Mucosal angiogenesis regulation by CXCR4 and its ligand CXCL12 expressed by human intestinal microvascular endothelial cells

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Chemokines are members of an extended family of 8- to 12-kDa secreted peptides with distinct chemoattractant properties, which are divided into subsets based on the number and spacing of amino acid cysteine residues (CC, CXC, CX3C, and CX4C) (36, 48). Originally defined by the site of action or production, chemokines have also been classified into an inducible/inflammatory group or a homeostatic/constitutive group (31, 53). Biological activity of chemokines is mediated by expression of chemokine receptors on target cells (36, 48). Evidence indicates that chemokines may have significant functional roles beyond the directed migration and activation of leukocyte subsets. Consistent with those reports, CXC chemokines bearing the NH2-terminal glutamate-leucine-arginine (ELR) motif have been identified as potent inductors of angiogenesis in vitro and in vivo (59). In contrast, the ELR-negative CXC chemokine ligands for the CXCR3 receptor have been shown to be angiostatic (4, 8, 25, 59).

Mice genetically deficient in either non-ELR motif CXC chemokine SDF-1 or its cognate receptor CXCR4, synonyma fusin, LESTR, or HUMSTER, die perinatally, reflecting marked defects in cardiac septal formation and vascularization of the gastrointestinal tract, suggesting a pivotal role for this chemokine-receptor pair in development and differentiation of the intestinal vasculature (37, 60). Subsequent studies of macrovascular endothelium seemed to corroborate these murine studies (21, 51). However, a conflicting report indicates SDF-1/CXCL12 invokes angiostatic mechanisms in growth factor-treated endothelial cells (8). Thus the variable role of the non-ELR motif CXC chemokine SDF-1/CXCL12 in angiogenesis appears to reflect marked tissue-specific endothelial microvascular heterogeneity (19, 32, 39, 43). Importantly, endothelial cells in established vascular beds are believed to undergo an angiogenic switch under various physiological and pathophysiological conditions (9).

Studies in genetically deficient mice suggest CXCR4 and SDF-1/CXCL12 play a role in developmental vascularization of the gastrointestinal tract. Similarly, endothelial cells from differing tissue subsets may be phenotypically distinct. We therefore sought to define the expression and angiogenic potential for CXCR4 and SDF-1/CXCL12 expressed in the human intestinal mucosa.
man gastrointestinal mucosa. We show herein that human intestinal mucosal microvessels in vivo and cultures of isolated human intestinal microvascular endothelial cells (HIMEC) in vitro express CXCR4 and SDF-1/CXCL12. Activation of CXCR4 by SDF-1/CXCL12 signals physiological characteristics of microvascular angiogenesis including proliferation, chemotaxis, and endothelial tube formation, through the ERK and phosphoinositide 3-kinase (PI3K) signaling pathways.

MATERIALS AND METHODS

Patients. HIMEC were isolated from histologically normal appearing colonic and ileal resection specimens from patients undergoing scheduled surgical bowel resection. The use of intestinal tissues and peripheral blood monocytes (PBMC) for this study was approved by the Institutional Review Board of the Medical College of Wisconsin.

Antibodies and reagents. Anti-human CXCR4 monoclonal antibody (clone 12G5) and an appropriate mouse isotype control were purchased from BD/Pharmingen (San Diego, CA). Monoclonal mouse anti-human SDF-1/CXCL12 antibody (clone K15C) was a kind gift from Dr. F. Arenzana-Seisdedos (Paris, France) (12). Clone K15C detects both SDF-1/CXCL12 isoforms with equal specificity. Synthetic human SDF-1α and endothelial cell growth supplement (ECGS) were from Upstate Biotech (Lake Placid, NY). Neutralizing murine monoclonal antibody to SDF-1/CXCL12 (clone O111:B4), Con-A, trypsin inhibitor (type II-S), pentostatin/streptovoxin/fungizone (PSF), and Brefeldin A were from Sigma (St. Louis, MO). DMEM and RPMI 1640 media were purchased from GIBCO (Grand Island, NY) and DMEM-F12 medium was obtained from BioWhittaker (Walkersville, MD). Collagenase type II was purchased from Worthington (Lakewood, NJ), and BSA were obtained from BioWhittaker (Walkersville, MD). DMEM and RPMI 1640 media that were supplemented with 0.5% (vol/vol) ECGS, porcine heparin, LPS (Escherichia coli O111:B4), Con-A, trypsin inhibitor (type II-S), pentostatin/streptovoxin/fungizone (PSF), and Brefeldin A were from Sigma (St. Louis, MO). DMEM and RPMI 1640 media were obtained from BioWhittaker (Walkersville, MD). Collagenase type II was purchased from Worthington (Lakewood, NJ), and BSA (Fraction V) was obtained from Fisher Scientific (Fair Lawn, NJ). Human plasma bronectin was purchased from Chemicon International (Temecula, CA). Matrigel was obtained from BD/Pharmingen.

