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The Chemokine CXCL13 (BCA-1) Inhibits FGF-2 Effects on Endothelial Cells

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Several chemokines, belonging to both the CXC and CC classes, act as positive or negative regulators of angiogenesis. We sought to investigate the role of CXCL13, B cell-attracting chemokine 1 (BCA-1), also known as B-lymphocyte chemoattractant (BLC), on endothelial cell functions. We tested the effect of CXCL13 on HUVEC chemotaxis and proliferation in the presence of fibroblast growth factor (FGF)-2 and found that such chemokine inhibits FGF-2-induced functions, while is not active by itself. To test whether other FGF-2-mediated biological activities may be affected, we evaluated the ability of CXCL13 to rescue HUVEC from starvation-induced apoptosis, as FGF-2 is a survival factor for endothelial cells, and found that CXCL13 partially inhibits such rescue. Multiple mechanisms may be responsible for these biological activities as CXCL13 displaces FGF-2 binding to endothelial cells, inhibits FGF-2 homodimerization, and induces the formation of CXCL13–FGF-2 heterodimers. Our data suggest that CXCL13 may modulate angiogenesis by interfering with FGF-2 activity. © 2001 Academic Press

Key Words: **angiogenesis; chemokine; FGF-2; endothelial cells.**

Several members of the chemokine family, belonging to both the CXC and CC classes, are known modulators of angiogenesis. The presence of ELR residues preceding the CXC motif, critical for the biological activities of CXCL8 (IL-8) in neutrophils, has been correlated with the angiogenic function of CXC chemokines (1, 2). In contrast, NON-ELR CXC chemokines are reported to be potent inhibitors of angiogenesis both *in vitro* and *in vivo* (3–6). Some exceptions from both classes of molecules have been described (7, 8).

Angiostatic chemokines inhibit growth factor-induced cell functions rather than showing a direct effect on these cells. CXCL4, platelet factor 4 (PF4), a well-studied CXC chemokine, interferes with FGF-2 binding and dimerization (9), inhibits FGF-2-induced DNA synthesis and entry into S phase (10) and prevents p21WAF1 downregulation in endothelial cells (11). Furthermore, CXCL4 inhibits vascular endothelial growth factor (VEGF) binding to its receptors on endothelial cells by affecting the interaction with heparan sulfates and by directly interacting with VEGF (6). However, since CXCL4 still retains its activity when its heparin binding sites are mutated, other unknown mechanisms of action are likely to be present (6). Even if the characterization of chemokine receptors involved in mediating the angiostatic activity is still under investigation, it has been demonstrated that the chemokine CXCL10, also known as interferon-inducible protein 10 (IP-10) inhibits endothelial cell proliferation by competing for FGF-2 binding to heparan sulfates (5) and by engaging its chemokine receptor CXCR3 (12).

CXCL13 (BCA-1) is a B cell chemoattractant (13, 14) and is highly induced in chronic *Helicobacter pylori*-gastritis and in gastric MALT lymphomas (15). Moreover, CXCL13 transgenic mice develop lymph node-like structures that contain B and T cell zones, high endothelial venules and stromal cells (16). CXCL13 was previously described as a ligand of the chemokine receptor CXCR5 (13, 14), a B cell homing receptor, and recently, it has been shown to bind with low affinity to the chemokine receptor CCR10 (17) and to displace CXCL10 binding to CXCR3 (18).

In this report we have identified a novel biological function for CXCL13 as an inhibitor of FGF-2-induced chemotaxis, proliferation, and rescue from apoptosis of endothelial cells that may have a role in the interplay between inflammation and angiogenesis.

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METHODS

Cell culture and reagents. Human umbilical vein endothelial cells (HUVEC) were obtained from Mascia Brunelli s.r.l. (Milan, Italy) and cultured in endothelial cell basal medium (EBM-2) supplemented with 2% fetal bovine serum (FBS) and human epidermal growth factor (hEGF), hFGF-2, hVEGF, insulin growth factor 1 (R3-IGF-1) (Biowittaker Italia s.r.l., Caravaggio, Italy). Human recombinant (hr) CXCL13 and macrophage inflammatory protein 1 (MIP-1α) CCL3 were purchased from Pepro Tech (London, UK) and hrFGF-2 from Endogen (Woburn, MA).

