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CXCR1 and CXCR2 Silencing modulates CXCL8-dependent Endothelial Cell Proliferation, migration and Capillary-Like Structure Formation

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Abstract

CXCR1 and CXCR2 are receptors for angiogenic ELR+ CXC chemokines and are differentially expressed on endothelial cells; however, their functional significance in angiogenesis remains unclear. In this study, we determined the functional significance of these receptors in modulating endothelial cell phenotype by knocking-down the expression of CXCR1 and/or CXCR2 in human microvascular endothelial cells (HMEC-1) using short-hairpin RNA (shRNA). Cell proliferation, migration, invasion and capillary-like structure (CLS) formation were analyzed. Our data demonstrate that knock-down of CXCR1 and/or CXCR2 expression inhibited endothelial cell proliferation, survival, migration, invasion and CLS formation. Additionally, we examined the mechanism of CXCL-8-dependent CXCR1 and/or CXCR2 mediated phenotypic changes by evaluating ERK phosphorylation and cytoskeletal rearrangement and observed inhibition of ERK phosphorylation and cytoskeletal rearrangement in HMEC-1-shCXCR1, HMEC-1-shCXCR2 and HMEC-1-shCXCR1/2 cells. Together, these data demonstrate that CXCR1 and CXCR2 expression plays a critical role in regulating multiple biological activities in human microvascular endothelial cells.

Keywords

Endothelial cells; CXCR1; CXCR2; Proliferation; Migration; Angiogenesis

Introduction

Angiogenesis, the formation of new blood vessels from an existing capillary bed, is a crucial process involved in various physiological and pathological conditions, including embryonic development, wound healing, chronic inflammation, and malignancies. It comprises of a cascade of events, emanating from endothelial cell proliferation, survival, migration, extracellular matrix remodeling, and maturation to form capillary tubes (1–7). In addition to cell proliferation and migration, endothelial cell survival is also an important component for

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tumor angiogenesis (8;9). Multiple key molecular regulators direct single or multiple steps of this process (10). Several reports suggest that members of CXC chemokine family of proteins mediate several steps during angiogenesis.

Members of the CXC (or α -chemokine) subfamily contain one non-conserved amino acid (X) between the first and second cysteine residues. Based on the presence or absence of a Glu-Leu-Arg (ELR) motif, the CXC chemokines can be further subdivided into two groups, ELR⁺ and ELR⁻ (11–13). The ELR motif is located at the N-terminus immediately before the first cysteine amino acid residue (14). Extensive investigations regarding the functions of the CXC subfamily have revealed that the presence/absence of the ELR motif determines if the chemokine is angiogenic or angiostatic (15;16). Recently, we and others have demonstrated that CXCL8, a member of ELR⁺ CXC chemokine family and its receptors, CXCR1 and CXCR2, are potent angiogenic regulators (5;6;17). CXCR1 and CXCR2 are widely expressed on various tumors and endothelial cells (18–21). Both receptors bind CXCL8 with high affinity, but CXCR2 also binds to other ELR⁺ CXC chemokines. CXCR1 and CXCR2 play an important role in endothelial cell proliferation (22–24). Thus, involvement of CXCR1 and CXCR2 and their ligands (ELR⁺ CXC chemokines) in different cell process makes this ligand-receptor axis of particular interest especially its functional role in modulating the angiogenic phenotype of endothelial cells.

To examine the mechanistic role of CXCR1 and CXCR2 on endothelial cells, we inhibited CXCR1 or CXCR2 expression using a gene knock-down strategy to modulate angiogenic phenotypes. Our results show that down-regulation of CXCR1 and/or CXCR2 inhibited endothelial cell growth, survival, migration, invasion, and CLS formation.

Material and methods

Endothelial cell culture

Human microvascular endothelial cells (HMEC-1) were obtained from Centers for Disease Control (CDC; Atlanta, GA). The cells were maintained in culture as an adherent monolayer in endothelial cell growth media (EGM-2 MV, Clonetics) containing fetal bovine serum (FBS), endothelial growth supplement, and gentamycin. Cells were grown at 37°C with 5% CO₂ in humidified atmosphere.