Cell culture. HIMEC were isolated as previously described (10, 22). Briefly, mucosal strips from resected normal colon were washed, minced, and digested in collagenase type II solution (2 mg/ml). Endothelial cells were mechanically extruded and plated onto fibronectin-coated tissue culture dishes in MCDB-131 growth medium supplemented with 20% (vol/vol) FCS (BioWhittaker), ECGS, porcine heparin (130 mg/ml), and 2.5% (vol/vol) PSF solution. This medium was found to be free of detectable SDF-1α or SDF-1β, as assessed by ELISA (data not shown). After 7–10 days of culture, microvascular endothelial cell clusters were physically isolated, and a pure culture was obtained. HIMEC cultures were recognized by microscopic features, modified lipoprotein uptake (Dil-ac-LDL, Biomedical Technology, Stoughton, MA), and expression of Factor VIII-associated antigen. All experiments were carried out using HIMEC cultures between passages 8 and 12. Human umbilical vein endothelial cells (HUVEC) were purchased from Clonetics (Walkersville, MD) and maintained in growth medium as described above. Human small intestinal smooth muscle cells were obtained from ATCC (Manassas, VA) and were cultured in DMEM supplemented with 5% (vol/vol) FCS and 2 mM l-glutamine. PBMC from healthy donors were purified by Ficoll-Hypaque (Amersham Pharmacia Bio- tech, Arlington Heights, IL) density centrifugation. Whole blood samples were aseptically drawn into heparinized vials, mixed (1:1) with PBS (150 mM NaCl, 10 mM sodium phosphate, pH 7.4), and centrifuged over a Ficoll density gradient. Cell viability was >95% as assessed by trypan blue exclusion. PBMC were washed and cultured overnight in RPMI 1640 containing 2 mM l-glutamine, 25 mM HEPES, 2 μg/ml LPS, 4 μg/ml Con-A, and 5% (vol/vol) FCS.

Immunohistochemistry. Full-thickness normal colonic specimens were fixed in 4% (wt/vol) paraformaldehyde/PBS overnight. Specimens were saturated in 20% (wt/vol) sucrose/PBS for 6 h, embedded in OCT compound (Sakura, Japan), and snap-frozen in liquid nitrogen. Eight-micrometer frozen sections were stained with either murine monoclonal antibodies to CXCR4 (clone 12G5), SDF-1/CXCL12 (clone K15C), or mouse IgG isotype control (1:100) at 4°C overnight using Cell and Tissue Staining Kit (R&D Systems). After immunodetection of horseradish peroxidase-conjugated antibodies with diaminobenzidine, sections were briefly counterstained with Mayer’s hematoxylin and mounted with Fluoromount-G (Southern Biotechnology, Birmingham, AL).

Immunofluorescence. HIMEC were grown to confluence on fibronectin-coated glass slides (LabTek II; Nunc, Naperville, IL). After incubation with Brefeldin A (10 μg/ml) for 4 h, cells were fixed in 4% (wt/vol) paraformaldehyde/PBS for 15 min on ice, permeabilized with 0.2% (vol/vol) Triton X-100/PBS, and washed three times with PBS. Cells were incubated with antibody to SDF-1/CXCL12 (clone K15C), CXCR4 (clone 12G5), or an isotype control IgG for 1 h at room temperature, washed, and incubated with FITC-labeled anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h in the dark. Separate cells were incubated with rabbit anti-human von Willebrand factor antibodies (Sigma) followed by Cy3-labeled donkey anti-rabbit antibodies (Jackson Immunoresearch, Westgrove, PA) to confirm the endothelial nature of the cells. To visualize nuclei, von Willebrand factor-immunostained cells were colabeled with DAPI (Sigma). Cells were washed, mounted with Fluoromount-G, and examined using a fluorescence microscope (Olympus BX-40).