Flow cytometric analysis. CXCR5 expression by FACS analysis was performed as previously described (19, 20). Briefly, 3×10^5 cells were permeabilized with 0.2% Triton X-100/PBS for 2 min on ice, washed with ice cold 0.1% BSA/PBS, and incubated for 30 min with anti-human CXCR5 (R & D, Abingdon, UK) or control IgG (BD, Erembodegem, Belgium). A fluorescein isothiocyanate-conjugated goat anti-mouse IgG (DAKO, Glostrup, Denmark) was used as secondary antibody, and samples were then analyzed by flow cytometry (FACScan, BD, Mountain View, CA).

Chemotaxis assay. Chemotaxis was performed by using a 48-well microchamber (Neuroprobe, Cabin John, MD) and 8-μm pore-size polycarbonate filters (Costar, Cambridge, MA) coated with murine collagen type IV (Becton–Dickinson, Bedford, MA) (21). Briefly, HUVEC, passages 4–6, were cultured for 48 h in RPMI 1640 medium supplemented with 15% FBS, then detached and resuspended in migration medium (MM): RPMI 1640, 0.01% BSA, 25 mM Hepes at 10^6/ml. After 4 h incubation at 37°C, the chemotaxis assay, in triplicate wells, was stopped and cells were fixed on the filters and stained using Diff Quik (Dade AG, Dudingen, Switzerland). Six random fields were counted at 400× magnification and migration index (MI) was calculated by dividing the number of migrated cells in the presence of chemoattractants by the cells migrated in migration medium alone. Percentage of inhibition was calculated by dividing the number of cells which migrated in response to FGF-2 + CXCL13 by those responding to FGF-2 × 100.

Cell proliferation assay. DNA synthesis was assessed by the level of [^{3}H]thymidine incorporation. Briefly, HUVEC (2×10^3/well) were seeded in 24-well plates, and quiesced for 24 h in serum- and growth factor-free EBM-2. Medium was then replaced with 0.5% FBS EBM-2 containing FGF-2 (0.12 nM) in the presence or absence of CXCL13 (50 nM), and cell proliferation was evaluated after 3, 5, or 7 days. At these time points [^{3}H]thymidine (1 μCi/ml) (Amersham–Pharmacia Biotech, UK) was added to the wells and cells pulsed for additional 4 h, then washed and treated with 10% Trichloroacetic acid for 1 h at 4°C. After solubilization with 0.2 M NaOH, radioactivity was analyzed by liquid scintillation counter (Packard). Each sample was assessed in triplicate and data represent the mean of three experiments ± SE. Fold induction of [^{3}H]thymidine incorporation was calculated by dividing the values (cpm) of treated samples by the values of control samples (medium alone).

Cell survival. HUVEC (4×10^4 cells/well) were plated in 6-well plates and were synchronized by culturing for 48 h in starvation medium (SM: 2% FBS EBM-2 medium). Cells were then incubated in SM either in the presence or in the absence of FGF-2 (0.6 nM) and CXCL13 (10–30 nM) for 16 to 120 h. Floating and trypsinized adherent cells were mixed together and stained with an hypotonic propidium-iodide (PI) solution containing 50 μg/ml PI, 0.01% Triton X-100 and 0.01% Sodium Citrate (pH 6.8), for 4 h in the dark at +4°C and then analyzed by flow cytometry (FACScan, BD, Mountain View, CA). The percentage of inhibition of FGF-2-induced rescue from apoptosis was calculated on hypodiploid and normal diploid DNA content values.

Binding and dimerization experiments. HUVEC (4×10^4 cells/well) were seeded on 24-well plates in triplicates and grown in complete EBM-2 medium for 24 h. After two washes with cold binding buffer (BB: RPMI 1640, 2% BSA, 10 mM Hepes, pH 7.4), cells were incubated with 0.125 nM ^{125}I-FGF-2, 1000 Ci/mmol specific activity (Amersham–Pharmacia Biotech) for 2.5 h at +4°C in BB in the presence or absence of unlabeled CXCL13 (4 to 400 nM). Cells were then washed with BB, lysed with PBS 2% SDS, radioactivity was counted in a gamma counter and data analyzed using the GraphPad Prism and LIGAND programs. The range of IC_{50} was calculated out of four independent experiments performed.

