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Vascular Endothelial Growth Factor and Basic Fibroblast Growth Factor Induce Expression of CXCR4 on Human Endothelial Cells

In Vivo *Neovascularization Induced by Stromal-Derived Factor-1α*

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The contribution of chemokines toward angiogenesis is currently a focus of intensive investigation. Certain members of the CXC chemokine family can induce bovine capillary endothelial cell migration *in vitro* and corneal angiogenesis *in vivo*, and apparently act via binding to their receptors CXCR1 and CXCR2. We used an RNAse protection assay that permitted the simultaneous detection of mRNA for various CXC chemokine receptors in resting human umbilical vein endothelial cells (HUVECs) and detected low levels of only CXCR4 mRNA. Stimulation of HUVECs with vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF) up-regulated levels of only CXCR4 mRNA. CXCR4 specifically binds the chemokine stromal-derived factor-1α (SDF-1α). Competitive binding studies using ^{125}I-labeled SDF-1α with Scatchard analysis indicated that VEGF or bFGF induced an average number of approximately 16,600 CXCR4 molecules per endothelial cell, with a $K_d = 1.23 \times 10^{-9}$ mol/L. These receptors were functional as HUVECs and human aorta endothelial cells (HAECs) migrated toward SDF-1α. Although SDF-1α-induced chemotaxis was inhibited by the addition of a neutralizing monoclonal CXCR4 antibody, endothelial chemotaxis toward VEGF was not altered; therefore, the angiogenic effect of VEGF is independent of SDF-1α. Furthermore, subcutaneous SDF-1α injections into mice induced formation of local small blood vessels that was accompanied by leukocytic infiltrates. To test whether these effects were dependent on circulating leukocytes, we successfully obtained SDF-1α-induced neovascularization from cross sections of leukocyte-free rat aorta. Taken together, our data indicate that SDF-1α acts as a potent chemoattractant for endothelial cells of different origins bearing CXCR4 and is a participant in angiogenesis that is regulated at the receptor level by VEGF and bFGF. *(Am J Pathol 1999, 154:1125–1135)*

Angiogenesis is the process by which new capillaries sprout from existing vessels. It involves a concerted sequence of events including activation of endothelial cells, degradation of the proximal basement membrane and extracellular matrix, directional migration and proliferation of endothelial cells, and canalization of endothelial cords penetrating surrounding tissue. Angiogenesis is fundamental to a variety of physiological activities, such as ovulation, menstruation, pregnancy, and wound healing. During these processes, angiogenesis occurs rapidly but transiently and is tightly regulated. In addition, angiogenesis also plays a role in pathological processes such as chronic inflammation and tumor growth.[1–4]

A number of mediators induce angiogenesis, including members of the fibroblast growth factor (FGF)[1] family, vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), tumor necrosis factor (TNF)-α, and certain members of the CXC chemokine family.[1–5] Chemokines are characterized by their ability to induce cell migration and are divided into four groups based on structural properties and primary amino acid sequence: CXC, CC, C, or CX3C.[6–10] The CXC chemokine subfamily

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includes interleukin (IL)-8, NAP-2, ENA-78, GRO, interferon-inducible protein (IP-10, MIG, PF4, and stromal-cell-derived factor (SDF)-1 among others. All of the genes encoding known CXC chemokines are clustered on human chromosome 4, with the exception of the SDF-1 gene, which is located on chromosome 10.[11] The presence of an amino-terminal proximal Glu-Leu-Arg (ELR) motif is a reported correlate of angiogenic chemokines, whereas CXC chemokines lacking this motif, such as PF4 and IP-10, are reported to be angiostatic.[12]

SDF-1α is highly conserved between human and mouse. It was originally isolated from murine bone marrow stromal cells, and it is highly expressed on stromal cells of several tissues, including pancreas, spleen, ovary, and small intestine, but not in peripheral blood leukocytes.[11] SDF-1α acts as pre-B-cell growth factor in the presence of IL-7[13] and as a chemoattractant for leukocytes[14] and hematopoietic progenitor cells.[15] SDF-1-deficient mice are grossly normal but die shortly after birth, lack B cell lymphopoiesis during embryonic development, have defective bone marrow myelopoiesis, and have a ventricular septal defect.[16] CXCR4, also known as LESTR, HUMSTER, or Fusin, specifically binds SDF-1α. CXCR4 is expressed on lymphocytes, monocytes, neutrophils, and epithelial cells[17,18] and was recently found on human microglia[19] and various endothelial cells.[20–22]

Although ELR-positive members of the CXC chemokine family are reported to induce corneal angiogenesis, expression of their receptors (CXCR1 and CXCR2) on endothelial cells, a prerequisite of endothelial cell chemotaxis and direct induction of angiogenesis, remains a controversial issue.[23,24] It has been recently reported that human umbilical vein endothelial cells (HUVECs) express CXCR4[21,22] and that this expression can be modulated by the inflammatory mediators interferon (IFN)-γ, TNF-α, IL-1β or lipopolysaccharde (LPS).[21] However, in this study, the investigators cultured their endothelial cells in media containing either VEGF or added basic (b)FGF.[21]

In this paper we investigated the ability of known angiogenic and/or inflammatory factors to induce expression of CXC chemokine receptors on endothelial cells. Under the conditions used, we found that either VEGF or bFGF induced elevated cell surface expression of CXCR4 only and substantially increased HUVEC migration toward SDF-1α. Furthermore, we demonstrate that SDF-1α induces local neovascularization *in vivo*. Thus, even though SDF-1 lacks an ELR motif, it nevertheless can act as a novel angiogenic factor in conjunction with other angiogenic growth factors.

Materials and Methods

Endothelial Cell Culture

HUVECs were isolated by treatment of umbilical cords with trypsin/EDTA (0.25%/0.02%) in PBS for 10 minutes at 37°C. After elution of the HUVECs with RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 20% FCS (Hyclone, Logan, UT), HUVECs were cultured on gelatin-coated (Sigma Chemical Co., St. Louis, MO) flasks in 199 medium (Life Technologies) or EBM medium (Clonetics, Walkersville, MD) containing 10% fetal calf serum, glutamine (2 mmol/L), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were characterized by morphological criteria and positive staining with CD31 or inducibility of vascular cell adhesion molecule (VCAM)-1 after TNF-α stimulation. Human aorta endothelial cells (HAECs) were prepared as described.[25] Experiments were performed on subcultures between the third and sixth *in vitro* passage.

Chemokines and Antibodies

Recombinant human SDF-1α, recombinant human VEGF, recombinant human bFGF, recombinant human epidermal growth factor (EGF), and recombinant human TNF-α were purchased from Pepro Tech (Rocky Hill, NJ). Recombinant human IFN-γ was purchased from Biogen (Cambridge, MA), and phorbol ester and LPS was purchased from Sigma. Monoclonal anti-human CXCR4 (12G5) was purchased from R&D Systems (Minneapolis, MN). Anti-human VCAM-1 and anti-human ELAM-1 were purchased from Immunotech (Westbrook, ME), and mouse IgG and Rabbit IgG (Coulter, Miami, FL) were used as negative controls.

