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Endothelial Cells Differentially Express Functional CXC-Chemokine Receptor-4 (CXCR-4/Fusin) under the Control of Autocrine Activity and Exogenous Cytokines

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Analysis of endothelial cell (EC) chemokine receptor expression by RT-PCR revealed that EC essentially do not express CC-chemokine receptors whereas they express all known CXC-chemokine receptors. Endotheliotropic functions of ligands for CXCR-1, CXCR-2, and CXCR-3 have previously been described. We have consequently performed a detailed analysis of endothelial CXCR-4 expression. CXCR-4 is constitutively expressed by quiescent, resting EC. Cytokine stimulation revealed that bFGF upregulates endothelial CXCR-4 expression, whereas TNFα downregulates endothelial CXCR-4 expression. Expression of CXCR-4 mRNA as well as protein is also upregulated in autocrine activated, migrating bovine aortic endothelial cells (BAEC). Furthermore, migrating BAEC preferentially present CXCR-4 on the cell surface as evidenced by cytochemistry and FACS analysis. Lastly, the monospecific CXCR-4 ligand SDF-1 was found to act as a potent inducer of EC chemotaxis. In summary, the data indicate that the CXCR-4/SDF-1 receptor ligand interaction may be an important regulator of activated endothelial cell functions as they occur during vascular remodeling and angiogenesis.

Key Words: **endothelial cell; chemokine; CXCR-4; SDF-1; fusin.**

Chemokines are a family of polypeptides (8-10 kDa) that act as potent chemoattractants (1,2). They are structurally grouped into two subfamilies, the α- or CXC-subfamily and the β- or CC-subfamily, based on the characteristic presence of four conserved cysteine residues. Additionally, lymphotactin has been identified as a C-chemokine that lacks two of the four conserved cysteine residues (3). Likewise, fractalkine has been identified as a member of a novel subfamily of CX_3C-chemokines (4). Chemokines are produced by a large number of different cell types and have distinct, but overlapping target cell specificities. Members of the α-chemokine subfamily act predominately on neutrophils, whereas β-chemokines attract monocytes, eosinophils, and basophils. Members of both subfamilies together with lymphotactin attract specific lymphocyte subpopulations.

Hematopoietic cells are the primary target of chemokines. Over the last few years, however, several CXC-chemokines have also been shown to either act as stimulators or as inhibitors of angiogenesis, supposedly by directly acting on endothelial cells (5-10). Interleukin-8 (IL-8) induces EC proliferation and chemotaxis *in vitro* and angiogenesis *in vivo* and appears to be a primary mediator of macrophage induced angiogenesis (6). In contrast, platelet factor-4 (PF-4) was reported to inhibit EC proliferation *in vitro* (7) and angiogenesis *in vivo* (8). Antiangiogenic activities have also been described for the CXC-chemokines interferon-γ-inducible protein (IP-10) (9) and growth-related oncogene β (GRO-β) (10). In a very elegant study, Strieter et al. showed that the pro- and antiangiogenic activities of CXC-chemokines are dependent on an amino terminal ELR motif which is expressed by the stimulators and not by the inhibitors (11).

Chemokines exert their effector functions through chemokine receptors which are seven-transmembrane domain, GTP-binding protein-coupled cell surface receptors (12). To date, 8 CC-chemokine receptors and 5 CXC-chemokine receptors have been identified. In view of the well studied effects of CXC-chemokines on angiogenesis, surprisingly little is known about the expression of chemokine receptors on endothelial cells. Recent experiments aimed at identifying novel chemokine receptors through a degenerate RT-PCR approach have identified several chemokine receptors in endothelial

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cells, including the HIV-coreceptor CXCR-4/fusin (13,14). Endothelial cells have also been reported to express the promiscuously chemokine binding, but not signal transducing Duffy antigen (DARC) (15). A number of studies have described the binding of chemokines, such as IL-8 and RANTES, by EC without actually showing expression of the corresponding receptor (16,17). Binding sites for the angioinhibitory IP-10 molecule on EC have been characterized as heparan sulfate proteoglycans (18). Corresponding to these findings, immobilization of chemokines by EC surface proteoglycans has been reported to facilitate the adhesion and extravasation of circulating hematopoietic cells (19). This type of EC chemokine binding is, thus, indicative of the presentation of chemokines to attract hematopoietic cells. It does, however, not correspond to the reported angiogenesis regulating functions of CXC-chemokines which would require binding and activation of signal transducing cell surface receptors.