FGF-2 dimerization experiments were performed as previously described (9) with some modifications. Briefly, 2 ng ^{125}I-FGF-2 plus 20 ng unlabeled FGF-2 were incubated for 1 h at room temperature with or without heparin (0.03 UI/ml) in the absence or presence of 5- to 100-fold molar excess of CXCL13 in PBS in a final volume of 35 μl. Then, 3.5 μl of the chemical crosslinker DSS (5 mg/ml) (Pierce, Rockford, IL) was added and the samples incubated for 30 min at room temperature. Boiled samples were loaded on a 10% SDS–PAGE, the gels dried and subjected to autoradiography at −80°C for 5–16 h using Hyperfilm (Amersham–Pharmacia Biotech).

Statistics. Results were analyzed by one-way ANOVA. Post hoc tests according to the Student–Newman–Keuls method allowed the assessment of statistically significant differences among groups. A value of $P < 0.05$ was considered statistically significant. Results are reported as mean values ± SE.

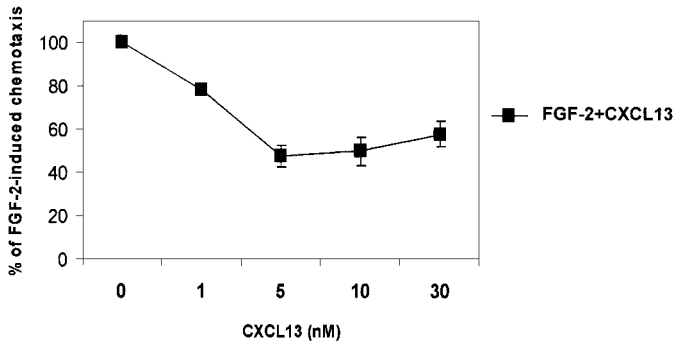

FIG. 1. Endothelial cell chemotaxis. HUVEC chemotaxis in response to FGF-2 (0.6 nM) was inhibited, by up to 50%, by treatment with 1–30 nM CXCL13. FGF-2 alone induced the migration of 29 ± 1.7 cells/field. Results represent means ± SE of three to five independent experiments.

RESULTS

CXCL13 Effect on Chemotaxis

We first tested the effect of CXCL13 on HUVEC chemotaxis and found that this molecule had no effect by itself in a wide range of concentration (1–100 nM) (not shown). As often NON-ELR CXC chemokines behave as inhibitors of growth factor functions on endothelial cells, we tested CXCL13 for such activity. We therefore performed HUVEC chemotaxis in the presence of FGF-2 (0.6 nM) with increasing amounts (0 to 30 nM) of CXCL13 (Fig. 1). We here show that the FGF-2-induced chemotaxis was inhibited by about 20% at 1 nM and by 40–50% at 5, 10, and 30 nM of CXCL13. The chemokine CCL3 (MIP-1α), used as an irrelevant chemoattractant, had no effect on FGF-2-induced chemotaxis (not shown).

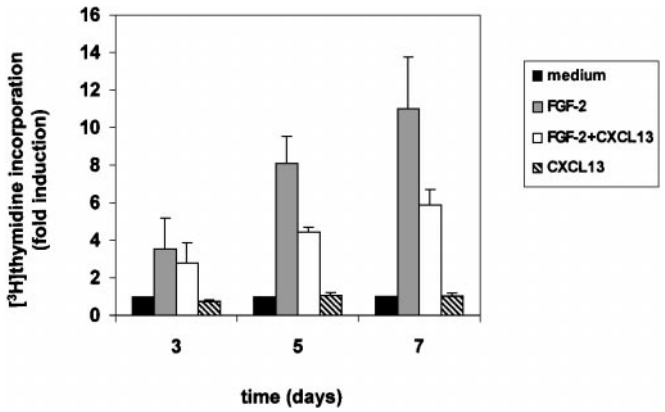

FIG. 2. Effect of CXCL13 on FGF-2-induced proliferation of HUVEC. CXCL13 (50 nM) strongly inhibits DNA synthesis of HUVEC at 5 and 7 days following stimulation with 0.12 nM FGF-2. Results are means of three independent experiments ±SE each performed in triplicate. Shown is fold induction of [^{3}H]thymidine incorporation following treatment with FGF-2, CXCL13, and FGF-2 + CXCL13.

CXCL13 Effect on Proliferation

We next investigated whether CXCL13, similarly to other angiostatic chemokines, may affect not only chemotaxis but other growth factor-induced cell functions. We therefore analyzed the CXCL13 effect on FGF-2-mediated DNA synthesis in HUVEC.