RNAse Protection Assay

After a 4-hour incubation of the cells with VEGF (50 ng/ml), bFGF (50 ng/ml), EGF (100 ng/ml), SDF-1α (100 ng/ml), TNF-α (200 U/ml), LPS (10 μg/ml), IFN-γ (100 U/ml), and phorbol ester (160 nmol/L) alone or in different combinations, RNA was isolated by the TRIZOL method as directed (Life Technologies) and thereafter used for analysis of mRNA expression using the Riboquant RNAse protection assay system (human CR6 probe set, PharMingen, San Diego, CA) according to the manufacturer's instructions. Briefly ^{33}P-labeled antisense RNA probes were synthesized from the human chemokine receptor 6 template by T7 RNA polymerase. The probe (1.5×10^6 cpm) was hybridized in solution overnight in excess to target RNA (10 μg total RNA/treatment) in a total reaction volume of 10 μl. The free probe and other single-stranded RNA were digested with RNAses A and T1 per instructions provided by the manufacturer. The remaining RNAse-protected probes were precipitated, dissolved in 5 μl of sample buffer (PharMingen), and resolved on denaturing polyacrylamide gels followed by autoradiography for 1 to 7 days at −70°C.

Immunofluorescence Flow Cytometry

HUVECs and HAECs were stimulated with escalating concentrations of VEGF, bFGF, TNF-α, and LPS. Optimal doses for each factor were then used in a kinetic assay at 12, 24, 48, and 72 hours for cell surface expression of CXCR4 by immunofluorescence. For TNF-α-stimulated cells, cells were exposed for approximately 40 minutes to 200 U/ml TNF-α, which was removed by washing. Treated cells were thereafter cultured as described. In-

direct immunofluorescence was performed by exposing cells to saturating amounts of mouse antibodies to human CXCR4, VCAM-1, or ELAM-1. As the second antibody, fluorescein-conjugated F(ab)$_2$ fragments of goat anti-mouse (Sigma) were used at a 1:20 dilution. After staining, cells were analyzed in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Mouse IgG was used as the negative control.

Endothelial Cell Migration Assay

Endothelial cell chemotaxis was performed using micro Boyden's chambers. Briefly, polycarbonate filters of 8-μm pore size (Nucleopore, NeuroProbe, Cabin John, MD) were coated with 0.5% collagen type I (Collaborative-Biomedical Products, Bedford, MA) overnight at 4°C. Binding buffer containing 1.0% bovine serum albumin in RPMI 1640 with or without the stimulus was placed in the lower compartment of the chamber, and 10^6 HUVECs or HAECs/ml resuspended in binding medium were then seeded into the upper compartment. The chambers were incubated for 2 hours at 37°C. After the filters were removed, the upper surface was scraped, fixed with methanol, and stained with Leukostat (Fisher Scientific). Membranes were analyzed using the BIOQUANT program (R & M Biometrics, Nashville, TN), and the results were expressed as the mean number of migrated cells/10 fields at $\times$10 magnification.

Receptor Binding Assay

Binding of chemokines to their receptors was assessed using 1 ng/ml of ^{125}I-labeled SDF-1α (New England Nuclear, Boston MA) in the presence of various concentrations of unlabeled SDF-1α (Pepro Tech), as previously described.[26] Stimulated HUVECs at 10^7/ml in RPMI 1640 containing 1% bovine serum albumin (w/v) and 25 mmol/L HEPES were incubated in the presence of SDF-1α for 45 minutes at room temperature and pelleted through 10% sucrose in PBS, and cell-pellet-associated radioactivity was determined in a gamma counter. Binding data were analyzed using the computer program LIGAND.

Assessment of Receptor Redistribution Using Confocal Laser Microscopy

HUVECs were grown on gelatin-treated tissue culture chamber slides (Nunc, Naperville, IL) in the presence or absence of VEGF (100 ng/ml) at 37°C in a humidified atmosphere containing 5% CO_2 for 24 hours. They were then washed three times with PBS and cultured in 199 medium containing SDF-1α (1 μg/ml) for 30 minutes, washed once with ice-cold PBS, and fixed in 2% paraformaldehyde/PBS. Cells were permeabilized in 0.15% saponin before incubation for 60 minutes with biotin-labeled 12G5 monoclonal antibody (MAb; R&D Systems). After washing, cells were incubated with streptavidin-conjugated rhodamine (Molecular Probes, Eugene, OR). Finally, cells were washed and stained with DAPI (Sigma) for 10 minutes, and slides were then examined using a Zeis 310 confocal laser scanning microscope. Nomarski, rhodamine (543 nmol/L, red), and DAPI (ultraviolet 364 nm, blue) images were prepared for each specimen and were subsequently superimposed using the Nomarski image as a base.

In Vivo *Angiogenesis Assay*

Female BALB/c mice were used at 6 to 8 weeks of age. Animal housing and management were in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences, Institute of Laboratory Animal Resources, National Research Council, 1996), and the protocol used was approved by the National Cancer Institute-Frederick Cancer Research and Development Center Animal Care and Use Committee. Angiogenesis was assessed using the technique of Weidner et al[27] as an increase in the number of microvessels in the subcutaneous tissue injected with chemokines. Mice, three per group per time point, were injected subcutaneously on days 0, 1, 2, and 3 with SDF-1α (1 μg) or VEGF (1 μg) or both in 0.1 ml of PBS. Injection sites were removed at necropsy on day 7 or day 14, fixed in Bouin's, and embedded in paraffin, and 5-μm-thick sections were prepared.

Immunohistochemical staining using rat anti-mouse CD31 (PharMingen), also known as PECAM-1, was performed at a dilution of 1:50 after pretreatment of the sections for 30 minutes with Sigma tissue-culture-grade trypsin diluted 1:10 in PBS. Biotinylated rabbit anti-rat IgG, mouse absorbed, was the secondary antibody used with the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Diaminobenzidine was used as a chromogen. Slides were also stained with hematoxylin and eosin (H&E) to evaluate inflammatory lesions.

ELISA

Growth factor release was quantitated by ELISA as directed by the manufacturer (R&D Systems). HUVECs were cultured (0.5×10^6 per/well) in six-well plates pretreated with gelatin as described above in medium containing VEGF (100 ng/ml) and bFGF (10 ng/ml). After 24 hours, the medium was removed, and the cells were washed eight times with PBS. Fresh 199 medium containing 2% fetal calf serum was added (2 ml/well), and treated wells received SDF-1α (1 μg/ml final concentration). Supernatant samples were collected at 24, 48, and 72 hours.