RT-PCR. Unlabeled oligonucleotides were purchased from Operon (Alameda, CA). Primers for SDF-1/CXCL12 were designed from published GenBank sequences; sense: 5′-TGA GCT ACA GAT GCC CAT GC-3′ and antisense: 5′-TTC TCC AGG TAC TCC TGA ATC C-3′ (product size 177 bp; GenBank Acc. No.: U16752). CXCR4 and β-actin primers were as described previously (11, 13). Total RNA was isolated from subconfluent HIMEC cultures by a single step guanidinium thiocyanate/phenol-chloroform extraction using TRizol reagent (Invitrogen, Carlsbad, CA). Genomic DNA was subsequently quenched by DNase I (Amplification Grade, Invitrogen). For reverse transcription, cDNA was generated using 1 μg of total RNA according to the manufacturer’s protocol (Superscript II Reverse Transcription Kit, Invitrogen) in a total volume of 20 μl. PCR was performed using RoboCycler (Stratagene, La Jolla, CA). For each reaction, 1.25 μl of cDNA were amplified using specific primers (25 pmol) in a final volume of 50 μl and subjected to 35 cycles of 94°C denaturation (60 s) and 62°C annealing (150 s), followed by a final 72°C extension step (6 min). PCR products were separated on a 1% agarose gel and visualized by ethidium bromide staining. DNase-treated total RNA samples (no RT) were used as a negative control, whereas cDNA of stimulated PBMC served as a positive control for CXCR4. cDNA generated from confluent cultured human intestinal smooth muscle cells was used as a positive control for amplification of SDF-1/CXCL12.

Flow cytometry. For detection of intracellular SDF-1/CXCL12, HIMEC were pretreated with Brefeldin A (10 μg/ml) for 4 h at 37°C. HIMEC monolayers were washed, briefly trypsinized on ice, and washed in FACS buffer [PBS, pH 7.4, containing 0.1% (wt/vol) Na3C and 2% (vol/vol) FCS]. Cells were fixed in 2% (wt/vol) paraformaldehyde in PBS for 5 min on ice and permeabilized with 0.1% (vol/vol) Triton X-100 in PBS. Cells were washed and incubated with monoclonal antibodies to CXCR4 (clone 12G5) or SDF-1/CXCL12 (clone K15C) for 1 h on ice. After being washed three times, bound primary antibody was detected by incubation with FITC-labeled anti-mouse IgG (Santa Cruz Biotechnology) for 1 h on ice in the dark. Cells were washed extensively, resuspended in formaldehyde in PBS, and analyzed by flow cytometry (FACScan, Becton Dickinson, Mountain View, CA). Murine IgG served as an isotype-negative control.
**Immunoblot analysis.** HIMEC were lysed in ice-cold modified RIPA buffer [50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.25% (wt/vol) sodium deoxeycholate, 1% (vol/vol) Igepal CA-630, 0.1% (wt/vol) sodium dodecylsulfate containing Protease Inhibitor Cocktail III (CalBiochem, San Diego, CA), sodium orthovanadate (10 mM), sodium fluoride (20 mM), and β-glycerophosphate (40 mM)]. Protein concentration of preclarified lysates was determined by a Bradford assay (Bio-Rad, Hercules, CA). For detection of ERK1/2, confluent HIMEC monolayers were serum-starved in MCDB-131 medium containing antibiotics for 16 h. HIMEC isolates were stimulated with SDF-1/CXCL12 as indicated. HIMEC were lysed in ice-cold lysis buffer, and 12 μg of total cellular protein per lane were separated by SDS-PAGE, electrotransferred to PVDF (Millipore), and analyzed by immunoblotting using phospho-specific polyclonal antibodies against ERK1/2 (Cell Signaling Tech., Beverly, MA). To ensure equal protein loading, membranes were stripped and reprobed using polyclonal antibodies against total ERK1/2. Phospho specificity of ERK1/2 antibodies was confirmed using an in vitro-phosphorylated recombinant ERK 2 (Cell Signaling).

**Endothelial cell chemotaxis assay.** Polycarbonate filters (8-μm pore size, BD Labware) were coated at 4°C overnight with human fibronectin (10 μg/ml). HIMEC were briefly trypsinized, washed with chemotaxis buffer [MCDB-131 + 1% (wt/vol) BSA] containing soybean trypsin inhibitor (10 mg/ml), and suspended with SDF-1/CXCL12 buffer, and 5 × 10^5 cells were seeded onto the coated filters. Chemotaxis buffer containing SDF-1/CXCL12 at indicated concentrations was added to the lower compartment of the 12-well plates. After 3 h of incubation at 37°C (5% CO_2–95% air), cell culture inserts were removed and the upper side of the membrane was gently wiped using a cotton swab. Filters were stained with DiffQuik (Baxter Scientific, McGraw, IL), air dried, and mounted onto glass slides. Migrated HIMEC adherent to the lower side of the membrane were counted [10 random high-power fields (HPF) per condition]. In separate studies, chemotaxis was assessed in the presence of the pharmacological inhibitors; PD-98059 (IC_50 = 5 μM) to attenuate MAPK (CalBiochem) (40), or wortmannin (IC_50 = 5 nM; CalBiochem) to block PI3K signaling (5). Cell viability was assessed using a colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium salt] assay (34). HIMEC (1 × 10^3 cells) were seeded to collagen-coated 96-well plates in endothelial cell medium containing antibodies, 1% (vol/vol) FBS, and devoid of endothelial growth supplement. HIMEC were allowed to adhere 16 h before stimulation with titrated doses of SDF-1/CXCL12. Stimulated cells were grown for an additional 16 h before the addition of 25 μl of sterile MTT/PBS solution (2.5 mg/ml; Sigma) to each well. After 6-h incubation, cells were lysed, the precipitated chromogen was released by the addition of 0.04 N HCl to each well, and absorbance at 575 nm was measured by microplate reader.