Figure 2 shows that the addition of CXCL13 (50 nM) inhibited FGF-2-induced [^{3}H]thymidine incorporation by HUVEC up to about 50% at different time points, while CXCL13 had no effect per se.

CXCL13 Effect on Starvation-Induced Apoptosis

To better characterize the activity of CXCL13 on FGF-2 functions we next investigated whether this molecule may modulate cellular apoptosis in the presence of such growth factor. In fact, serum-starved HUVEC undergo apoptosis that may be rescued by the addition of growth factors such as FGF-2. Our results show that CXCL13, at 10 nM, partially inhibited FGF-2-induced HUVEC survival, as measured by FACS analysis of propidium iodide-stained nuclei (Fig. 3). Maximal inhibition, of about 25% ($P = 0.025$), was reached 24 h following treatment, then declining at later time points. Similar results were obtained by using a cell death ELISA kit (not shown).

FGF-2 Binding on HUVEC and Heterodimers Formation

We then sought to characterize the mechanisms involved in the angiostatic activity of CXCL13. At first we performed heterologous displacement experiments in order to investigate whether FGF-2 binding to cells could be affected by this chemokine.

We found that unlabeled CXCL13 (4–400 nM) is indeed able to compete with ^{125}I-FGF-2 (0.125 nM) binding to HUVEC with an IC_{50} = 12–25 nM (Fig. 4A).

We then tested whether CXCL13 could affect FGF-2 dimer formation that is required to induce FGF receptor dimerization and activation (22). As both FGF-2 and CXCL13 have heparin binding sites and heparin is known to modulate growth factor activities (23), we analyzed the CXCL13/FGF-2 interaction *in vitro* both in the absence (Fig. 4B, lanes 1–4) and in the presence (Fig. 4B, lanes 5–8) of heparin. Interestingly, CXCL13 induced a dose-dependent decrease of FGF-2 homodimers (MW = 34 kDa) only in the presence of heparin. A scanning densitometric analysis of the 34-kDa band showed that the decrease in the homodimeric form was 13, 33, and 75% at 175, 875, and 3500 nM CXCL13 (Fig. 4B, lane 6–8), respectively, when compared to the levels of homodimers in the absence of chemokine (Fig. 4B, lane 5). Furthermore, a molecular weight species of about 28 kDa, likely representing CXCL13/FGF-2 heterodimers (Fig. 4B, right panel) was induced upon addition of increasing amounts of CXCL13 to the reaction. Differently from CXCL13, the CC chemokine CCL3 (3500 nM) had no such activity (not shown). In the presence of heparin, we retained the upper part of the gel and observed, interestingly, that CXCL13 induced the formation of very high molecular weight complexes unable to enter the SDS gel, in a dose–response manner (Fig. 4B, right panel). In contrast, in the absence of exogenously added heparin, similarly to what has been described for CXCL4 (9), a slight increase in the FGF-2 homodimer (34-kDa species) formation but no appearance of the CXCL13/FGF-2 heterodimer (28-kDa species) was observed (Fig. 4B, left panel). The amount of CXCL13 required to alter dimer formation was higher than the biologically active concentrations of CXCL13 on HUVEC, possibly due to crosslinker efficiency.

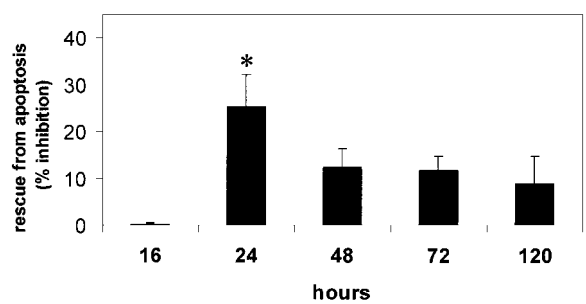

FIG. 3. FGF-2-induced rescue of HUVEC from apoptosis. Inhibitory effect of CXCL13. CXCL13 (10 nM) partially inhibits FGF-2-induced rescue from apoptosis of 24-h serum-starved HUVEC ($P = 0.025$). Ten experiments by FACS analysis of propidium-iodide stained nuclei were performed at 24 h, 4 to 8 at the other time points.

