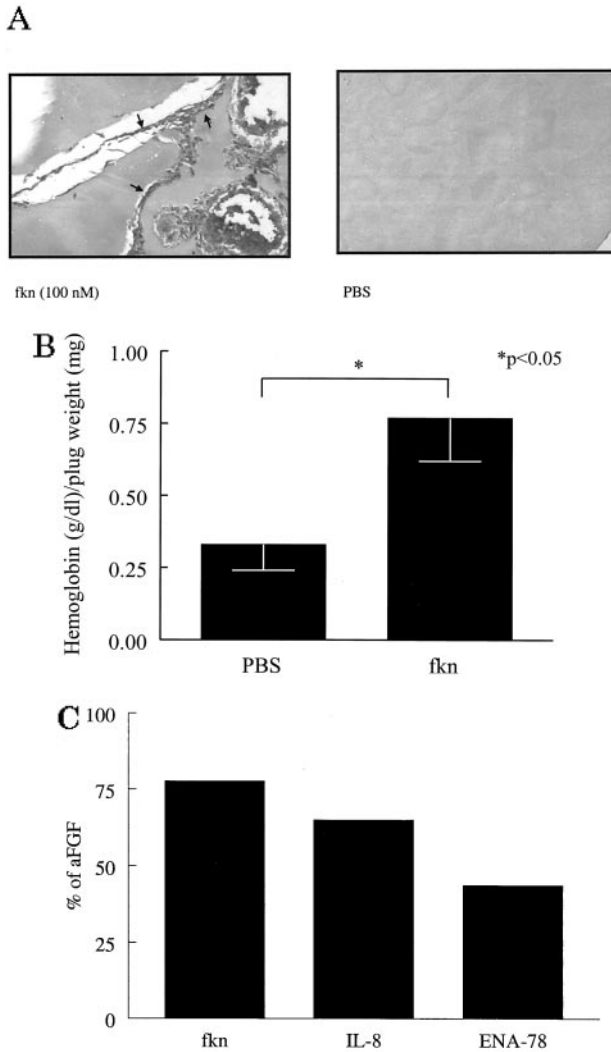


Figure 6. Fkn induces angiogenesis in Matrigel plugs *in vivo*. **A:** Representative assay showing Masson trichrome staining of blood vessels in Matrigel plugs. Fkn-induced blood vessel formation compared to PBS control (original magnification, $\times 66$). Blood vessels are shown in fractalkine-treated well (arrows). **B:** Values represent the concentration of hemoglobin (g/dl)/Matrigel plug weight (mg) \pm SE for 18 assays. **C:** Values represent the concentration of hemoglobin (g/dl)/Matrigel plug weight (mg) as a percentage of the positive control aFGF-induced hemoglobin (g/dl)/Matrigel plug weight (mg) for between seven to nine assays. *, $P < 0.05$ significantly different from vehicle control.

form tubes in Matrigel *in vitro* at the same rate as the angiogenic chemokines, IL-8 and ENA-78 (10 μ mol/L) (data not shown), and PMA (50 μ mol/L), a strong inducer of EC differentiation and EC tube formation (Figure 4B).²⁵ We also show the angiogenic properties of fkn *in vivo*, as fkn induced angiogenesis in Matrigel plugs inserted in mice comparable in potency to the known angiogenic chemokines IL-8 and ENA-78 (Figure 6). Thus, fkn is the first CX₃C chemokine shown to function as an inducer of EC migration and angiogenesis.

Because fkn contains both a chemokine domain and a mucin stalk resembling an adhesion molecule, we questioned which domain was responsible for its EC migration and tube-forming properties. We found that the chemokine domain of fkn is necessary and that the mucin domain is not sufficient for inducing EC migration and tube

Table 2. Migration of HMVECs in Response to RA SF Incubated in the Presence and Absence of Anti-Fkn

Patient	Mean cells/well*		% Suppression [†]
	Goat IgG ₁	Anti-fkn	
1	35	17	51.4
2	31	15	51.6
3	28	11	60.7
4	25	9	64.0
5	32	16	50.0
6	29	12	58.6
Mean \pm SE			56.1 \pm 2.4

*RA SFs were assayed for their ability to induce migration of HMVECs. The results represent mean number of cells per well as measured in three high-power fields ($\times 400$). Each sample was tested in two to four wells. The ability of anti-fkn antibodies (25 μ g/ml) to neutralize the migratory properties of RA SF was determined as percent suppression of migration compared with that of isotype control antibody. Positive control migration in response to bFGF (60 nmol/L) was a mean of 44 cells/well. Negative control migration in response to PBS was 12 cells/well.