**cAMP enzyme immunoassay.** To assess CXCR4 intracellular signal activation, HIMEC monolayers grown on fibronectin-coated 12-well plates were used to measure cAMP levels following addition of SDF-1/CXCL12. As described previously (15), cells were incubated for 30 min with 10 mM 3-isobutyl-1-methylxanthine (IBMX) to inhibit phosphodiesterase activity and then treated for 10 min at 37°C with SDF-1/CXCL12 (100 ng/ml) in Hanks’ balanced salt solution (HBSS) containing IBMX and subsequently incubated 10 min with 1 μM forskolin (Sigma) to stimulate cAMP production. Baseline cAMP levels were assessed in cells that remained unstimulated with SDF-1/CXCL12 or forskolin. Separate controls independently received SDF-1/CXCL12 or forskolin alone. The HBSS/IBMX solution was aspirated, the cells were washed and solubilized, cAMP was assayed using a commercially available competitive enzyme immunoassay (Biotrak, Amersham), and percent of control cAMP production was calculated.

**Blood vessel tube formation assay.** Endothelial tube formation was assessed using Matrigel, a solubilized extracellular basement membrane matrix extracted from the Engelbreth-Holm-Swarm mouse sarcoma, as described previously (54). Multiwell dishes (24-well) were coated with 250 μl of complete medium containing 5 mg/ml Matrigel, and HIMEC resuspended in complete growth medium were seeded at a density of 1 × 10^4. The growth medium was supplemented with 5 μg/ml of neutralizing antibodies against CXCR4 or SDF-1/CXCL12 or a murine isotype control. Separate wells received 2 μM MAPKK inhibitor PD-98059. Control cells remained free of antibody and kinase inhibitor. Cells were cultured on Matrigel for 16 h and endothelial tube formation was enumerated by an observer blinded to treatment regimen by inverted phase-contrast microscopy using previously established protocols (3, 30). Five HPF per condition were examined and experiments were repeated in three independent HIMEC cultures.

**Statistical analysis.** Statistical analysis was performed by ANOVA using StatView for Macintosh (version 4.51; Abacus Concepts, Berkeley, CA). *P* < 0.05 was considered statistically significant.

**RESULTS**

**CXCR4 and SDF-1/CXCL12 are expressed in intestinal mucosal microvessels in vivo.** Immunohistochemistry was used to determine whether CXCR4 and/or its ligand SDF-1/CXCL12 are expressed in human gastrointestinal mucosal microvessels in vivo. As shown in Fig. 1, both CXCR4 (A) and its ligand SDF-1/CXCL12 (B) could be detected in mucosal intestinal microvessels. Consistent with prior reports, we also noted, as an internal control, positive CXCR4 and SDF-1/CXCL12 immunostaining in normal colonic crypt epithelium (not shown) (1, 13). No specific immunostaining was observed using the appropriate IgG isotype control antibody (Fig. 1C).

**HIMEC constitutively express CXCR4 and SDF-1/CXCL12.** We next sought to define expression of CXCR4 and SDF-1/CXCL12 on primary cultures of HIMEC. As an initial step, we performed RT-PCR to assess mRNA expression of CXCR4 and SDF-1/CXCL12. HIMEC isolates from three different patients uniformly expressed high constitutive levels of CXCR4 mRNA in vitro (Fig. 2A, top). All three HIMEC isolates variably expressed detectable levels of SDF-1/CXCL12 mRNA (Fig. 2A, middle). Comparable data on CXCR4 and SDF-1/CXCL12 expression were noted in macrovascular HUVEC cells, as described previously (21, 50, 51, 55) and used as a control (not shown). Consistent with the literature, SDF-1/CXCL12 was not expressed by stimulated PBMC, so as a separate positive control we amplified SDF-1/CXCL12 mRNA from the mesenchymal human intestinal smooth muscle cell line.