In order to further clarify the role of chemokines on the regulation of endothelial cell functions during angiogenesis, we decided to systematically analyze the EC expression of chemokine receptors. These experiments revealed that EC do not express CC-chemokine receptors, whereas they essentially express all CXC-chemokine receptors. As the most abundantly expressed chemokine receptor, we identified the CXC-chemokine receptor-4 (fusin). We consequently hypothesized that endothelial CXCR-4 expression and activation through its ligand SDF-1 may play an important role in regulating EC functions and decided to study CXCR-4 expression and endotheliotropic functions of SDF-1 in detail.

MATERIALS AND METHODS

Cytokines, antibodies, and reagents. rhSDF-1α (aa 22-89), rhSDF-1β (aa 22-89), and rhMCP-1 were obtained from R&D Systems (Minneapolis, MN). rhbFGF and rhTNFα were from Promega (Madison, WI). Neutralizing monoclonal mouse anti-bovine bFGF antibody was purchased from Upstate Biotechnology (Lake Placid, NY) and polyclonal rabbit anti-human CXCR-4 antibody was from Calbiochem (San Diego, CA). Endothelial cell growth medium (ECGM) and endothelial cell growth supplement (ECGS/H) were obtained from PromoCell (Heidelberg, Germany). Dulbecco's modified Eagle's medium (DMEM) and RPMI1640 were from Gibco BRL (Eggenstein, Germany). Fetal bovine serum (FBS) was from Biochrom (Berlin, Germany).

Cell culture and chemotaxis experiments. Human umbilical vein endothelial (HUVE) cells and bovine aortic endothelial (BAE) cells were isolated by collagenase digestion from human umbilical veins and bovine thoracic aortas, respectively. Human dermal microvascular endothelial (HDMVE) cells were obtained from Clonetics (Walkersville, MD). Control U937 cells were from ATCC. Cells were cultured at 37°C in ECGM containing 0.4% ECGS/H (HUVEC, HDMVEC) or DMEM containing 10% heat-inactivated FBS (BAEC). Endothelial cells were used up to passage 4 to 6 (HUVE, HDMVE) or passages 20 to 25 (BAE). Subconfluent migrating cells were harvested at approximately 50% confluence 48 h after seeding. Quiescent, postconfluent BAE cells were refed once and harvested three days after growing to confluence.

In vitro chemotaxis was assessed in 48-well chambers (NeuroProbe, Cabin John, MD) using polyvinylpyrrolidone-free polycarbonate membranes with 12-μm pores (Costar, Cambridge, MA). Membranes were coated overnight in 1% ECM in PBS (Sigma-Aldrich, Deisenhofen, Germany). Chemokines were dissolved in medium (RPMI1640 containing 0.5% FBS and 0.1% BSA) and loaded into the lower compartment of the chemotaxis chamber. The membrane was inserted and 50 μl with HUVE cells, suspended in the same medium (5×10^5cells/ml), were seeded into the upper compartment. Chambers were incubated for 4 h at 37°C in 5% CO_2. Following incubation, the cells on the upper side were removed with a cell scraper and the membrane was fixed and stained with hematoxyline. Migration of cells across the membrane was quantitated by counting the cells in at least five high power fields per well (400×).

RNA isolation and Northern blot analysis. Cells were washed twice with PBS and harvested with a cell scraper. RNA was isolated according to the single step guanidium thiocyanate-phenol-chloroform extraction procedure using Trizol (Gibco BRL, Eggenstein, Germany). For Northern blot analysis, 10 μg (BAE) or 20 μg (HUVE) of total RNA was electrophoresed through a 1% agarose gel, capillary transferred onto nylon membranes (HybondN, Amersham, Germany), and used for hybridization with the corresponding random prime labeled cDNA (DNA labeling kit, Pharmacia, Uppsala, Sweden). Hybridization with an 18S rRNA oligonucleotide was performed to confirm equal loading of the different lanes (20). All Northern blot experiments have at least been performed 3 times with similar results.