[†]P value for percent suppression compared to isotype control matched IgG in all patient samples assayed was < 0.05 .

formation on Matrigel, as an antibody specific for the chemokine domain completely inhibited fkn-induced HMVEC migration and tube formation (Figure 2 and Figure 4C). Because fkn does not contain an ELR motif, the mechanism by which fkn induces EC migration seems unique from that of the other angiogenic chemokines.

Chemokines have been shown to induce EC chemotaxis through binding their EC chemokine receptors. Moore and co-workers^{36,37} showed that angiogenesis induced by the CXC chemokines, IL-8, KC, MIP-2, and ENA-78 was mediated through the EC chemokine receptor CXCR2. Fiel and Augustin³⁸ showed that SDF-1-CXCR4 interactions are involved in bovine aortic EC chemotaxis. Weber and co-workers¹⁰ inhibited MCP-1-induced EC chemotaxis with a CCR2, the MCP-1 receptor, antagonist. Here we demonstrate CX₃CR1 mRNA and protein expression in HMVECs in culture (Figure 5). We have also demonstrated by immunohistochemistry EC expression of CX₃CR1 in ST in adjuvant-induced arthritic rats.¹³ Thus, it is possible that

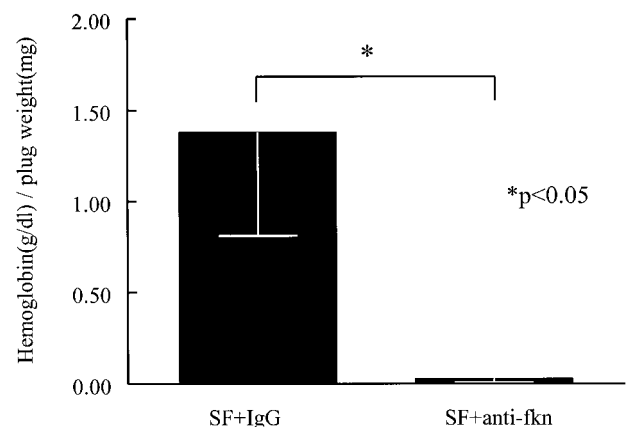


Figure 7. RA SF angiogenic activity is decreased by immunodepletion of fkn *in vivo*. SFs from six RA patients were pooled, immunodepleted of fkn, and assayed for their angiogenic ability in Matrigel plugs implanted in mice. Results represent the mean concentration of hemoglobin (g/dl)/Matrigel plug weight (mg) \pm SE. *, $P < 0.05$, significantly different from IgG control.

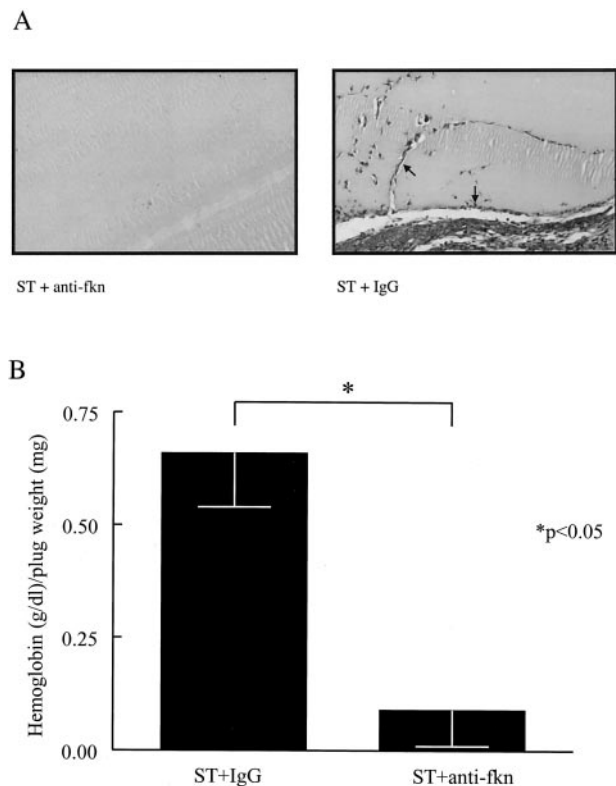


Figure 8. RA ST angiogenic activity is decreased by immunodepletion of fkn *in vivo*. ST homogenates prepared from five RA patients were pooled, immunodepleted of fkn, and assayed for their angiogenic ability in Matrigel plugs implanted in mice. **A:** Representative assay showing Masson trichrome staining of blood vessels in Matrigel plugs. Lack of RA ST-induced blood vessel formation after immunodepletion of fkn with anti-fkn compared to IgG control (original magnification, $\times 66$). Blood vessels are indicated by **arrows**. **B:** Results represent the mean concentration of hemoglobin (g/dl)/Matrigel plug weight (mg) \pm SE. *, $P < 0.05$, significantly different from IgG control.

fkn-induced EC migration is mediated through interaction of fkn with its EC receptor CX₃CR1.