Having detected CXCR4 and SDF-1/CXCL12 mRNA in isolated HIMEC, we next analyzed protein expression in HIMEC using two parallel approaches, flow cytometry and immunofluorescence. As shown in Fig. 2B, HIMEC expressed surface CXCR4 receptors by flow cytometry. Western blot
analysis confirmed expression of CXCR4 protein in HIMEC (not shown). To detect intracellular SDF-1/CXCL12 protein using flow cytometry, permeabilized HIMEC were immunostained with monoclonal antibodies and shown to constitutively express SDF-1/CXCL12 (Fig. 2C).

We next used immunofluorescence to confirm CXCR4 and SDF-1/CXCL12 protein expression in cultured HIMEC. Phase-contrast microscopy revealed the prototypical cobblestone appearance of cultured HIMEC (Fig. 3A). Endothelial morphology of HIMEC was further verified by immunofluorescence microscopy using the endothelial cell marker von Willebrand factor (Fig. 3B), as we previously defined (10). Next, parallel HIMEC cultures were immunostained with an-
tibodies to CXCR4 and SDF-1/CXCL12. CXCR4 surface expression on cultured HIMEC was confirmed by speckled immunofluorescence localized at the cell membrane (Fig. 3D). Perinuclear cytoplasmic SDF-1/CXCL12 immunofluorescence was observed, suggestive of accumulated synthesized protein in the Golgi apparatus and the endoplasmic reticulum (Fig. 3E). No specific immunofluorescence was observed using murine isotype control antibodies for von Willebrand factor (Fig. 3C) or CXCR4 and SDF-1/CXCL12 (Fig. 3F). Taken together, these findings indicate in vivo expression of CXCR4 and SDF-1/CXCL12 was paralleled in cultured human intestinal mucosal microvessels. Furthermore, CXCR4 and SDF-1/CXCL12 appear to be localized to the correct cellular compartments for autocrine or paracrine signaling within the gastrointestinal mucosa.

CXCR4 expressed on HIMEC is functional. We next sought to determine if CXCR4 localized to the HIMEC cell surface was functionally activated on engagement with its cognate ligand. Chemokine receptors such as CXCR4 are predominantly linked to G protein in leukocytes and receptor-transfected cell types (36, 48). The functional paradigm for G protein is the inhibition of adenyl cyclase-mediated formation of cAMP (57). Thus, to determine if HIMEC-expressed CXCR4 was functionally coupled to intracellular signaling machinery, we measured levels of cAMP in HIMEC stimulated with the adenyl cyclase activator forskolin. Consistent with functional CXCR4 localized to the HIMEC cell surface, 10 ng/ml of SDF-1/CXCL12 significantly inhibited forskolin-evoked cAMP formation (56.4% ± 14.2 of forskolin control, \( P < 0.05, n = 4 \)).

SDF-1/CXCL12 evokes chemotaxis and proliferation in HIMEC. Angiogenesis and neovascularization are a multistep process requiring the activation, directed migration (chemotaxis), and enhanced proliferation of endothelial cells. As a first step to determine the role for CXCR4 in angiogenesis of human intestinal microvasculature, we employed a Transwell chamber system to investigate the chemotactic response evoked by SDF-1/CXCL12 in HIMEC. As expected, stimulation with SDF-1/CXCL12 significantly evoked a concentration-dependent chemotactic response in HIMEC, with the highest number of migrated cells observed at a concentration of 10 ng/ml (Fig. 4A). This concentration of SDF-1/CXCL12 stimulated comparable chemotactic responses in macrovascular HUVEC (21) or a Jurkat cell line expressing CXCR4 (data not shown) and indicates that angiogenic mechanisms are regulated by SDF-1/CXCL12 in HIMEC. As expected, higher concentrations of SDF-1/CXCL12 were less robust at stimulating chemotaxis (44).

Prior reports in leukocytes and receptor-transfected cell systems suggested that MAPK and PI3K signaling pathways play an essential role in chemotaxis of those cell types (18, 58). We therefore tested this paradigm in nontransfected HIMEC cultures. Cells incubated with the MAPKK inhibitor PD-98059 (40) dose dependently attenuated chemotaxis of SDF-1/CXCL12-stimulated HIMEC (Fig. 4B). Comparable inhibition was noted in cells incubated with wortmannin, a specific inhibitor of PI3K signaling (Fig. 4B). These data suggest that SDF-1/CXCL12 binds HIMEC-localized CXCR4 to initiate chemotaxis in a MAPK- and PI3K-dependent fashion.