RT-PCR analysis. Poly(A)$^+$ RNA was extracted from 10 μg of total RNA using the Dynabead mRNA purification kit (Dynal, Hamburg, Germany), and cDNA was synthesized with cDNA synthesis kit (Boehringer Mannheim, Mannheim, Germany) using random hexamer primers. For the PCR-reaction, 0.5 μl of the cDNA was mixed with specific primers (20 pmol each), 2 U of *Taq* DNA polymerase (Gibco BRL, Eggenstein, Germany), 5 μl of 10× reaction buffer, 200 μM dNTPmix in a final volume of 50 μl. The samples were sealed with mineral oil and the PCR reaction was carried out in a Perkin-Elmer thermocycler (Norwalk, CT) as follows: First 5 min at 94°C for denaturation, followed by 1 min at 94°C, 1 min at 55°C, 1 min at 72°C for 40 cycles. The last cycle was terminated with 7 min at 72°C. The following primer pairs were used to amplify CC- and CXC-chemokine receptor transcripts in EC:

CCR-1 (forward [fw]: 5′-AGAAGGTGAACGAGAGG-3′;
reverse [rev]: 5′-AGCCTGAAACAGCTTCC-3′)

CCR-2a (fw: 5′-CATTCTCCTGAACACCTTCC-3′,
rev: 5′-GTCTCTCTCCTCTCAGCCTT-3′)

CCR-2b (fw: 5′-AGAAGGTGAACGAGAGG-3′,
rev: 5′-GTGCCTGTCACATTCTCTTC-3′)

CCR-3 (fw: 5′-CTGATACCAGAGCACTG-3′,
rev: 5′-AGTGTGGAAATGCCTCC-3′)

CCR-4 (fw: 5′-CGGATATAGCAGATACCACC-3′,
rev: 5′-ACCGCCTTGTTCTTCTTCTC-3′)

CCR-5 (fw: 5′-CCAGAAGAGCTGAGACATCC-3′,
rev: 5′-CAGGTGTAATGAAGACC-3′)

CCR-6 (fw: 5′-GATGTTACTGTGCTCCTTGC-3′,
rev: 5′-CCAACATCAGCAGCTTCCAC-3′)

CXCR-1 (fw: 5′-TGATCTCTGACTGCAGCTCC-3′,
rev: 5′-GTAACGGTCCACACTGATGC-3′)
CXCR-2 (fw: 5′-CTGGAGGTGTCCTACAGGTG-3′,
rev: 5′-GTAACGGTCCACACTGATGC-3′)
CXCR-3 (fw: 5′-CCACTGCCAATACAACTTCC-3′,
rev: 5′-GCAAGAGCAGCATCCACATC-3′)
CXCR-4 (fw: 5′-GTTACCATGGAGGGGATCAG-3′,
rev: 5′-TCCTTGGCCTCTGACTGTTG-3′)
DARC (fw: 5′-CTTCATCCTCACCAGTGTCC-3′,
rev: 5′-AATCCAGTCCTAGAACCACC-3′).

All amplification products were also cloned, sequenced, confirmed for identity and used as cDNA probes for Northern blot experiments.

Immunoprecipitation and Western blot analysis. Cells were washed twice with PBS, harvested with a cell scraper, and lysed in 1% NP40 containing lysis buffer. Nuclei were removed by centrifugation. Protein samples (400 μg) were immunoprecipitated via incubation with anti-CXCR-4 antiserum (1:100) overnight at 4°C and with protein G-agarose (Sigma-Aldrich, Deisenhofen, Germany) for an additional 1 h. The beads were then washed three times in lysis buffer, resuspended in Laemmli buffer, and subjected to SDS-polyacrylamide gel electrophoresis (12.5%). PAGE-separated proteins of immunoprecipitates and total cell lysates were transferred to cellulose nitrate membranes (Schleicher und Schüll, Dassel, Germany), blocked overnight with 2% BSA in PBS, and probed with anti-CXCR-4 antiserum (1:1000). Bound antibody was visualized by incubation with a HRP-labeled anti-rabbit Ig second antibody (1:2000; Dako, Hamburg, Germany), which was detected by chemiluminescent detection (ECL Detection System, Amersham, Braunschweig, Germany).

Flow cytometric analysis. For FACS analysis, endothelial cells were harvested by trypsinization, fixed for 10 min in 1% paraformaldehyde, washed, blocked for 30 min with 3% BSA in PBS, and incubated with anti-CXCR-4 antiserum (1:50) for 2 h at 4°C. After washing, cells were labeled with FITC-conjugated anti-rabbit IgG antibody (Dako, Hamburg, Germany), and CXCR-4 expression was analyzed with a FACScan cytofluorometer (Becton-Dickinson, Mountain View, CA).