Generation of shRNA-expression plasmids and cell lines

Silencing of gene expression was achieved using short hairpin RNA (shRNA) technology. shRNAs targeting CXCR1 (1sh- 5'-CCC GCG TCA CTT GGT CAA GTT TGT-3'), CXCR2 (2sh-5'-CCC CAA TAC AGC AAA CTG GCG GAT-3'), and CXCR1/2 (1/2sh- 5'-CCC CTT CTA TAG TGG CAT CCT GCT-3') and scrambled (control) were generated using a CXCR1 or CXCR2 specific sequence with *Bgl*III and *Hind*III overhangs to allow for cloning into the pSuper.neo vector (Oligoengine, Seattle, WA). HMEC-1 cells were transiently transfected with pSuper.neo/scrambled (HMEC-1-control), pSuper.neo/shCXCR1 (HMEC-1-shCXCR1), pSuper.neo/shCXCR2 (HMEC-1-shCXCR2) or pSuper.neo/shCXCR1/2 (HMEC-1-shCXCR1/2) plasmid using Lipofectamine reagent (Invitrogen, Carlsbad, CA) following the manufacturers protocol. Forty-eight hours later, cells were used for different assays.

mRNA analysis

CXCR1 and CXCR2 expression was determined using semi-quantitative RT-PCR as described previously (25). Briefly, cDNA was synthesized from 5 µg total RNA using SuperScript™ II Reverse Transcriptase (Invitrogen) and oligo(dT) primer. Two micro liter of first strand cDNA (1:10 dilution) was amplified using PCR primer sets (Table 1) and a

DNA thermal cycler (Perkin Elmer, Foster City, CA). Amplified products were resolved through a 1.5% agarose gel containing ethidium bromide, visualized and photographed using a gel documentation system (Alpha-Innotech, San Leandro, CA). Relative intensity of specific gene expression was determined using ImageQuant 5.1 software (Molecular Dynamics, Inc., Sunnyvale, CA).

Immunohistochemistry

For confirming the down-regulation of CXCR1 and/or CXCR2, cells (10,000 cells) were seeded overnight on coverslips, fixed in ice cold 4% formaldehyde, blocked and incubated with the following primary antibodies: mouse monoclonal anti-CXCR1 (1:100; R&D systems, Minneapolis, MN) and mouse monoclonal anti-CXCR2 (1:50; R&D systems) as described previously (25). Corresponding biotinylated secondary antibody was used at room temperature. Immunoreactivity was detected using the ABC Elite kit and DAB substrate (Vector Laboratories, Burlingame, CA) per the manufacturer's instructions. A reddish brown precipitate in the cytoplasm indicated a positive reaction. Negative controls had all reagents included except the primary antibody.

In vitro growth and apoptosis assay

Transfected cells were serum and growth factor starved overnight. Following trypsinization and washing different HMEC cells were seeded in 96-well plate at low density (1000 cells/well). Following overnight adherence, cells were incubated with media alone or media containing CXCL8 (10ng/ml) for 72 h. Cell growth was determined by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay as previously described (26;27). Growth increase was calculated as percent (%) = $[(A / B) - 1] \times 100$, where A and B are the absorbance of treated (CXCL8 stimulated) and untreated cells (media alone), respectively.

To determine whether knockdown of CXCR1 and/or CXCR2 induces apoptosis, cells were with medium alone or medium containing CXCL8 (10 ng/ml) for 24 h. Cells were stained for apoptosis using the CaspACE FITC-VAD-FMK *in situ* marker kit (Promega, Madison WI) and mounted with antifade Vectashield mounting medium (Vector Laboratories). The number of apoptotic cells was determined by counting immunostained cells using Nikon fluorescence microscope in ten independent high-power fields (200 $\times$) with each field containing 50–100 cells.

Endothelial cell migration and invasion assay

To investigate the effect of silencing CXCR1 and/or CXCR2 expression on endothelial cell migration, cells (1×10^6 cells/well) in serum free media were plated in the top chamber of noncoated polyethylene terephthalate membranes (six-well insert; 8 μ m pore size; Becton Dickinson, Franklin Lakes, NJ) in a transwell chambers. For invasion assay, cells (1×10^4 cells/wells) were plated onto Matrigel-coated transwell chambers (24-well insert; 8 μ m pore size; Corning Costar Corp., Cambridge, MA) in serum free media. The bottom chamber contained 1.0 ml serum free media with CXCL8 (10 ng/ml). The cells were incubated for 24 h at 37°C. Cells that did not pass through the membrane pores were removed using cotton swab. Migrated cells were stained using Hema 3 kit (Fisher Scientific Company L.L.C., Kalamazoo, MI) as per the manufacturer's instructions and migrated cells were counted in ten random fields (200 $\times$) and expressed as the average number of cells per field of view. The data is represented as the average of three independent experiments.