The angiogenic properties of fkn are similar in potency to other angiogenic mediators. Fkn induced a doubling in the amount of EC migration, a technical indicator of potent migration, at 1 nmol/L and reached statistical significance at concentrations as low as 10^{-6} nmol/L. Fkn induced angiogenesis *in vivo* at 100 nmol/L. These concentrations of fkn are comparable to concentrations of the CXC chemokines, IL-8, ENA-78, and growth-related oncogene- α (GRO- α), shown to induce EC chemotaxis and angiogenesis. We previously showed IL-8 to induce a doubling in human umbilical vein EC chemotaxis at 1.25 nmol/L and to induce angiogenesis at 10 nmol/L.⁶ ENA-78 induced bovine adrenal gland capillary EC chemotaxis at as low as 5 nmol/L and ENA-78 and GRO- α induced angiogenesis in the rat cornea neovascularization assay at 10 nmol/L.⁹ Thus, fkn is a powerful chemoattractant for ECs and is angiogenic *in vivo* in the nmol/L range, similar to other angiogenic CXC chemokines.

Angiogenic factors function to form intact microvessels by inducing EC migration, proliferation, elongation, orientation, and differentiation resulting in lumen formation, re-establishment of the basement membrane and anastomosis with other vessels. We and others have reported

ELR motif-containing CXC chemokines such as IL-8 and GRO- α to induce both EC chemotaxis and proliferation.^{6,39} We also have recently shown the cytokine IL-13 to be chemotactic for ECs but not to induce EC proliferation.²³ Here we show that fkn induces EC migration (chemotaxis and chemokinesis), but not proliferation. In this way, fkn acts in the same manner as other angiogenic mediators by inducing some facets of the angiogenic process while having no effect on others. In this work, we also demonstrated that fkn can induce ECs to form tubes on Matrigel *in vitro* and to form functional blood vessels in Matrigel plugs *in vivo*, thus establishing its angiogenic properties.

RA ST is replete with newly formed blood vessels in response to the increased demand for nutrients and oxygen by the proliferating pannus tissue.¹⁻³ The level of RA ST vascularity correlates with more severe clinical and inflammatory scores and is greater than degrees of vascularity seen in osteoarthritis ST.^{1,40,41} RA SF and ST homogenates are potent EC chemotactic agents and contain several mediators that are chemotactic for ECs including the chemokines, IL-8, ENA-78, and GRO- α .^{4,42-47} In another report, we have shown RA SF and ST contain greater levels of antigenic fkn than SF and ST from patients with osteoarthritis or other forms of arthritis.¹³ We report here that RA SF immunodepleted of fkn has significantly reduced chemotactic activity for ECs and that RA SF and ST homogenates immunodepleted of fkn have significantly reduced angiogenic activity. The complete nature of the reduction in RA SF- and ST-induced migration and angiogenesis with anti-fkn treatment is possibly because of the complex sharing of chemokine receptors and signaling molecules between different chemokines or possibly synergy between the different angiogenic mediators. In this manner, immunodepletion of an individual factor may have a profound impact on the total angiogenic response, because of the unique dynamics of stimulating cells with intricate biological tissues. Our findings suggest an important role for fkn in inducing EC migration (chemotaxis and chemokinesis) and angiogenesis in RA and identify a new potential target for treating the disease.

In summary, fkn, the sole member of the CX₃C chemokine family, induces EC migration (chemotaxis and chemokinesis), EC tube formation, and blood vessel formation *in vivo*. RA SF and ST homogenates' angiogenic activities are in part because of fkn. We hypothesize that in a disease state such as RA, fkn may act in an autocrine manner. Specifically, two prominent proinflammatory cytokines in RA, IL-1 β , and tumor necrosis factor- α , activate ECs to produce fkn on their surface. Next, EC surface fkn is released by enzymatic cleavage and the resulting soluble fkn binds EC CX₃CR1 inducing EC migration and synovial angiogenesis.

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