Rat Aortic Ring Assay

Rat aortic rings were prepared as previously described[28] with modifications. The thoracic and abdominal aorta was obtained from 100- to 150-g male Sprague-Dawley rats (Taconic Farms, Germantown, NY). Excess perivascular tissue was removed, transverse sections (1 to 2 mm) were made, and the resulting aortic rings were then ex-

tensively washed in medium 199 (Mediatech). The rings were then embedded in 2 μg/ml rat tail collagen in Nunc eight-well chamber slides (Nalgene Nunc International) so that the lumen was parallel to the base of the slide. After the collagen I gelled (by adjustment of pH to neutral with NaOH), serum-free medium (endothelial basal medium supplemented with antibiotics and e-aminocaproic acid, 0.3 μg/ml) was added to each well, and the slides were incubated at 37°C, 5% CO_2, for 3 days. Once sprouts began to appear, SDF-1α was added at concentrations of 1 to 100 ng/ml (n = 6 per dose). ECGS was used as the positive control at concentrations of 200 μg/ml. The rings were incubated for 3 additional days and then fixed, stained, and photographed. The ring assay was repeated two times.

Results

bFGF- and VEGF-Induced CXCR4 mRNA Expression in Endothelial Cells

In an effort to identify chemokines that might be direct inducers of angiogenesis, we first established the capacity of unstimulated HUVECs and HUVECs preincubated with a variety of stimuli to express known CXC subfamily receptor genes. We used the RNAse protection assay, which allows for the detection of multiple chemokine receptor messages in a single RNA preparation. Freshly isolated HUVECs (passage 4) were grown in basal (199) medium and subsequently stimulated for 4 hours, as described in Materials and Methods, with the following combinations: bFGF, VEGF, and EGF together; TNF-α, LPS, and IFN-γ together; or phorbol ester alone. As shown in Figure 1A, HUVECs constitutively expressed low levels of CXCR4 mRNA, and this expression was increased approximately 10-fold after treatment either with phorbol ester or the combination of VEGF, bFGF, and EGF.

We next investigated the effect of the individual stimuli. HUVECs were stimulated with either bFGF, VEGF, EGF, SDF-1α, LPS, TNF-α, or IFN-γ under the same conditions as described above. As shown in Figure 1B, bFGF treatment increased CXCR4 mRNA twofold and VEGF fourfold; however, neither EGF nor SDF-1α had any stimulatory effect. In addition, TNF-α, IFN-γ, and LPS did not have any effect on CXCR4 mRNA levels within 4 hours when tested alone (data not shown). Despite reports of endothelial cell CXC chemokine receptor expression and that the ligands for CXCR1 and CXCR2 are angiogenic and for CXCR3 are angiostatic, we did not detect CXCR1, CXCR2, or CXCR3 mRNAs in HUVECs under basal conditions or after stimulation with any of the stimuli used above by using the RNAse protection assay (Figure 1).

Cell Surface Expression of CXCR4 on Endothelial Cells

Consistent with the low constitutive levels of CXCR4 mRNA in cultured endothelial cells, we detected low expression of CXCR4 protein on the cell surface. After combined stimulation with VEGF (50 ng/ml) and bFGF (10 ng/ml), CXCR4 expression on HUVECs and HAECs was significantly enhanced, as detected by FACS analysis (Figure 2A). No detectable increase in cell surface expression of this receptor was observed after either EGF, SDF-1α, or LPS treatment, whereas IFN-γ reduced CXCR4 levels (Figure 2B). The highest expression of CXCR4 was detected using HUVECs cultured for 24 hours in the presence of 10 to 50 ng/ml VEGF. This high level of expression was maintained beyond 48 hours of stimulation but returned to basal levels by 72 hours (data not shown). We also observed that TNF-α induced increased CXCR4 expression on HUVECs; however, this effect required 48 hours or more to become evident, whereas the effect of VEGF or bFGF peaked at 24 to 48 hours (data not shown).

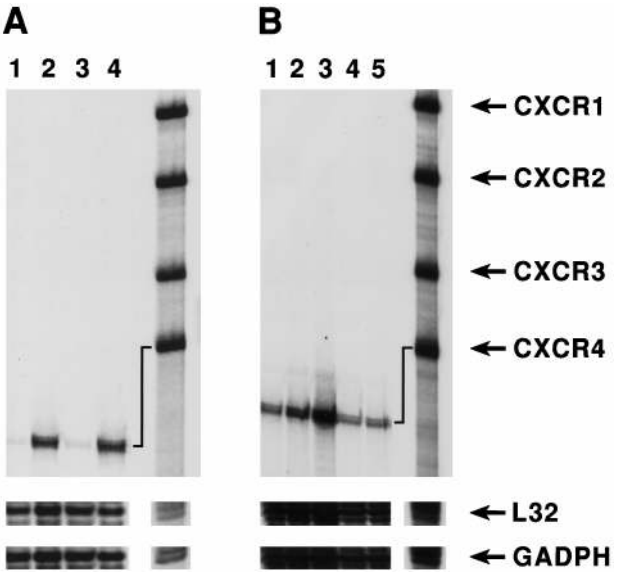

Figure 1. Stimulated HUVECs express elevated levels of CXCR4 mRNA. A: RNAse protection assay of HUVEC RNA was performed on unstimulated (lane 1) cells or cells stimulated for 4 hours with bFGF (50 ng/ml), VEGF (50 ng/ml), and EGF (100 ng/ml) together (lane 2), LPS (10 μg/ml) and IFN-γ (100 U/ml) together (lane 3), or phorbol ester (160 nmol/L) (lane 4). B: Expression of CXCR4 was detected at 4 hours after no stimulation (lane 1) or bFGF (50 ng/ml) (lane 2), VEGF (50 ng/ml) (lane 3), EGF (100 ng/ml) (lane 4), or SDF-1α (100 ng/ml) (lane 5) stimulation. The fold increase in mRNA expression was assessed by densitometric analysis after normalization using GADPH and L32 as controls. A representative experiment is shown.

SDF-1α Binds Specifically and with High Affinity to Stimulated HUVECs and HAECs

To investigate the binding capacity of CXCR4 on bFGF- and VEGF-stimulated HUVECs, we performed competitive binding studies with radio-iodinated SDF-1α. The ^{125}I-labeled SDF-1α (10 nmol/L) bound rapidly (after 30 minutes) to HUVECs, and unlabeled SDF-1α competitively reduced the binding of labeled SDF-1α (Figure 3). Scatchard analysis for SDF-1α binding indicated that stimulated HUVECs expressed an average number of 16,608 receptors/cell, which bound SDF-1α with high affinity ($K_d = 1.23 \times 10^{-9}$ mol/L; Figure 3; Table 1).