Immunocytochemistry. A two dimensional lateral sheet migration assay was used to study differential CXCR-4 expression of migrating and resting EC by imunocytochemistry (21). Cells were released from growth arrest and allowed to migrate for 24 h after which they were fixed with 3.7% formaldehyde in PBS. Fixed cells were blocked with 2% BSA in PBS and used for cytochemical detection of CXCR-4 expression applying the same antibodies as used for Western blotting experiments. Binding of the secondary peroxidase labeled antibody was visualized using DAB as substrate.

RESULTS

RT-PCR analysis of endothelial chemokine receptor expression. To systematically investigate chemokine receptor expression of endothelial cells, we performed a broad RT-PCR analysis of confluent and subconfluent human umbilical vein (HUVEC) and human dermal microvascular endothelial cells (HDMVEC) (Table 1). Using a panel of monospecific primer pairs, no transcripts for CC-chemokine receptors could be identified in both EC populations with the standard PCR conditions applied in this study (40 cycles). This was in contrast to the prominent amplification products generated for all CC-chemokine receptors when using cDNA from either buffy coat or monocytic U937 cells as template which served as positive controls. The screening character of these experiments does not fully rule out endothelial cell CC-chemokine receptor expression (e.g., variable results obtained for CCR-5; see Table 1). It does, however, correspond to the fact that no endotheliotropic effector functions of CC-chemokines have been described so far, despite the fact that endothelial cells are an important cellular source for CC-chemokines (22,23).

Variable RT-PCR results were obtained when analyzing the expression of the interleukin- 8 receptors CXCR-1 and CXCR-2. Transcripts for these receptors were weakly detectable in both EC populations with some differences between confluent and subconfluent cells. Interestingly, amplification products for both receptors were prominently detectable in confluent and subconfluent HUVEC and HDMVEC when the cells were stimulated with TNFα (data not shown). Transcripts for CXCR-3 were found in all analyzed EC populations (Table 1). However, TNFα was found to downregulate endothelial cell CXCR-3 expression (data not shown). Lastly, both migrating and resting HUVEC and HDMVEC expressed transcripts for the CXC-chemokine receptor-4. In fact, a comparative semiquantitative RT-PCR identified CXCR-4 as the most abundantly expressed EC chemokine receptor, being still detectable at 28 cycles which was not sufficient to generate a PCR amplification product of any of the other analyzed chemokine receptors.

Transcriptional regulation of CXCR-4 expression in endothelial cells. The abundance of endothelial cell CXCR-4 expression as detected by RT-PCR prompted us to analyze endothelial cell CXCR-4 expression in more detail. Northern blot experiments of HUVEC identified CXCR-4 as a differentially expressed EC gene with higher steady state levels of mRNA in quiescent, resting HUVEC compared to subconfluent, migrating HUVEC (Fig. 1A). Stimulation of HUVE cells with TNFα for 4 h induced a downregulation of CXCR-4 expression in both, subconfluent and confluent HUVE cells (Fig. 1A).

Following the identification of the differential expression of CXCR-4 in subconfluent and confluent HUVEC, we next decided to study differential CXCR-4 expression in other EC populations. Bovine aortic endothelial cells (BAEC) have a high degree of autocrine activity and have been used extensively by us and

TABLE 1

RT-PCR Analysis of Endothelial Cell Chemokine Receptor Expression

Receptor	Buffy coat	U937	HUVEC		HDMVEC	
			Confl.	Sub.	Confl.	Sub.
CCR-1	++	++	−	−	−	−
CCR-2	++	++	−	−	−	−
CCR-3	++	++	−	−	−	−
CCR-4	++	++	−	−	−	−
CCR-5	++	++	+	−	−	−
CCR-6	++	++	−	−	n.d.	n.d.
CXCR-1	++	++	−	+	+	+
CXCR-2	++	++	+	+	−	+
CXCR-3	++	++	++	++	++	++
CXCR-4	++	++	++	++	++	++
DARC	++	−	++	++	++	++

Note. confl., confluent; sub., subconfluent; DARC, Duffy antigen; HUVEC, human umbilical vein endothelial cells; HDMVEC, human dermal microvascular endothelial cells; n.d., not done; − = expression not detectable, + = variable and/or weak expression, ++ = prominent expression.

other laboratories to study the contribution of autocrine EC activation to the induction of angiogenesis (24-26). BAE cells express prominent steady state levels of CXCR-4 mRNA (Fig. 1B). In contrast to the findings obtained with HUVEC, however, CXCR-4 expression was higher in subconfluent than in confluent cells (Fig. 1B). Autocrine activity of subconfluent BAEC is to a large degree determined by the endogenous expression of bFGF (20,23,26,27). Correspondingly, exogenous stimulation with bFGF strongly upregulated CXCR-4 expression in BAEC, whereas addition of neutralizing anti-bFGF antibodies resulted in a moderate downregulation of CXCR-4 expression (Fig. 1B).