To further assess the angiogenic properties of SDF-1/CXCL12 in HIMEC, cellular proliferation rates were measured by a \(^{3}H\)-thymidine uptake assay. As shown in Fig. 4C, stimulation with SDF-1/CXCL12, with an optimal concentration of 100 ng/ml, led to a marked increase in HIMEC proliferation. Of note, SDF-1/CXCL12-evoked proliferation was comparable to VEGF used as a positive control (51). Separate experiments using a MTT assay verified the dose response for HIMEC proliferation observed using \(^{3}H\)-thymidine uptake (data not shown) and agree with prior reports of SDF-1/CXCL12 mitogenic properties (7, 25). Although 10 ng/ml of SDF-1/CXCL12 alone minimally upregulated proliferation, in accordance with a prior report (25), we noted that concentration of chemokine attenuated the mitogenic response induced by 50 ng/ml VEGF. The exact mechanism for this phenomenon remains to be elucidated, but interference (e.g., heterologous receptor desensitization) of intracellular signaling pathways may serve as a possible explanation. Taken together, HIMEC chemotaxis and proliferation in response to SDF-1/CXCL12 stimulation are consistent with a role for CXCR4 activation in regulating angiogenesis of human intestinal microvessels.

SDF-1/CXCL12 stimulates MAPK phosphorylation in HIMEC. MAPKs have been shown to be a pivotal signaling pathway in chemokine activation of target cells and in growth factor-regulated angiogenesis (18). SDF-1/CXCL12 has previously been shown in murine pre-B cells stably transfected with human CXCR4 to rapidly induce sustained phosphorylation of ERK1/2 (p44/42 MAPK), but not JNK kinase or p38 MAPK (18). To characterize these pathways in our nontransfected primary HIMEC isolates, we performed immunoblot analysis of whole cell lysates using phospho-specific antibodies raised...
against human ERK1/2. As shown in Fig. 5A, SDF-1/CXCL12 alone induced the phosphorylation of ERK1/2, comparable to that evoked by VEGF as well as to lower doses of chemokine (1 and 10 ng/ml; data not shown). Although we noted that SDF-1/CXCL12 attenuated VEGF-stimulated proliferation, a 30-min stimulation of SDF-1/CXCL12 (10 ng/ml) combined with VEGF (50 ng/ml) had only a minimal effect on phosphorylation of ERK1/2 (Fig. 5A). These data suggest ERK1/2 is minimally if at all involved in the inhibition of VEGF-induced HIMEC proliferation by exogenous SDF-1/CXCL12. As shown in Fig. 5B, maximal ERK1/2 phosphorylation by mitogenic doses of SDF-1/CXCL12 (100 ng/ml) peaked at 5 and 15 min, compared with unstimulated HIMEC, and remained elevated above unstimulated control levels after 30 and 60 min. Similar to a prior report, SDF-1/CXCL12 did not significantly activate JNK kinase or p38 MAPK (18) in parallel experiments using the same time course and chemokine concentration (data not shown). These findings are consistent with the pharmacological inhibition of HIMEC migration.

Spontaneous endothelial tube formation of HIMEC in Matrigel is inhibited by blockade of endogenous CXCR4, SDF-1/CXCL12, or ERK1/2. We further assessed the angiogenesis activity of SDF-1/CXCL12 and CXCR4 using a tube formation assay employing Matrigel as a three-dimensional extracellular matrix. HIMEC seeded to the Matrigel cushion rapidly initiated tube formation within a few hours, displaying formation of strongly branched endothelial tubes after 16 h (Fig. 6A). To assess the role of HIMEC-expressed CXCR4 in this activity, cells were seeded to the Matrigel in the presence of neutralizing monoclonal antibodies against human SDF-1/CXCL12 and CXCR4 (5 μg/ml each). As shown in Fig. 6, endothelial tube formation was inhibited, with only few poorly developed capillary sprouts assembling in cultures incubated with anti-CXCR4 (B) or anti-SDF-1/CXCL12 (C). This inhibition was especially prominent with anti-CXCR4, where endothelial...
tubes were disrupted and cells were cohered in spherical clusters. Furthermore, inhibition of ERK1/2 phosphorylation using the specific inhibitor PD-98059 (40) resulted in a similar disruption of endothelial tube formation (Fig. 6D). We previously noted similar inhibition of endothelial tube formation in HIMEC cultured with PI3K inhibitors (22). In contrast, tube formation was not inhibited in cultures incubated with mouse IgG (5 μg/ml; Fig. 6E). Enumeration of endothelial tube branches verified the morphological assessment (Fig. 6F). Thus constitutive HIMEC tube formation was significantly attenuated in cultures incubated with neutralizing antibody to CXCR4 (12G5) or SDF-1/CXCL12 (K15C) or the MAPKK inhibitor PD-98059 (Fig. 6F). Unstimulated HIMEC-seeded Matrigel cultures possessed a comparable number of endothelial tube branches as cultures treated with an isotype control antibody. These findings support a prominent role for auto- and paracrine SDF-1/CXCL12 signaling through CXCR4 (12G5) and other growth-related cytokines, regulates chemokine-dependent angiogenic signaling in the human intestinal microvasculature.