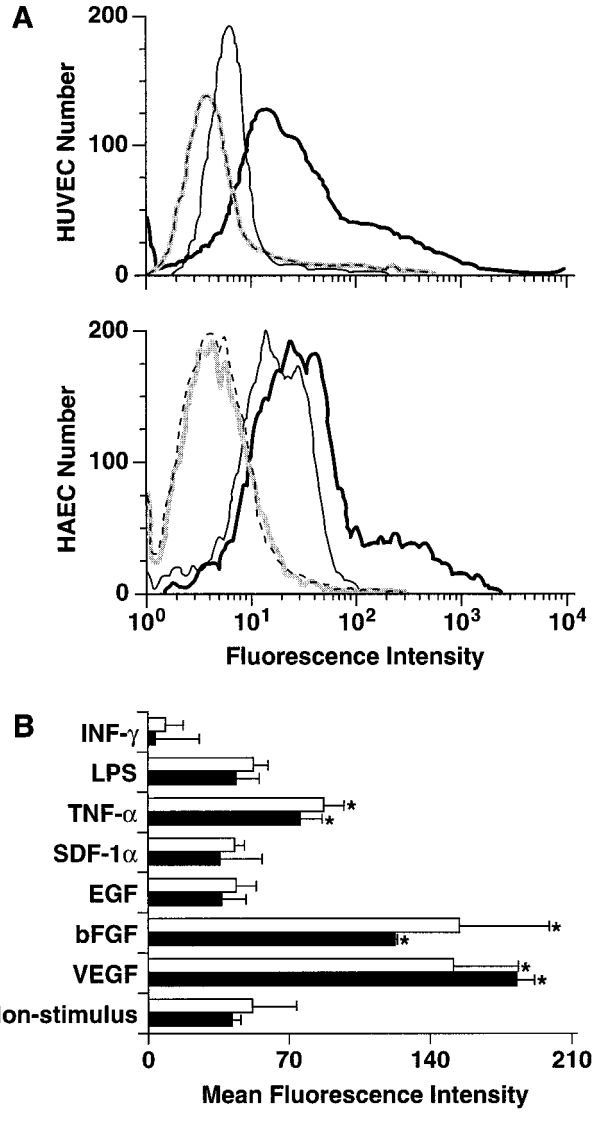

Figure 2. Induction of CXCR4 cell surface expression after stimulation with bFGF and VEGF. A: Flow cytometric analysis of HUVECs and HAECs at 24 hours after stimulation as described in Materials and Methods. Resting endothelial cells: Ab control (broken line) and 12G5 MAb (thin line); VEGF- and bFGF-stimulated endothelial cells: Ab control (gray line) and 12G5 MAb (thick line). B: Mean fluorescence intensity on resting and stimulated HUVECs (filled bars) and HAECs (open bars) at 48 hours after stimulation. The mean and SEM of three experiments is shown. $^*P < 0.01$

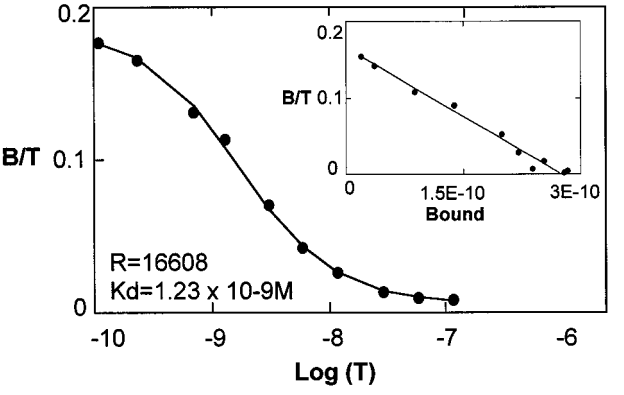

Figure 3. SDF-1α binds to VEGF- and bFGF-stimulated HUVECs with high affinity. HUVECs were stimulated, and binding of ^{125}I-labeled SDF-1α (1 ng/ml) in the presence of the indicated concentrations of unlabeled SDF-1α was performed as described in Materials and Methods. The bound/total ratio is shown. HUVECs express an average of approximately 16,600 CXCR4 receptors per cell (inset). A representative experiment is shown.

SDF-1α Induced Redistribution of CXCR4 on Stimulated HUVECs

Interaction of chemokines with their receptors typically results in receptor redistribution and/or internalization. To analyze the effect of SDF-1α binding to CXCR4 on activated HUVECs, immunostaining for CXCR4 followed by confocal microscopy was performed. As shown in Figure 4, CXCR4 on VEGF-stimulated HUVECs was primarily localized on the cell surface and in intracellular pools (Figure 4A). After SDF-1α treatment, reduction of cell surface CXCR4 with an increase in the intracellular pools of this receptor was observed (Figure 4B). Indeed, cell surface expression of CXCR4 as assessed by flow cytometric analysis, was decreased after SDF-1α treatment. Furthermore, permeabilized SDF-1α-treated cells were clearly CXCR4 positive (data not shown). These findings are in agreement with the effects of phorbol myristate acetate and SDF-1, both of which also cause the internalization of CXCR4 by T cells.[29]

SDF-1α Induced Endothelial Cell Chemotaxis via CXCR4

We next assessed the ability of SDF-1α to induce endothelial cell migration *in vitro*. SDF-1α was capable of inducing chemotaxis of VEGF- and bFGF-activated HUVECs and HAECs in a dose-dependent fashion. The dose-response curve had the characteristic bell shape typical of other chemokines with an optimal chemotactic dose between 10 and 100 ng/ml (Figure 5A). Interestingly, the chemotactic index for VEGF was approximately threefold lower than SDF-1α (data not shown).

To distinguish between chemokinetic and chemotactic effects of SDF-1α on HUVECs, we performed assays by placing different concentrations of SDF-1α in the upper and/or lower wells of the chemotaxis chamber. Checker-

Table 1. CXCR4 Expression on Human Endothelial Cells

CELLS	SDF-1α (K_d in nmol/L ± SEM)	SDF-1α (binding sites/cell ± SEM)
Nonstimulated HUVECs	1.32 ± 0.22	0.20 ± 0.21 × 10^4
VEGF- and bFGF-stimulated HUVECs	1.76 ± 0.33	2.05 ± 1.08 × 10^4
Nonstimulated HAECs	1.25 ± 0.325	0.828 ± 0.38 × 10^4
VEGF- and bFGF-stimulated HAECs	1.58 ± 0.75	3.14 ± 0.68 × 10^4