Expression of CXCR-4 protein in endothelial cells. To determine if the differential transcriptional regulation of endothelial cell CXCR-4 expression as determined by Northern blot experiments also reflected different levels of CXCR-4 protein, we next performed immunoprecipitation and Western blotting experiments. Western blots of total cellular lysates readily detected a single protein band in HUVEC and BAEC corresponding to the molecular weight of CXCR-4 (46 kDa) (Fig. 2). In contrast to the Northern blot experiments,

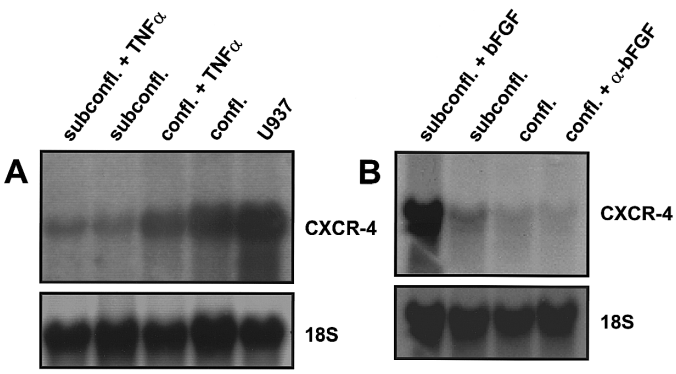


FIG. 1. Northern blot analysis of CXCR-4 expression in HUVEC (A) and BAEC (B). Cells were harvested as confluent and subconfluent cells as described in Materials and Methods. HUVE cells were stimulated with 0.5 ng/ml TNFα for 4 h. Subconfluent BAE cells were stimulated 10 ng/ml bFGF for 4 h and confluent BAE cells were exposed to 1 μg/ml neutralizing anti-bFGF-antibodies for 6 h. Monocytic U937 cells served as positive control.

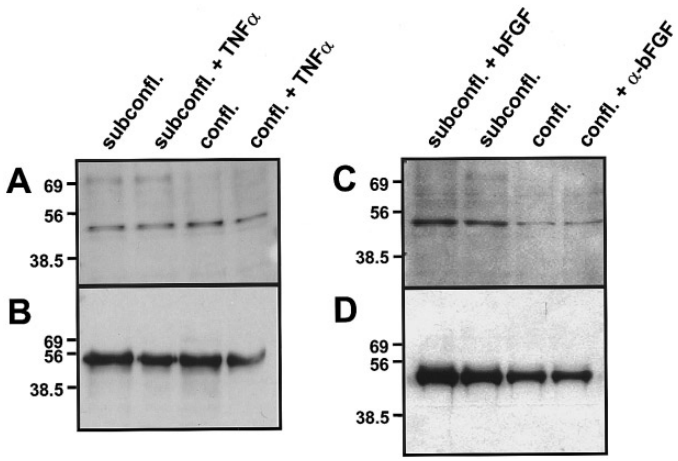


FIG. 2. Analysis of endothelial cell CXCR-4 protein expression by HUVEC (A,B) and BAEC (C,D). Cells were harvested as confluent and subconfluent cells as described in Materials and Methods. HUVE cells were stimulated with 0.5 ng/ml TNFα for 4 h. Subconfluent BAE cells were stimulated 10 ng/ml bFGF for 4 h and confluent BAE cells were exposed to 1 μg/ml neutralizing anti-bFGF-antibodies for 6 h. CXCR-4 protein was either detected directly by Western blots from whole cell lysates (5 μg protein per lane) (A,C) or following immunoprecipitation (400 μg protein of total cell lysate used per IP) and subsequent Western blot (10% of total immunoprecipitate loaded per lane) (B,D).

immunoprecipitation of CXCR-4 from lysates of confluent and subconfluent HUVEC identified similar levels of CXCR-4 protein in both cell populations. The downregulation of HUVEC CXCR-4 expression by TNFα, as determined by Northern blot experiments (Fig. 2A), however, was also detectable on the protein level, identifying lower levels of CXCR-4 protein in the immunoprecipitates of TNFα stimulated HUVEC (both subconfluent and confluent; Fig. 2A).