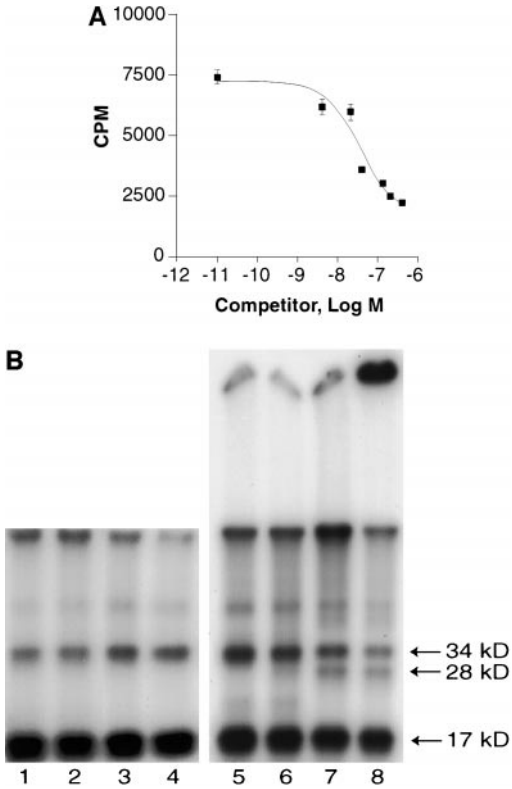

FIG. 4. CXCL13 inhibition of FGF-2 binding to HUVEC and of FGF-2 homodimer formation. (A) Representative competition binding experiment showing that CXCL13 inhibits ^{125}I-FGF-2 binding to endothelial cells. Unlabeled CXCL13 (4–400 nM) induced displacement of FGF-2 (0.125 nM) binding with an IC_{50} = 12–25 nM. Similar results were obtained in four independent experiments. (B) Chemical crosslinking was evaluated both in the absence (lanes 1–4) and in the presence (lanes 5–8) of heparin (0.03 UI/ml). The addition of increasing amount of CXCL13 to 2 ng ^{125}I-FGF-2 plus 20 ng unlabeled FGF-2, in a 35 μl volume, induced a strong inhibition of FGF-2 homodimers (MW approx. 34 kDa, indicated by the arrow) and the formation of CXCL13-FGF-2 heterodimers (MW approx. 28 kDa) in the presence, but not in the absence of heparin. Also shown is the ^{125}I-FGF-2 monomer (MW approx. 17 kDa). CXCL13 (nM): lanes 1 and 5, 0; lanes 2 and 6, 175; lanes 3 and 7, 875, lanes 4 and 8, 3500. A similar effect was obtained in four different experiments. In the presence of heparin, CXCL13 induced the formation of very high-molecular-weight complexes (right panel, top of the gel). Both stacking and resolving gels were processed and shown in lanes 5–8.

CXCR5 Expression on HUVEC

Since CXCR5 is regarded as the high affinity receptor for CXCL13, we analyzed whether HUVEC may express such molecule. As shown in Fig. 5, a shift in the fluorescence intensity of subconfluent cells treated with a specific antibody was observed, showing the presence of the CXCR5 receptor on HUVEC.

DISCUSSION

Angiogenesis, the generation of newly formed vessels from the preexisting ones, is under the control of a balance of positive and negative regulators (24).

As chemokines have been shown to regulate angiogenesis we sought to clone and characterize novel molecules belonging to this superfamily that may affect endothelial cell functions. Our analysis of dEST databases for chemokines possessing the CXC motif yielded a cDNA with a high degree of similarity with CXCL8 (GenBank Accession No. AF029894). While such factor was described as a specific B cell chemoattractant, named BCA-1/BLC/CXCL13, and shown to bind to the CXCR5 chemokine receptor (13, 14), we characterized its putative function on endothelium.

The NON-ELR motif present in CXCL13 predict an inhibitory role for this molecule on endothelial cell functions, as other angiostatic chemokines that affect growth factor-mediated activities on endothelium (2). This inhibition was previously described for example for the NON-ELR chemokines CXCL10 and CXCL9 (Mig) that negatively regulate CXCL8- and FGF-2-induced endothelial cell chemotaxis and angiogenesis *in vivo* (2, 3).

In our study we show that chemotaxis, proliferation and survival of HUVEC, regarded as important steps in the angiogenic process, were not affected by CXCL13 per se. Therefore we tested the ability of CXCL13 to modulate FGF-2 functions on these cells.