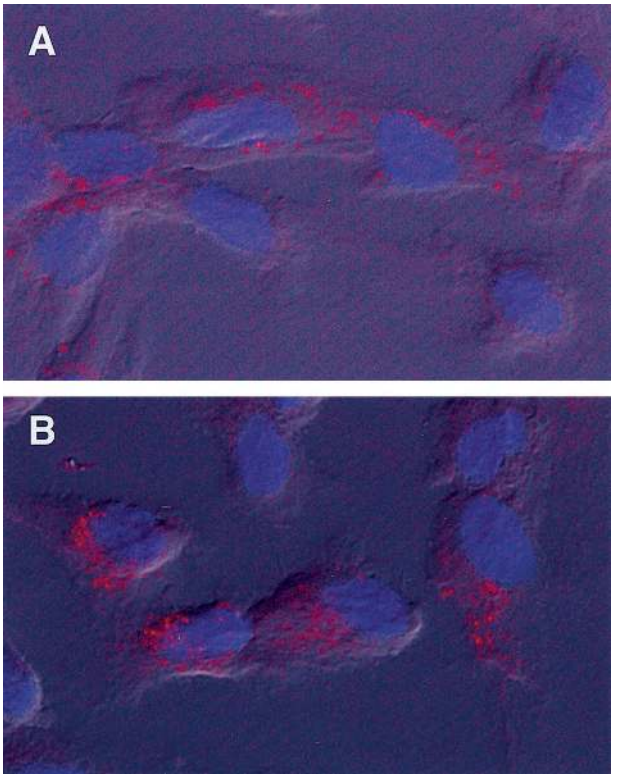

Figure 4. SDF-1α induced CXCR4 redistribution on HUVECs. Cells were stimulated with VEGF (100 ng/ml) for 24 hours. The 12G5 MAb was used to detect CXCR4 as shown in confocal photomicrographs. A: VEGF only. B: VEGF-stimulated cells were washed and exposed to SDF-1α (1 μg/ml for 30 minutes), and redistribution of its receptor was observed.

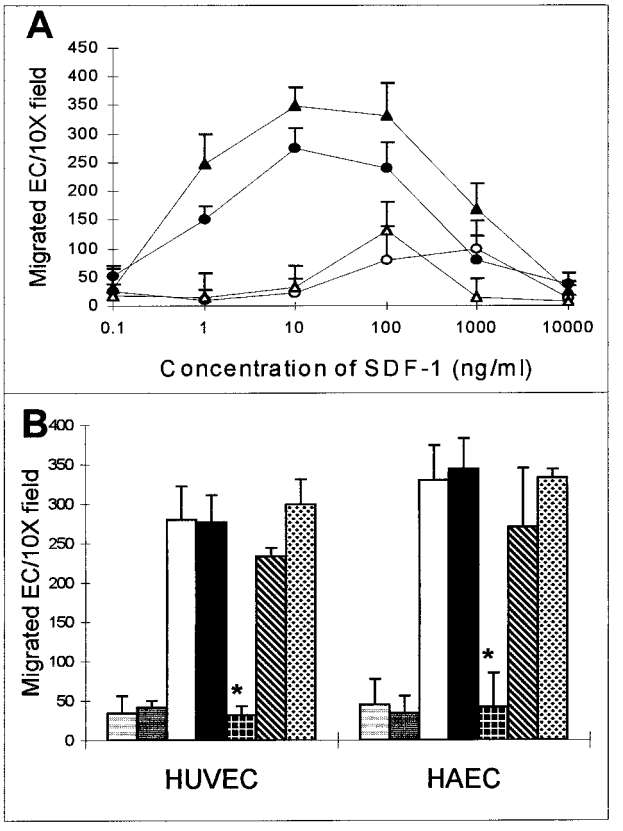

Figure 5. *In vitro* chemotaxis of HUVECs and HAECs toward SDF-1α is inhibited by CXCR4 antibody. The number of migrated HUVECs and HAECs per 10× field was quantitated as described in Materials and Methods. A: Unstimulated HUVECs (○), VEGF- and bFGF-stimulated HUVECs (●), unstimulated HAECs (△), VEGF- and bFGF-stimulated HAECs (▲). B: Inhibition of the chemotactic response of HUVECs and HAECs toward SDF-1α (10 ng/ml) by MAb 12G5: Migration was toward medium alone (basal migration; stripes) or in the presence of 12G5 at 10 μg/ml (gray) and migration toward SDF-1α in the absence of antibody (open bars), mouse IgG (10 μg/ml; filled bars), 12G5 MAb (10 μg/ml; checkerboard bars), 12G5 MAb (1 μg/ml; hatched bars), and 12G5 MAb (0.1 μg/ml; dotted bars); $^*P < 0.001$. The mean and SEM of three experiments are shown.

board analysis revealed that the response of bFGF- and VEGF-stimulated HUVECs to SDF-1α was chemotactic rather than chemokinetic (data not shown).

As a test of specificity, we determined the effects of a MAb directed against human CXCR4 on SDF-1α-induced migration of activated HUVECs and HAECs. The results demonstrated that 12G5, which recognizes an epitope located in the second extracellular loop of CXCR4, inhibited HUVEC and HAEC migration by more than 80% (Figure 5B). The CXCR4 antibody, however, did not inhibit the chemotactic response of endothelial cells toward VEGF, indicating that the effect of this growth factor on HUVECs and HAECs is direct and not dependent on SDF-1α (data not shown).

SDF-1α Induced Neovascularization in Vivo

In our hands, SDF-1α had a low but significant proliferative effect on VEGF-pretreated HUVECs or HAECs (data not shown). Nonetheless, our *in vitro* chemotaxis findings suggested that SDF-1α might act as an angiogenic factor. To test this possibility, subcutaneous injections (day 0) in mouse skin of 1 μg of either SDF-1α, VEGF (positive control), or PBS daily for 4 consecutive days were performed, as described in Materials and Methods. At day 7, significant induction of microvessel formation was observed using SDF-1α alone, which was equivalent to the VEGF-induced response (Figures 6A and 7). At 14 days after the initial injection the average number of microvessels was reduced, but the number of microvessels in both the SDF-1α- and VEGF-stimulated skin sections was still significantly elevated relative to the PBS control (Figure 6B). We did not observe a significant increase in microvessel formation when both agents were used in combination relative to VEGF alone ($P \leq 0.375$). Furthermore, an inflammatory reaction consisting of neutrophils and mononuclear cells, including CD3-positive lymphocytes was observed at day 7 in both VEGF- and SDF-1-stimulated skin sections (data not shown).