Western blotting and immunoprecipitation experiments with BAE cells confirmed the differential expression of CXCR-4 in this cell population as determined by Northern blot analysis (Fig. 2B): Both, Western blots of total cell lysates as well as immunoprecipitation experiments identified higher levels of CXCR-4 protein in subconfluent, migrating compared to confluent, resting BAE monolayers. Exogenous addition of bFGF to subconfluent BAEC enhanced CXCR-4 expression, whereas addition of neutralizing anti-bFGF antibody to confluent BAE cells downregulated CXCR-4 protein levels.

Cell surface expression of CXCR-4. Using a lateral sheet migration assay (21), we next studied the subcellular localization of CXCR-4 protein in BAE cells. In this assay, cells are grown to confluence in the confines of a rectangular silicon template. Upon reaching confluence, the silicon template is removed and the cells are allowed to laterally migrate into the space that was previously occupied by the silicon template (Fig. 3A). This approach allows the controlled release of cells from growth arrest without wounding the cells at the migration front and supplies populations of migrating and resting cells that can be studied simultaneously, e.g., for cytochemical applications (28). Comparative cytochemical detection of CXCR-4 expression in autocrine-activated, migrating and quiescent, resting BAE cells identified distinctly different expression patterns of CXCR-4 in these two cell populations (Fig. 3A-C): Resting BAEC exhibited a patchy perinuclear staining, indicative of an accumulation of CXCR-4 protein in intracellular storage granules (Fig. 3C). In contrast, migrating BAE cells showed an intense diffuse cell surface staining in addition to the perinuclear staining of intracellular granules, suggesting that the activation associated with the migratory EC phenotype leads to the cell surface expression of intracellularly stored CXCR-4 (Fig. 3C). The differential cell surface expression of CXCR-4 protein by migrating and resting BAE cells as determined by cytochemistry could also be shown by comparative flow cytometric analysis of populations of confluent and subconfluent BAE cells, which demonstrated an upregulated surface expression of CXCR-4 of subconfluent BAE cells (Fig. 3D).

Induction of endothelial cell chemotaxis by SDF-1. In order to determine if the endothelial cell CXCR-4 expression data correspond to functional activity of endothelial cells, we next examined, if SDF-1, which has, hitherto, been identified as the only CXCR-4 ligand (29,30), is able to induce EC chemotaxis. We used HUVE cells for these experiments 1.) because they expressed similar intensities of CXCR-4 protein in the confluent as well as in the subconfluent state and 2.) because recombinant bovine SDF-1 for the study of BAEC chemotaxis is not yet available. In these experiments, both SDF-1α and SDF-1β dose-dependently induced HUVEC chemotaxis, with SDF-1α being slightly more potent than SDF-1β (Fig. 4A). Concentrations of

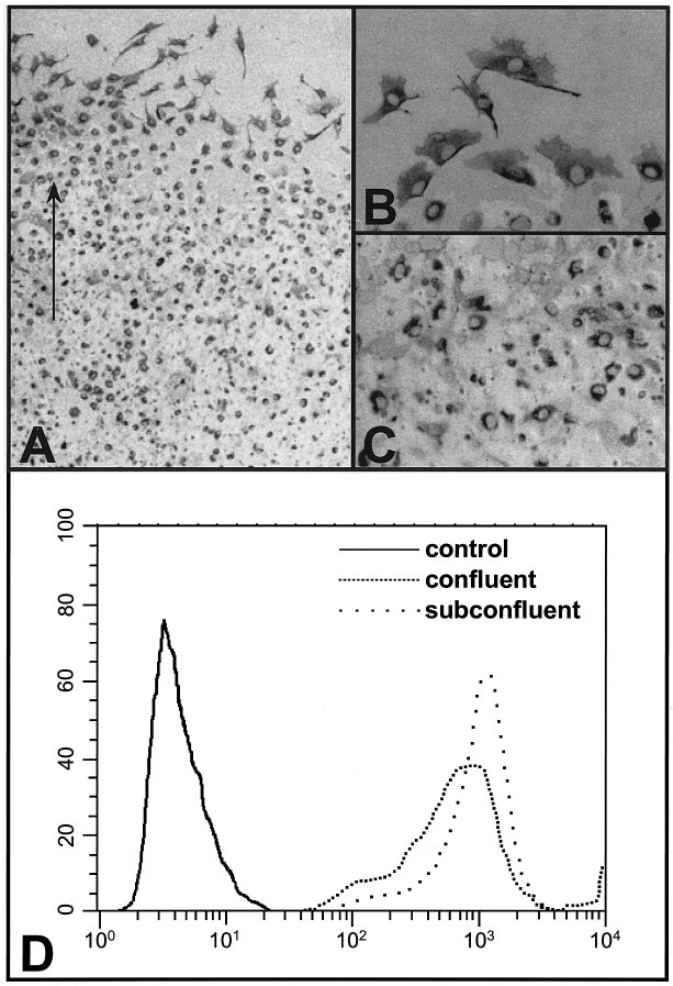
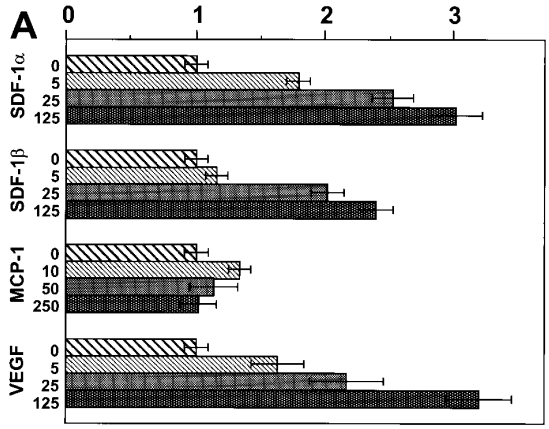