**DISCUSSION**

Our data herein are the first to describe the expression of the CXC chemokine receptor CXCR4 and its sole ligand SDF-1/CXCL12 in the microvasculature of the adult human intestinal mucosa. We found that angiogenic chemotactic and proliferative responses and corresponding endothelial tube formation in isolated human intestinal microvascular cells are accompanied by increased ERK1/2 activation. Mice lacking either the SDF-1 or CXCR4 gene die perinatally with characteristic defects in cardiac septum formation and hematopoiesis as well as marked deficiencies in vascularization of the gastrointestinal tract (29, 37, 60). Our data support the notion from murine studies that CXCR4 and SDF-1/CXCL12 play an angiogenic role in vascularization of the adult human gastrointestinal tract.

Within the gastrointestinal mucosa, chemokines are known mediators of inflammation and immune responses to enteric pathogens and have been shown to be upregulated in colorectal cancer (14, 41). We previously showed that the cells of the colonic epithelium express CXCR4, as well as several other chemokine receptors (13, 23, 24). Our subsequent analyses indicate that epithelial cell-expressed CXCR4 is coupled to G protein-proteins that negatively regulate adenylyl cyclase-mediated production of cAMP and epithelial ion transport dependent on that second messenger (15). Data herein expand on those findings and indicate that HIMEC-expressed CXCR4 is similarly coupled to Gαi-α-proteins, suggesting this chemokine receptor G protein couple is conserved among leukocytes as well as epithelial and endothelial cells. Moreover, expression of both CXCR4 and SDF-1/CXCL12 by normal and malignant colonic epithelial cells (1, 13, 24) propounds the concept of a potential epithelial/endothelial cross-talk communication (27), which may be important in mucosal homeostasis or inflammation. In agreement with that notion, the expression of the CXCR4 ligand SDF-1/CXCL12 by HIMEC noted herein is paralleled by epithelial cells of the human small and large intestine in vivo (1).
Given that the intra- and intercellular communication mechanisms responsible for tissue morphogenesis also regulate tissue repair in mucosal inflammatory diseases or following injury (61), it is likely that autocrine or paracrine CXCR4 and SDF-1/CXCL12 signaling plays a role in maintaining endothelial homeostasis in the intestinal mucosa. This hypothesis is further supported by recent observations that VEGF, a prototypical cytokine crucially involved in wound healing and tissue homeostasis, was found to strongly upregulate the expression of the SDF-1/CXCL12 receptor CXCR4 in human endothelial cells (51). Apart from this angiogenic mechanism being involved in tissue homeostasis, VEGF-promoted upregulation of CXCR4 in breast carcinoma cells is now appreciated as an autocrine loop involved in cancer invasion (6). Importantly, hypoxia, as might be expected in inflammatory and cardio- and cerebrovascular diseases, pulmonary disease, and cancer, is also a potent stimulant for upregulated CXCR4 expression and increased chemotactic responsiveness of those tissues (55), further implicating a role for this signaling axis in angiogenesis and wound healing in the intestinal mucosa.

As the gastrointestinal mucosa is considered physiologically inflamed, it is likely that cytokines within the mucosal milieu may modulate CXCR4 or SDF-1/CXCL12 expression or signaling, with likely repercussions in mucosal wound healing dependent on normal angiogenic function. For example, angiogenic effects of prostaglandin E2 have been linked to the ability of that potent mediator of inflammation to upregulate CXCR4 expression on dermal microvascular endothelial cells (52). Consistent with this notion, we observed that IL-8, a prototypic inducible/inflammatory chemokine (31) markedly upregulated in gut inflammatory diseases (22, 41), also stimulates HIMEC angiogenesis (22). Alternatively, in addition to regulation of CXCR4 or SDF-1/CXCL12 expression, signaling via CXCR4 may modulate physiological as well as pathophysiological inflammation through upregulation of expression of intestinal addressin molecules critical to mucosal lymphocyte trafficking (62). Although those data suggest a role for CXCR4 in trafficking of lymphocytes to the mucosa, we found that signaling via that receptor negatively regulated epithelial ion transport evoked by inflammatory mediators (15), suggesting SDF-1/CXCL12 and its receptor may have varying, cell type-specific roles in immune regulation of mucosal functions.