F-actin immunostaining

Cells were grown at low density (1×10^4 cells) in media containing 10 ug/ml of CXCL8 overnight on coverslips, fixed in ice cold 4% formaldehyde, permeabilized in 0.3% Triton X-100 and stained with Texas Red-phalloidin (Molecular Probes, Eugene, OR) for 30 min at room temperature. Cells were further washed with PBS-T (PBS containing 0.1% Tween 20) and mounted with antifade Vectashield mounting medium (Vector Laboratories). The stained cells were analyzed using a confocal microscope (UNMC core facility).

Western blot analysis

Transiently transfected HMEC-1 cells treated with 10 ug/ml of CXCL8 were processed for protein extraction and Western blotting using standard procedures. Briefly, the cells were washed twice with PBS and scraped in Triton X-100 buffer [1% Triton X-100, 50 mmol/L TBS (pH 7.4), 10 mmol/L EDTA with protease inhibitors (Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitors (5 mM NaF and 5 mM Na_3VO_4 ; Sigma Chemicals, St. Louis, MO)]. Cell lysates were passed through the needle syringe to facilitate the disruption of the cell membranes and were centrifuged at 14,000 rpm for 20 min at 4°C, and supernatant were collected. The proteins (50 µg) were resolved by electrophoresis on 8% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. Subsequently, the membranes were subjected to standard immunodetection procedure using specific antibodies: anti-GAPDH, anti-pERK1/2 and anti-ERK1/2 (1:1000, rabbit monoclonal, Cell Signaling Technology, Beverly, MA). Secondary horseradish peroxidase-conjugated antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used at 1:2000 dilutions. All the blots were processed with ECLPlus Western Blotting detection kit (GE Healthcare, Piscataway, NJ), and the signal was detected by a Typhoon 9410 Variable Mode Imager.

In vitro capillary-like structure formation assay

An *in vitro* CLS formation assay was performed as described earlier (23). Briefly, 2×10^4 HMEC-1 cells pretreated with MAPK kinase inhibitor (PD98059; 20 µM; Calbiochem, Gibbstown, NJ) or CXCR2/1 small molecule antagonists (SCH-527123; 10 ng/ml; Schering Plough, Kenilworth, NJ) (19) for 2h. Pretreated HMEC-1 cells or transiently knock-down cells (HMEC-1-control, HMEC-1-shCXCR1, HMEC-1-shCXCR2 and HMEC-1-shCXCR1/2) were plated in a Matrigel-coated 48-well plate. The growth media contained CXCL8 (10 ng/ml) and remained free of inhibitors. After 4 hr of incubation, the plate was examined for CLS formation. CLS were evaluated by counting the number of completely enclosed endothelial networks in each well. Three high-power fields were randomly selected and the total number of enclosed networks was evaluated (29;30). Each assay was done in duplicate and each experiment was repeated three times.

Statistical analysis

All values are expressed as mean $\pm$ SEM. Differences between the groups were compared using the unpaired two-tailed *t*-test in SPSS software (SPSS Inc., Chicago, Illinois). *In vivo* analysis was performed using Mann-Whitney *U*-test for significance. A *p* value of equal or less than 0.05 was considered statistically significant.

Results

Knock-down of CXCR1 and CXCR2 inhibits endothelial cell growth and modulates survival

To knock down CXCR1 and/or CXCR2 expression, plasmids containing shRNA sequences were transiently transfected into HMEC-1 cells (HMEC-1-shCXCR1, HMEC-1-shCXCR2 or HMEC-1-shCXCR1/2) along with HMEC-1-control. Expression of CXCR1 and/or CXCR2 was inhibited from 48-78% in HMEC-1-shCXCR1, HMEC-1-shCXCR2, and

HMEC-1-shCXCR1/2 respectively (Figure 1A). We confirmed CXCR1 and/or CXCR2 knock-down at protein level by immunocytochemical analyses (Figure 1B).