SDF-1α-Induced Rat Aortic Endothelial Cell Sprouting

As SDF-1α is chemotactic for lymphocytes and VEGF is chemotactic for mononuclear cells, which were found at sites of cutaneous injections of either SDF-1α or VEGF, we sought to investigate the possibility that the angiogenesis we observed was leukocyte dependent. To elucidate the role of SDF-1α in angiogenesis in the absence of

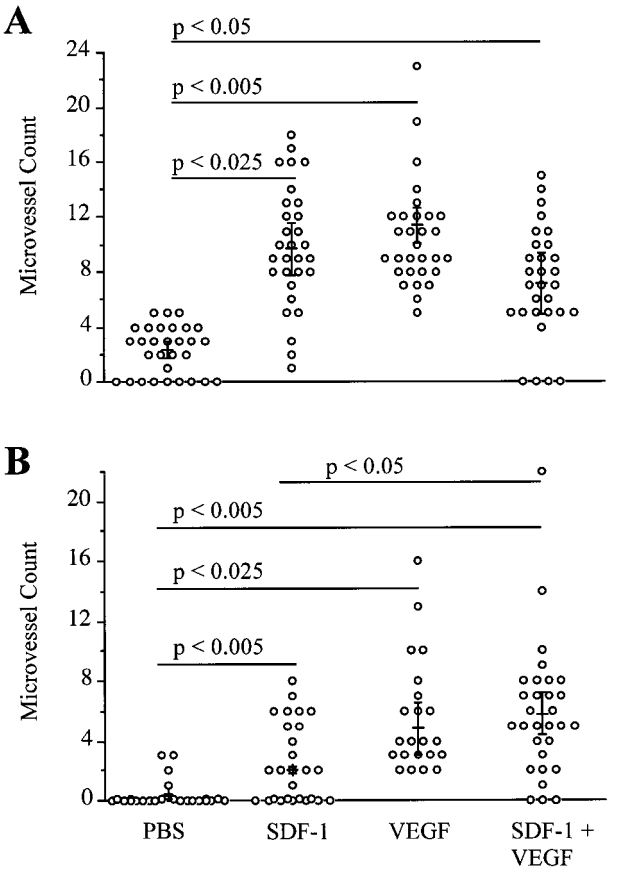

Figure 6. SDF-1α can induce neovascularization *in vivo*. The number of microvessels is shown per cluster within a section. Ten sections were analyzed per mouse skin section set at day 7 (A) or day 14 (B) after initiation of four daily injections. The mean, SEM and *P* value for each group of three mice are presented.

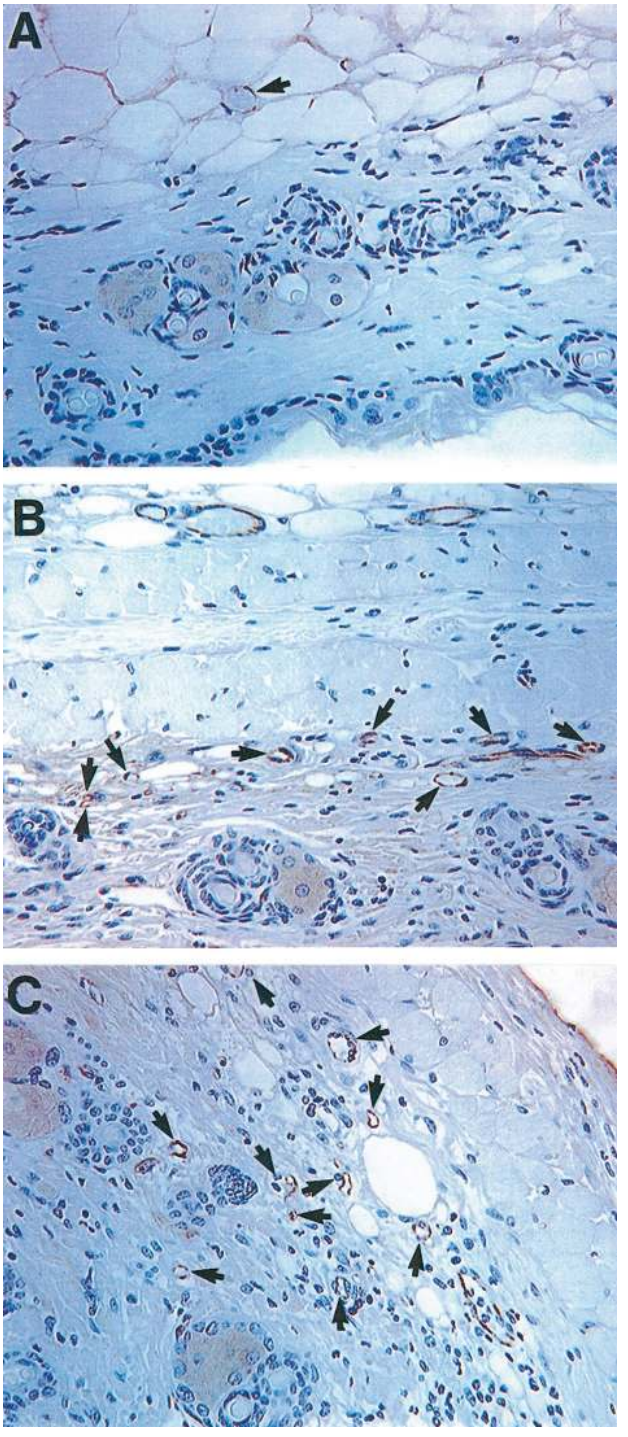

Figure 7. Composite photomicrographs showing neovascularization in SDF-1α-injected mouse skin. Mice received four daily injections of PBS (A), SDF-1 (1 μg; B), or VEGF (1 μg; C) and were biopsied on day 7, and sections were stained for PECAM-1 (CD31), a marker for endothelial cells. Microvessels are indicated by arrows.

inflammatory cell infiltrates, SDF-1α was tested using the *ex-vivo* rat aortic ring sprouting assay. Transverse sections of rat aorta embedded in collagen were stimulated with SDF-1α, as described in Materials and Methods, and thereafter examined for the degree of sprouting vessels. Cell culture medium and endothelial cell growth supplement medium were used as negative and positive controls, respectively. As shown in Figure 8, SDF-1α stimulated numerous long capillary sprouts at a concentration as low as 1 ng/ml (0.125 nmol/L). Thus, the data indicated that SDF1-α can induce angiogenic sprouting at subnanomolar concentrations from rat aortic rings in the absence of inflammatory cell infiltrates.

SDF-1α-Induced VEGF Release from Cultured HUVECs

To learn whether SDF-1α-CXCR4 interaction on HUVECs induced the production of angiogeneic factors, ELISA of culture supernatant samples was performed as described in Materials and Methods. Relative to resting cultures, VEGF- and bFGF-conditioned HUVECs, in response to SDF-1α, produced from 7- to 13-fold more VEGF, depending on the donor of origin (Figure 9). VEGF production was diminished by 48 hours and returned to basal levels by 72 hours (data not shown). In contrast, no significant increase in bFGF or IL-8 was observed after addition of SDF-1α, under these conditions, at any of the time points examined (data not shown).