FIG. 3. Expression of CXCR-4 protein in migrating and resting BAE cells. (A) Low power magnification of a BAEC monolayer with migrating (top) and resting BAE cells stained for CXCR-4 expression (arrow: directionality of migration). (B) Higher magnification of BAE cells at the migrating front showing an intense, diffuse cell surface expression of CXCR-4 protein. (C) Higher magnification of resting BAE cells stained for CXCR-4 expression. CXCR-4 protein shows a patchy perinuclear staining pattern indicative of an accumulation in intracellular storage granules. (D) Flow cytometric analysis of CXCR-4 expression in BAE cells showing an upregulated surface expression of CXCR-4 in subconfluent cells.



B

SDF-1α in lower well (ng/ml)	SDF-1α in upper well (ng/ml) 0	5	25	125
0	100	94	72	62
5	132	87	66	62
25	192	161	104	92
125	288	258	183	76

FIG. 4. Induction of endothelial cell chemotaxis by SDF-1. (A) Agonists were loaded in the lower compartment of a 48-well chemotaxis chamber (concentrations given in ng/ml) and HUVE cells were seeded into the upper compartment as described in Materials and Methods. The compartments were separated by 12 μm pore chemotaxis membranes. Data are expressed as the percentage of migrated cells compared to the corresponding control cell population. The figure shows the mean ± SEM of five independent experiments performed in triplicate. (B) Checkerboard analysis of SDF-1 induced HUVEC migration to differentiate between chemotactic and chemokinetic activity of SDF-1. Data are expressed as percentage of migrated cells compared to the control. The figure shows representative results of a single experiment performed three time with similar results.

less that 1 nM SDF-1α (5 ng/ml) significantly induced chemotaxis of HUVE cells with maximal effect being at 125 ng/ml. The migration inducing capacity of SDF-1α was strictly chemotactic and not chemokinetic as determined by checkerboard analysis (Fig. 4B). MCP-1 served in all of these experiments as negative control and did not induce chemotaxis of HUVE cells even at a concentration of 250 ng/ml. VEGF which is known to induce HUVE cell chemotaxis (31) served as positive control and was on an equimolar basis similarly potent in inducing chemotaxis of HUVE cells than SDF-1α (Fig. 4A).

DISCUSSION

Chemokines act primarily as chemoattractive molecules to direct the trafficking of circulating hematopoietic cells (1,2). A number of chemokines, however, has also been shown to act on endothelial cells (5-10,13,14). These studies have convincingly demonstrated that CXC-chemokines have direct endotheliotropic effector functions and are capable to stimulate (IL 8) and to inhibit angiogenesis (PF-4, IP-10, GRO-β). The molecular basis of these endotheliotropic functions are still poorly understood.