To specifically determine the role of CXCR1 and/or CXCR2 in growth, endothelial cell proliferation was examined by MTT assay. Serum starved, growth factor deprived HMEC-control, HMEC-shCXCR1, HMEC-shCXCR2 and HMEC-shCXCR1/2 cells were incubated with media alone or media containing CXCL8 (10 ug/ml) for 72 h. HMEC variants incubated with media alone had extremely poor viability and no significant difference was observed in cell viability between vector control cells and CXCR1/2 knock-down cells (Figure 2A). We observed significant increase in cell proliferation in HMEC-control cell treated with CXCL8 (Figure 1A). The number of viable cells was significantly reduced in HMEC-1-shCXCR1, HMEC-1-shCXCR2 and HMEC-1-shCXCR1/2 cells incubated with 10 ug/ml of CXCL8 as compared with HMEC-1-control cells (Figure 2A) (Table 2).

Next we examined the role of CXCR1 and CXCR2 in the regulation of CXCL8-mediated endothelial cell survival. Cells (1×10^6) were plated onto the coverslips in 6 well plates and allowed to adhere overnight. The cultures were washed and incubated in media containing CXCL8 (10ng/ml) for 24h. Apoptotic cells showing positive CaspACE-FITC stain in the cytoplasm were counted using fluorescence microscopy. The levels of apoptotic cells significantly increased in HMEC-1-shCXCR1, HMEC-1-shCXCR2 and HMEC-1-shCXCR1/2 cells compared with HMEC-1-control cells (Figure 2B).

To determine the effect of knock-down of CXCR1 and/or CXCR2 expression on survival-associated gene, total RNA was isolated from transiently transfected cells treated with CXCL8 (10 ug/ml) and Bcl-2, Bcl-x and Bax mRNA expression was examined by semi-quantitative RT-PCR (Figure 2C). The expression index is shown under specific bands to demonstrate the quantitative differences in the expression levels. In HMEC-1-shCXCR1, HMEC-1-shCXCR2 and HMEC-1-shCXCR1/2 cells, we observed decreased mRNA levels for anti-apoptotic genes Bcl-2 and increased mRNA levels for pro-apoptotic genes Bax compared with control cells (Figure 2C and D). In addition, the ratio of Bax to Bcl-2 increased in HMEC-1-shCXCR1 HMEC-1-shCXCR2 and HMEC-1-shCXCR1/2 cells favoring apoptosis (Figure 2D). These data suggest that CXCR1 and CXCR2 are important in regulating CXCL8-mediated endothelial cell survival and apoptosis.

Silencing of CXCR1 and CXCR2 inhibited HMEC-1 migration, invasion and CLS formation by reducing stress fibers

Cells were placed on the upper chamber of non coated or Matrigel-coated transwells. After 24h cultures, migrated and invaded cells were determined as average number of cells per field of view. The average number of migrated cells in HMEC-1-shCXCR1, HMEC-1-shCXCR2 and HMEC-1-shCXCR1/2 were significantly reduced to 2.4–2.6 fold ($p < 0.05$) (Figure 3A), similarly number of invading cells were also reduced to 2.3–2.6 fold ($p < 0.05$) (Figure 3B). These data suggest that CXCR1 and CXCR2 may play critical roles in endothelial cell migration and invasion.

Cytoskeletal reorganization is prerequisite for migration. Having shown that transiently-transfected cells have decreased migration, we next assessed F-actin polymerization of cells with phalloidin. Cells transiently knocked down for CXCR1 and/or CXCR2 show reduced stress fiber compared with prominent stress formation in control cells, following stimulation with CXCL8 (Figure 4A). It clearly shows different F-actin distribution pattern in CXCR1 and/or CXCR2 knock-down cells treated with CXCL8 as compared to vector control transfected cells.

Next, we determined the role of CXCR1 and/or CXCR2 in angiogenesis by examining the CLS formation of knockdown cells into capillary tube structures. Matrigel-coated wells were plated with 2×10^4 HMEC-1-transfected cells cultured with medium containing CXCL8 (10 ug/ml). After 4h of incubation, plates were examined for CLS formation under an inverted microscope. Control cells formed capillary structures, whereas diminished CLS formation in HMEC-1-shCXCR1, HMEC-1-shCXCR2 and HMEC-1-shCXCR1/2 cells was observed (Figure 4B and C).