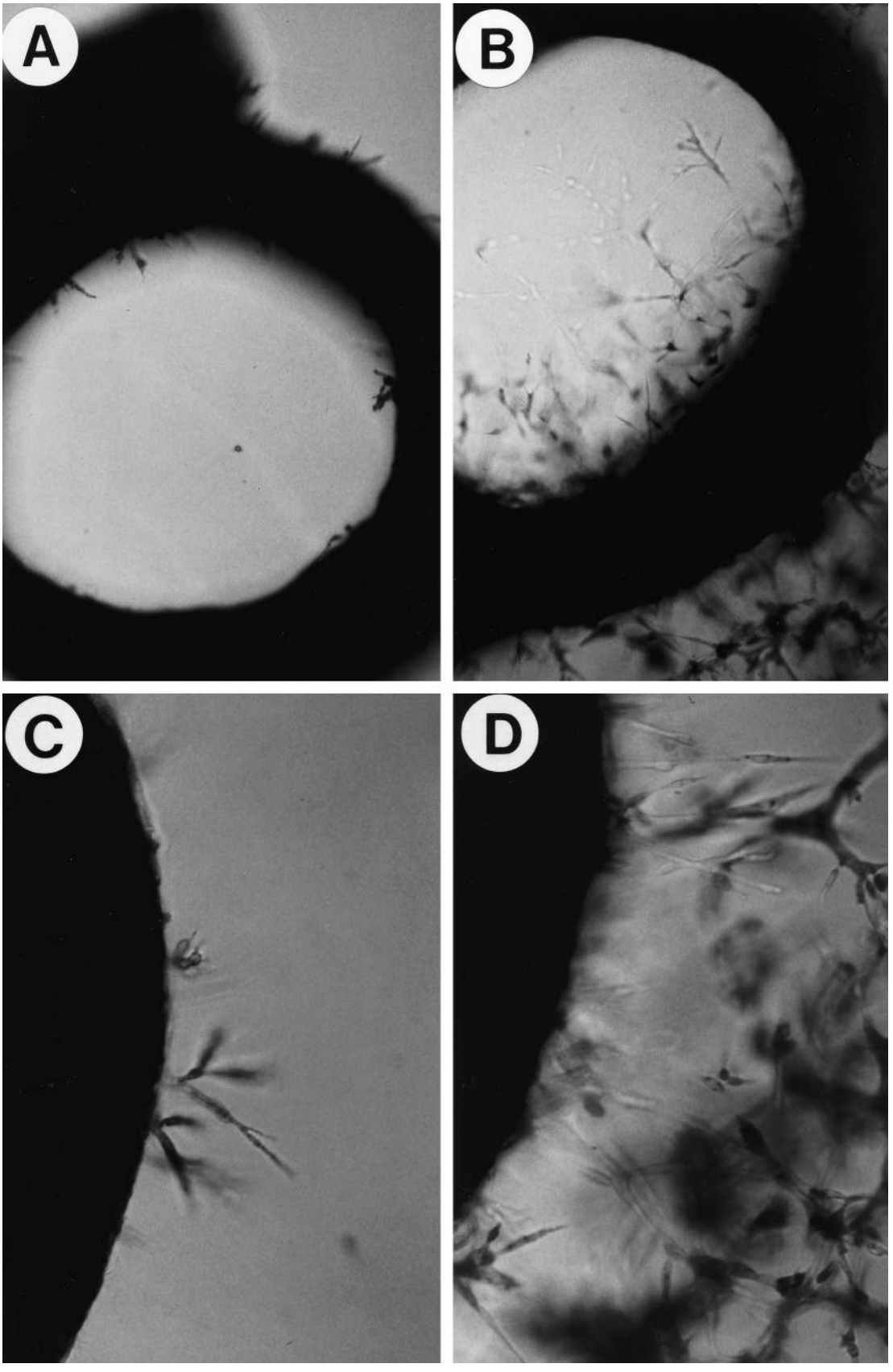

Figure 8. Rat aortic ring assay, showing rat aortic ring capillary sprouting in response to SDF-1α (1 ng/ml). Capillary sprouting occurred from the edge of the ring. A and C: Negative control; B and D: SDF-1α (1 ng/ml). Magnification, ×4 (A and B) and ×40 (C and D). Note that SDF-1α induced formation of long sprouts.

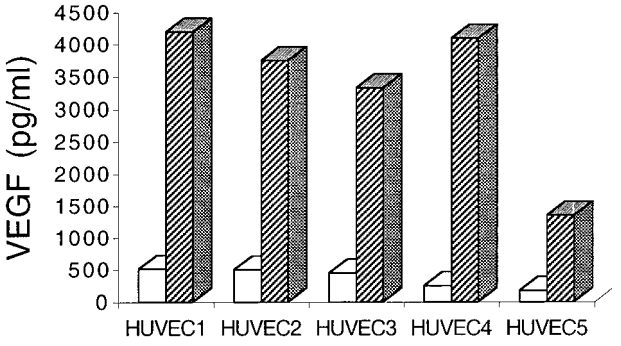

Figure 9. SDF-1α-CXCR4 interaction enhanced VEGF release from HUVECs. Cells were cultured as described in Materials and Methods and stimulated for 12 hours with VEGF and bFGF. Thereafter, cells were washed and treated with SDF-1α (1 μg/ml final) for 24 hours, and supernatant samples were collected at 24 hours for ELISA. Open bars, without SDF-1 treatment; hatched bars, with SDF-1α treatment. Concentrations of VEGF produced by different HUVECs are shown. A representative experiment is shown.

Discussion

The detection of chemokine receptor expression on endothelial cells has been controversial. This is reflected by ongoing attempts to establish models based on direct or indirect pathways of angiogenesis for CXC chemokines. Stimulation of endothelial cells with bacteria, TNF-α, IL-1, or LPS induces the release of IL-8, GRO-α and -β, and ENA-78 and expression of chemokine receptors such as CXCR1 and CXCR2.[30–32] However, under our culture conditions for fresh HUVECs, mRNA for CXCR1, CXCR2, and CXCR3 receptors were not detected even after stimulation with either inflammatory mediators (ie, LPS, TNF-α, or IFN-γ) or angiogenic factors (ie, VEGF, bFGF, or EGF) or with phorbol ester. Our RNAse protection assay results obtained using HUVECs in the absence of leukocytes after stimulation with either bFGF, VEGF, or phorbol ester showed inducible expression only of CXCR4 mRNA by 4 hours and by FACS analysis CXCR4 protein expression by 24 hours. In agreement with published results,[21] IFN-γ, TNF-α, and LPS, acting alone, failed to induce CXCR4 mRNA synthesis by 4 hours after stimulation. We observed late CXCR4 expression by 48 to 72 hours on TNF-α-stimulated HUVECs, and at a lower level than on VEGF- or bFGF-stimulated cells. TNF-α is also reported to be an angiogenic factor[33] and was capable of inducing VEGF and bFGF production from endothelial cells.[32,34] Indeed, antibodies to VEGF blocked TNF-α-induced neovascularization in the rabbit cornea.[32] We therefore tested the possibility that the capacity of TNF-α to induce delayed expression of CXCR4 was mediated by these angiogenic factors. Antibodies to VEGF and bFGF, when used in combination, inhibited up to 43% of the CXCR4 expression on TNF-α-stimulated HUVECs (data not shown). This suggests that the induction of CXCR4 by TNF-α is only in part mediated by VEGF and bFGF, and additional mediators are involved.