To further clarify the role of chemokines on EC effector functions, we decided to analyze EC chemokine receptor expression. The results of these experiments represent the first systematic analysis of EC chemokine receptor expression. Based on these RT-PCR experiments, EC do not express CC-chemokine receptors which corresponds to the fact that no endotheliotropic effector functions of CC-chemokines have been reported so far. In contrast to the lack of detectable CC-chemokine receptor expression, all CXC-chemokine receptors were expressed by EC, albeit with some distinct differences: The IL-8 receptors CXCR-1 and CXCR-2 were hardly detectable in unstimulated EC, but amplification products could be generated from TNFα stimulated EC. This finding is in surprising contrast to the regulation of IL-8 receptors in other cells in which TNFα stimulation has been reported to downregulate IL-8 receptor expression (32), but it could well correspond to the differing responsiveness of EC to IL-8 which is highly dependent on cell culture conditions (33). Furthermore, we determined that endothelial cell CXCR-3 expression is reciprocally regulated to the expression of CXCR-1 and CXCR-2, being expressed by unstimulated EC and downregulated in TNFα stimulated cells. The reciprocal regulation of these receptors is intriguing in light of the antagonistic pro- and anti-angiogenic functions of the corresponding ligands. Finally, of all chemokine receptors analyzed in the present study, CXCR-4 was most consistently and abundantly expressed by the different EC populations, which corresponds to the findings of recently described experiments aimed at identifying novel endothelial cell chemokine receptors by degenerate RT-PCR (13,14).

CXCR-4 was originally isolated from monocytes as an orphan chemokine receptor and found to be expressed by a large number of different leukocytes populations (34). This orphan receptor was subsequently identified to be identical to the HIV coreceptor fusin (35). Meanwhile, the CXC-chemokine SDF-1 was cloned and characterized as the ligand for CXCR-4 (29,30). In contrast to the relatively promiscuous ligand specificity of most chemokine receptors, SDF-1 is presently the only CXCR-4 ligand and the SDF-1/CXCR-4 interaction could reflect a monospecific ligand/receptor interaction.

Expression of endothelial cell CXCR-4 and induction of EC chemotaxis by SDF-1 was very recently described by Volin et al. (13) and Gupta et al. (14). Our data independently confirm and extend these findings to

identify CXCR-4 as a constitutively expressed endothelial cell molecule, whose expression is regulated under autocrine control as well as under the control of exogenous cytokines. In HUVEC, steady state mRNA expression of CXCR-4 was upregulated in quiescent, resting cells compared to subconfluent, migrating cells. This differential transcriptional regulation did, however, not correspond to differences in protein levels. In contrast, TNFα stimulation led to a downregulation of CXCR-4 in HUVE cells on the mRNA as well as on the protein level which has also been described by Gupta et al. (14). In BAE cells, CXCR-4 expression displays a different pattern: Its expression is upregulated on the mRNA and on the protein level by migrating EC. bFGF was identified as the primary exogenous cytokines that regulates CXCR-4 expression in BAE cells. Most notably, the differential expression also corresponded to a differential cell surface expression of the receptor with CXCR-4 being preferentially expressed on the cell surface by autocrine activated, migrating BAE cells. This differential cellular localization suggests that CXCR-4 in endothelial cells is functionally not just regulated on the transcriptional and translational level, but also through differential cellular distribution of the receptor. Corresponding to these findings, the functional experiments described in this study indicate that the effect of SDF-1 on endothelial cells is chemotactic rather than chemokinetic.

In summary, the present study demonstrates that CXCR-4 is a differentially expressed EC molecule with varying expression pattern in different EC populations. Furthermore, the induction of chemotaxis by stimulation with exogenous SDF-1 suggests that EC express functional CXCR-4 receptors and point to a role of SDF-1 in the regulation of endothelial cell function. The CXCR-4/SDF-1 interaction possible acts as important regulator of vascular functions *in vivo*: The absence of an ELR motif in the SDF-1 molecule would predict an antiangiogenic function of SDF-1 (11). This prediction would, however, be in conflict with the observed chemotaxis inducing capacity of SDF-1 on endothelial cells, which usually reflects a proangiogenic function (31). On the other hand, the phenotype of SDF-1-deficient mice that have distinct cardiovascular defects (ventricular septal defects) in addition to defects of B-cell lymphopoiesis and bone-marrow myelopoiesis (36) strongly indicates that SDF-1 plays a critical role in regulating cardiovascular functions. Future ex-periments will have to reveal the role of SDF-1 and endothelial cell CXCR-4 expression for vascular functions *in vivo* and particularly if the functional relevance of the SDF-1/CXCR-4 interaction in situations of endothelial cell activation as they are associated with vascular remodelling and angiogenesis.

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