Knock-down of CXCR1 and/or CXCR2 inhibits CXCL8-mediated ERK1/2 phosphorylation

We next, assessed the ability of CXCL8 to induce MAPK phosphorylation in CXCR1 and CXCR2-expressing endothelial cells by immunoblotting analysis. CXCL8 (10ng/ml) leads to a marked phosphorylation of ERK1/2 with in 30 min of stimulation in HMEC-1-control cells (Figure 5A). Knock-down of CXCR1 and/or CXCR2 significantly inhibited CXCL8-induced ERK1/2 phosphorylation (Figure 5B). Furthermore, inhibition of ERK1/2 phosphorylation using an inhibitor abrogated CXCL8-mediated endothelial cell migration, demonstrating important role of ERK1/2 phosphorylation in CXCL8-mediated CXCR1 and/or CXCR2-dependent endothelial cell migration (Figure 5C).

In addition, we examined the effect of MAPK kinase inhibitor and CXCR2/1 small molecule antagonists on CLS formation. MAPK kinase and CXCR2/1 antagonists significantly reduced the CLS formation in HMEC-1 cells compared with control treated cells (Figure 5D). All together, our data suggest an important role of CXCR1 and CXCR2 in endothelial cell functions.

Discussion

In the present study, we have demonstrated that silencing of CXCR1 or CXCR2 modulated endothelial cellular growth, migration, survival and neovascularization and ERK1/2 phosphorylation. ELR+ CXC chemokines including CXCL8 have been known to have angiogenic properties (22;31). It has also been shown that endothelial cells are major source of CXCL8 which is significantly enhanced during inflammation, infection, stress and tumor formation (32–35). Our previous study has suggested that endothelial cells constitutively express CXCR1 and CXCR2 (6). However the functional role of CXCR1 and CXCR2 in angiogenesis is unclear.

Autocrine and paracrine functions of CXCL8 have been shown to play an important role in angiogenesis, tumor growth and metastasis (22;36;37). CXCL8 stimulate endothelial cell proliferation and capillary tube organization which can be blocked by neutralizing anti-CXCL8 antibodies. Earlier reports have also demonstrated the important role of CXCR2 but not CXCR1 in mediating ELR+ CXC chemokine dependent angiogenesis (38–40). The importance of CXCR2 in mediating tumor angiogenesis was also substantiated by our *in vivo* studies using CXCR2 knock-out mice (24). Similarly, tumor in CXCR2^{-/-} mice demonstrated reduced growth, increased tumor necrosis, decreased tumor angiogenesis and metastasis in lung cancer (39). In the present study, silencing of either CXCR1 or CXCR2 on endothelial cells alters CLS formation. Interestingly the use of small molecule antagonist for CXCR2/1 (39) also diminishes capillary tube formation. These results are in agreement with our recent report where use of this inhibitor in melanoma mouse model inhibited vessel density in tumors (19). In addition, we have shown host CXCR2-dependent CXCL8-mediated angiogenesis in regulation of melanoma growth and metastasis. (24).

CXCR1 and CXCR2 activation and function are known to be involved in Rho, Rac (41) and Mitogen-activated Protein (MAP) kinase signaling pathways (42) which are linked to cell growth (43) and migration (44–46). In addition, ERK1/2 phosphorylation has been shown to

upregulate Bcl-2 and enhanced survival of human macrophages (28). Endothelial cell survival and programmed cell death are critical for maintenance of vascular structure in angiogenesis (47). Differential expression of Bcl-2 family members, which include anti-apoptotic (Bcl-2 and Bcl-x_L) and pro-apoptotic (Bax and Bcl-x_S) proteins, regulates apoptosis (48;49). The Bcl-2 and Bcl-x_L proteins interact with Bax to suppress apoptosis, while Bcl-x_S promotes cell death by inhibiting Bcl-2 and Bcl-x_L (48) and constitutive expression of bcl-x has been shown in HUVEC (50). In the present study, we observed significantly increased apoptotic cells and decreased anti-apoptotic gene expression by silencing CXCR1 and/or CXCR2. These results further support our previous observation where neutralization of CXCL8, CXCR1 or CXCR2 resulted in imbalance between anti-apoptotic and pro-apoptotic genes (23). Together, these data suggest that CXCR1 and CXCR2 regulate angiogenesis by modulating endothelial cell anti-apoptotic pathway.