Although CXCR4-SDF-1/CXCL12 have been characterized as prototypic molecules of the constitutive/homeostatic chemokine group, as suggested above, they have been shown to participate in a cell type- and tissue-specific manner in inflammation and infectious diseases. Biological activities of SDF-1/CXCL12 include chemoattractant properties for CXCR4-positive leukocytes and CD34+ hematopoietic progenitor cells and stimulation of pre-B cell growth in the presence of IL-7 (37). Consistent with those reports, we noted that SDF-1/CXCL12 induces a potent chemotactic and proliferative response in nonreceptor-transfected HIMEC cells in culture. In addition, SDF-1/CXCL12 has been shown to inhibit entry of human immunodeficiency virus (HIV) into T lymphocytes by ligation and internalization of its cognate receptor CXCR4 (37). Entry of HIV-1 through jejunal enterocyte-expressed chemokine receptor and mucosal macrophages, together with our data, suggests possible roles for those cells in entry or latency of mucosal HIV-1 (33). Although SDF-1/CXCL12 and its sole receptor CXCR4 have been described as regulators of homeostatic leukocyte functions, it has been previously reported that cytokine mediators such as tumor necrosis factor-α and basic fibroblast growth factor are effective modulators of CXCR4 expression in endothelial cells (16).

Reflecting endothelial cell heterogeneity, varying human macro- and microvascular cell subsets, including HUVEC and human endothelium derived from coronary arteries (21) and skin (49, 51, 52), variably express CXCR4 or SDF-1/CXCL12 (38). Likely reflecting expression patterns, data on the angiogenic properties of SDF-1/CXCL12, a CXC chemokine lacking the ELR motif, were inconclusive. One report suggested SDF-1/CXCL12 possesses angiostatic properties by counteracting the angiogenic activity induced by ELR+ CXC chemokines, basic fibroblast growth factor, and VEGF (25). Conversely, SDF-1/CXCL12 was found to induce angiogenesis in rat aortic ring assays in vivo, independent of the presence of inflammatory leukocyte infiltrates (51), as well as dermal microvascular cells (52) or HUVEC in vitro (21, 50, 51, 54, 55). In agreement with prior reports (21, 50, 51, 54, 55), we found SDF-1/CXCL12 to stimulate chemotaxis of macrovascular endothelial HUVEC. Thus CXCR4-SDF-1/CXCL12 interactions appear to heterogeneously modulate angiogenesis, dependent on the tissue microenvironment of angiogenic and angiostatic factors. Consistent with those reports, we noted that some intestinal microvessels in vivo and in vitro did not immunostain with antibody to CXCR4. Nonetheless, our data demonstrate that SDF-1/CXCL12 activation of CXCR4 evokes a potent angiogenic response in intestinal mucosal microvascular endothelial cells.

Despite the ubiquity of SDF-1/CXCL12 and CXCR4 expression in immune responses, little is known of the ability of those molecules to participate in wound repair following insult or in intestinal inflammatory disease or colorectal tumorigenesis. CXCR4 and its ligand have been implicated in the pathogenic process of tumor-associated neovascularization by colocalization in human glioblastoma multiforme, showing increased expression in areas with high angiogenesis and in tumors of higher grade (45). CXCR4-SDF-1/CXCL12 interactions were similarly found to be pivotal in pancreatic cancer progression through tumor cell migration and induction of angiogenesis (26), as well as patterns of metastasis and tumor growth in breast cancer and kidney carcinoma cells (35, 42, 47, 56). Moreover, tumors and ischemic tissue (55) may initiate neo-vascularization by the selective mobilization of both resident microvessels or bone marrow-derived endothelial precursors (20, 28), suggesting wound healing shares comparable mechanisms of angiogenesis with tumor neovascularization. A prior report of SDF-1/CXCL12 regulating gastric ulcer repair supports that notion (2). Consistent with these reports, it is plausible that the homeostatic chemokine SDF-1/CXCL12 plays a broad role in mucosal tissue morphogenesis as well as remodeling, wound repair, and tumorigenesis.

Our findings, combined with data regarding the chemokine receptor CXCR2 (22), suggest that the multitude of angiogenic factors likely use similar intracellular signaling pathways, notably the ERK1/2 cascade. These findings bear clinical significance, as CXCR4-SDF-1/CXCL12 interactions and endothelial ERK1/2 activation represent a potential combinatory target for antiangiogenic therapy in the setting of colorectal adenocarcinoma and other diseases leading to pathophysiological gastrointestinal neo-vascularization. As several small pep-
tide receptor agonists and antagonists have been developed, CXC4 expressed by human intestinal microvessels may provide, in combination with additional angiogenesis inhibitors, a new therapeutic target for regulating pathophysiological angiogenesis. Taken together, these reports coupled with our data herein strongly implicate a role for SDF-1/CXCL12 and its cognate receptor CXCR4 in angiogenic responses that may play an important role in gut homeostasis as well as repair within the physiologically inflamed human intestinal mucosa.

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