The induction of CXCR4 expression presumably precedes responsiveness to its ligand, and therefore the angiogenic effect of the ligand for CXCR4, SDF-1α, was investigated. *In vitro* chemotaxis, receptor binding, and receptor redistribution data indicated that VEGF- and bFGF-induced CXCR4 on HUVECs was indeed responsive to SDF-1α, which led us to experimentally demonstrate that injections of this chemokine can induce angiogenesis *in vivo*.

We observed that endothelial cell responsiveness to SDF-1α is not restricted to freshly isolated HUVECs; HAECs and spontaneously immortalized endothelial cell lines derived from human umbilical cords (HUVEC B019809 and HUVEC B019810) also migrated toward SDF-1α after VEGF and bFGF stimulation (Figure 5; data not shown). Furthermore, as SDF-1α induced endothelial cell proliferation and microvessel formation at peripheral injection sites, mouse capillary endothelial cells are also SDF-1α responsive. Moreover, rat aorta endothelial cells are also responsive to SDF-1α. Interestingly, CXCR4 knockout mice lack the ability to properly vascularize the intestine during fetal development.[35] These data indicate that SDF-1α-CXCR4 interaction is necessary for some, but not all, types of neovascularization. The capacity of SDF-1 knockout fetuses to vascularize tissues and heal wounds has not been evaluated.

Despite the reports that only ELR-containing CXC chemokines have a role in angiogenesis,[12,36] we found that SDF-1α, a member of the CXC chemokine family and ligand for CXCR4, even though it does not have an ELR motif, acted as a direct chemoattractant for endothelial cells *in vitro* and as an angiogenic factor *in vivo*. The presence of leukocytic infiltrates prompted the assessment of angiogenic activity using cross sections of rat aorta. The ability of SDF-1α to induce capillary sprouting is therefore leukocyte independent.

It is well established that IL-8, which binds to CXCR1 and CXCR2, can act as an angiogenic factor.[24,37–39] In addition, Yoshida et al[32] reported inducibility of CXCR2 by TNF-α on human microvascular endothelial cells. However, our observation on the lack of receptors for IL-8 on endothelial cells is in accordance with the data of others[21,23] showing that HUVECs and human dermal microvascular endothelial cells lack these receptors. The lack of CXCR1 and CXCR2 expression is consistent with an indirect model as proposed by Hu et al[39] in which pro-inflammatory cytokines derived from leukocytes contribute to angiogenesis triggered by IL-8 *in vivo*. Consequently, cytokines released by leukocytes at inflammatory sites, such as TNF-α, by inducing bFGF and VEGF, might be important indirect stimulants of the angiogenic response.

Under the conditions used, we were unable to induce detectable levels of CXCR3 mRNA after multiple stimuli (shown above). The ligand for CXCR3, IP-10, is a known potent angiostatic factor.[38] The lack of IP-10 receptor expression on HUVECs is puzzling and suggests that the effect of IP-10 may be indirect, or an as yet unidentified receptor for IP-10 might exist on endothelial cells. The inhibitory ability of mediators such as IFN-γ on angiogenesis might exert their effects *via* an IP-10-independent mechanism and might be based on the down-modulation of CXCR4.[21]

The fact that all three known VEGF, acid and basic FGF, and SDF-1 genes are widely expressed in normal organs of adult mice and humans and that their receptors

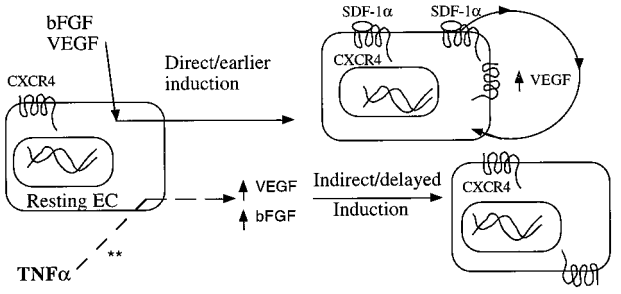

Figure 10. Hypothetical model of VEGF-, bFGF-, and TNF-α-induced angiogenesis. VEGF or bFGF amplify their angiogenic effects by inducing CXCR4 expression on endothelial cells. TNF-α acts indirectly (**) by inducing the production and release of VEGF and bFGF.[32] CXCR4-positive endothelial cells migrate toward SDF-1α to develop new vessels, and SDF-1-CXCR4 interaction increases VEGF production by endothelial cells, thus amplifying this response.

(flk, flt-1, flt-4, FGFR1–4, and CXCR4) are expressed on vascular endothelial cells[1,11,20] suggests that these interactions contribute to maintenance of the endothelium. Angiogenesis is therefore indirectly triggered by up-regulation of receptor levels by inflammatory mediators such as TNF-α[32] or by enhanced levels of angiogenic factors such as VEGF and bFGF. Indeed, SDF-1α-CXCR4 interaction further amplifies angiogenesis by inducing more VEGF release from CXCR4-bearing HUVECs. In addition, SDF-1 might contribute to angiogenesis during pathological neovascularization induced by angiogenic factors, such as bFGF or VEGF, which by increasing the expression of CXCR4 render endothelial cells more responsive to SDF-1α.

Consideration of the potential for interactions between SDF-1α and VEGF led to the possibility that these factors can act in an additive or synergistic manner. However, additivity and synergy are best determined when a single outcome is assessed, in this case, mitogenesis *versus* chemotaxis. The mitogenic influence of SDF-1α, in contrast to the proliferative effects of VEGF, appears limited at best, whereas VEGF is a less potent chemoattractant than SDF-1α. The two factors appear to act in a complementary fashion by performing different functions. Taken together, our results are consistent with a model for angiogenesis in which mediators such as TNF-α act indirectly by inducing the release of factors such as VEGF and bFGF.[32] Elevated levels of these growth factors in turn and in the absence of IFN-γ[22,40] promote enhanced expression of CXCR4 on endothelial cells, which can then respond to stromal-cell-derived SDF-1α. SDF-1α, in turn, and in addition to its role as a chemoattractant, acts as an amplifying factor by enhancing VEGF release (Figure 10).

Acknowledgements

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