Endothelial cell proliferation and migration are important steps towards angiogenesis. Several reports have demonstrated that CXCL8 modulates proliferation of endothelial cells (6;22;51–53). In the present study we demonstrated that functional blockade of CXCR1 and/or CXCR2 on endothelial cells decreases cell proliferation suggesting that CXCL8 may function as an autocrine/paracrine growth factor through CXCR1 and CXCR2. Our results support our previous observation that CXCL8 stimulates proliferation of endothelial cells, which can be modulated by neutralizing anti-CXCL8 antibodies (6).

Cell motility and invasiveness are associated with actin filament organization which are organized on the lamellipodia (54). We have observed decreased migration and invasion of CXCR1 and/or CXCR2 knock-down endothelial cells in response to CXCL8. We next chose to focus on the effect of down-regulation of CXCR1 and/or CXCR2 on actin cytoskeleton organization. Staining of CXCL8 treated HMEC-1-shCXCR1, HMEC-1-shCXCR2 and HMEC-1-shCXCR1/2 cells showed the presence of fewer lamellipodial structures as compared to control cells. This suggests that down-regulation of CXCR1 and/or CXCR2 reduces CXCL8 induced actin reorganization, thereby affecting the motility and, in turn, the invasiveness of endothelial cells. These behaviors are not restricted to endothelial cells, but also applied to cells that express these receptors constitutively. A375SM- human melanoma cells, express both CXCR1 and CXCR2 (20). Our recent report showed that silencing of either of the two receptors causes a decrease in F-actin content and distribution pattern, and thereby affecting the motility and invasion of melanoma cells (55). All together, these results suggest a role of CXCR1 and CXCR2 in endothelial cell signaling.

ERK1/2 phosphorylation is an important signaling pathway involved in the control of growth signals, cell survival and invasion (56). Our recent observation along with other studies supports the idea that stimulation of CXCR1 and CXCR2 leads to activation of MAP kinase (25;57).

In conclusion, our data provide direct evidence for the role of CXCR1 and CXCR2 on endothelial cells proliferation, survival, migration and angiogenesis. Therefore the development of strategies to block CXCR1 and CXCR2 may hold promise in limiting the deleterious effects of CXCR1- and CXCR2-mediated pathways in various diseases, including malignant tumors.

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Figure 1. CXCR1 and/or CXCR2 knock-down in endothelial (HMEC-1) cells

Cells were stably transfected with HMEC-1-control, HMEC-1-shCXCR1, HMEC-1-shCXCR2 or HMEC-1-shCXCR1/2 plasmid construct containing scrambled, CXCR1, CXCR2 or CXCR1/2 shRNA. (A), RT-PCR shows decreased expression of CXCR1 or CXCR2 mRNA in pooled sublines. GAPDH was used as a loading control. (B), immunocytochemistry shows decreased expression of CXCR1 or CXCR2 in HMEC-1-shCXCR1, HMEC-1-shCXCR2 and HMEC-1-shCXCR1/2 cells. Photomicrographs are representative of at least three experiments.

Figure 2. Knock-down of CXCR1 and CXCR2 inhibits endothelial cell growth and modulates endothelial cell expression of apoptosis-associated genes

Endothelial cells (HMEC-1-control, HMEC-1-shCXCR1, HMEC-1-shCXCR2 or HMEC-1-shCXCR1/2) were used for *in vitro* cellular proliferation and apoptosis assay. (A), cellular proliferation was determined at 72h by MTT assay. The values are mean absorbance $\pm$ SEM of cells incubated in medium alone or medium containing CXCL8 (10 ug/ml); *significantly different from HMEC-control cells. (B), the frequency of CaspACE-positive cells was determined by counting in ten fields (200 $\times$) for each treatment. The values are expressed as average number of cells per field view. (C&D), total RNA from endothelial cells were analyzed by RT-PCR. To facilitate the comparison between control and knockdown cells, expression levels of apoptosis-associated genes and mRNA production was normalized to controls. The mRNA expression index was calculated as the ratio of the optical densities for specific mRNA transcripts and the housekeeping gene, GAPDH, prior to normalization to controls. The values shown are fold increase/decrease in protein or mRNA levels compared to controls. This is representative of three experiments with similar results. *Significantly different from controls ($p < 0.05$).

Figure 3. Down-regulation of CXCR1 and/or CXCR2 reduces cell motility and invasion
Cells were seeded on non-coated or Matrigel-coated membranes for motility (**A**) and invasion (**B**), assays overnight. Migrated cells were stained and photographed at 200 $\times$ magnification. Migrated (**A**, lower panel) and invaded (**B**, lower panel) cells were counted in ten random fields (200 $\times$) and expressed as the average number of cells per field of view $\pm$ SEM. This is a representative of three experiments done in triplicate. *Significantly different from HMEC-1-control cells ($p < 0.05$).

Figure 4. Effect of CXCR1 and/or CXCR2 down-regulation on actin reorganization and capillary tube organization

(A), Cells were grown on coverslips and stained with Texas Red phalloidin. Decreased lamellipodial structures were observed in HMEC-1-shCXCR1, HMEC-1-shCXCR2 and HMEC-1-shCXCR1/2 cells compared with the HMEC-1-control cells. (B); For capillary tube formation, cells were plated on Matrigel-coated 48-well plates and incubated with medium containing CXCL8 for 4 h. CLS formation was examined by inverted microscope and photographed. C. Knock-down of CXCR1 and CXCR2 significantly diminished ($*p<0.05$) CXCL8-induced CLS formation in HMEC cells. This is a representative of three experiments with similar results.

Figure 5. Knock-down of CXCR1 and/or CXCR2 inhibits CXCL8-mediated ERK1/2 phosphorylation, cell migration, and CLS formation

(A) CXCL-8 enhanced phosphorylation of ERK1.2 as determined by Western blot analysis.

(B) To determine ERK1/2 phosphorylation, cell lysates (50ug) were fractionated by SDS-PAGE and subjected to Western blotting using pERK1/2, ERK1/2 and GAPDH antibody.

(C), Cells were seeded on non-coated or Matrigel-coated membranes for migration in presence and absence of CXCL8 and MAPK inhibitor. Migrated cells were counted in ten random fields (200 $\times$) and expressed as the average number of cells per field of view $\pm$ SEM.

(D), Capillary tube formation was disrupted by both MAPK and CXCR2/1 inhibitor as compared to control cells. *Significantly different from control cells ($p < 0.05$).

Table 1

Primers used for RT-PCR analysis.

Gene	Orientation	Sequence	Product size (bp)
CXCR1	Sense	5'-TGG GAA ATG ACA CAG CAA AA-3'	115
	Antisense	5'-AGT GTA CGC AGG GTG AAT CC-3'	
CXCR2	Sense	5'-ACT TTT CCG AAG GAC CGT CT-3'	92
	Antisense	5'-GTA ACA GCA TCC GCC AGT TT-3'	
Bcl-x	Sense	5'-GACGAGTTTGAAGTGCAGTA-3'	Bcl-XL 378, Bcl-Xs 190
	Antisense	5'-CACAGTCATGCCCGTCAG-3'	
Bcl-2	Sense	5'-TCCATGTCTTTGGACAACCA-3'	203 bp
	Antisense	5'-CTCCACCAGTGTCCCATCT-3'	
Bax	Sense	5'-TCTGACGGCAACTTCAACTG-3'	188 bp
	Antisense	5'-TTGAGGAGTCTACCCAACC-3'	
GAPDH	Sense	5'-TGAAGGTCGGAGTCAACGGATTTGGT-3'	983
	Antisense	5'-CATGTGGGCCATGAGGTCCACCAC-3'	

Table 2

Inhibition of CXCL8 induced CXCR1 and/or CXCR2 knock-down endothelial cell proliferation.

Cells	% cell proliferation	
	No serum	1.25% serum
HMEC-control	26.23 ± 0.21	43.15 ± 0.84
HMEC-shCXCR1	16.00 ± 0.21 *	24.29 ± 0.82 *
HMEC-shCXCR2	12.00 ± 0.45 *	22.50 ± 0.59 *
HMEC-shCXCR1/2	11.00 ± 0.50 *	20.30 ± 0.60 *

Endothelial cell proliferation was determined at 72h by MTT assay. The values are mean percent increase in cell proliferation ± SEM determined by CXCL8-induced cell proliferation (%) = $[(A / B) - 1] \times 100$, where A and B are the absorbance of treated (CXCL8 stimulated) and untreated cells (media alone), respectively.

* Significantly different from HMEC-control cells.