FGF-2 is involved in the control of myelopoiesis, megakaryocytopoiesis (25, 26), neurite outgrowth, differentiation, and survival (27). Further, such a molecule is a known modulator of endothelial cell chemotaxis, proliferation, differentiation, and survival in fact

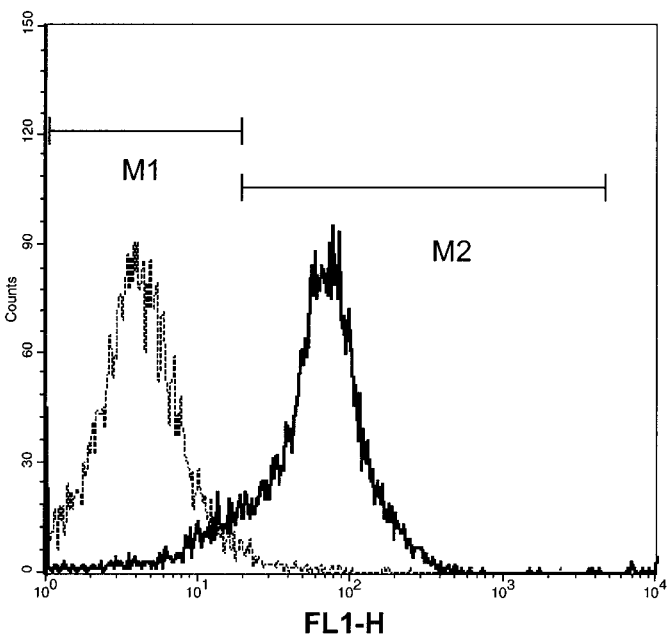

FIG. 5. Expression of CXCR5 on HUVEC. Flow cytometry of CXCR5 expression on HUVEC. Stainings with a specific monoclonal antibody anti-CXCR5 (solid line) and control antibody (dotted line) are shown. Fluorescence intensities (FL1-H) are indicated on the *X* axis and counts on the *Y* axis.

serum-starved HUVEC undergo apoptosis that can be rescued by the addition of FGF-2 (28).

In this study we show that CXCL13 inhibited FGF-2-induced chemotaxis, proliferation and survival of endothelial cells, thus behaving as a novel angiostatic chemokine.

FGF-2 exerts its biological functions as a homodimer, binding to specific cell surface tyrosin kinase receptors and heparan sulfate proteoglycans (HGSP) (22, 23). HGSPs can act stabilizing the FGF-2 dimer formation and allowing a proper interaction of FGF-2 with high affinity receptors on the endothelium. Similarly, chemokines bind both to high affinity receptors as well as to proteoglycans and a role for HGSP in the interaction between chemokines and their specific receptors has been proposed (29). Proteoglycans are thought to present chemokines on endothelial cells to circulating leukocytes contributing to the formation of a gradient critical for trans-endothelial migration and recruitment to sites of inflammation (30).

Our results show that CXCL13 displaced FGF-2 binding to endothelial cells in a dose-dependent manner. Examples from well-known angiostatic chemokines such as CXCL4 and CXCL10 indicate that they bind heparan sulfate molecules with intermediate affinity and that inhibit growth factor-induced endothelial cell activation, competing for their binding sites on such cells (5).

The displacement by CXCL13 of FGF-2 binding to HUVEC and the heparin-dependent inhibition of the FGF-2 homodimer formation, represent potential mechanisms responsible of the angiostatic activity shown by this chemokine. The appearance of the CXCL13/FGF-2 heterodimers in the presence of heparin is similar to what was described for CXCL4 (9) suggesting that, at least *in vitro*, the formation of complexes between angiostatic chemokines and growth factors may be heparin-dependent.

The direct involvement of chemokine receptors in regulating angiogenesis is still not well clarified. Recently, Salcedo *et al.* (31) showed the presence of CXCR3 on microvascular endothelial cells and Romagnani *et al.* (12) provided evidence that CXCR3 expression on microvascular endothelial cells is limited to the S/G2-M phase of cell cycle. The authors (12) show that the antiproliferative activity of the CXCR3 ligands CXCL10, CXCL9, and CXCL11 was blocked by the use of an anti-CXCR3 neutralizing antibody, pointing at a direct involvement of such chemokine receptor in the angiostatic activity. A recent study demonstrated that CXCL13 competes for CXCL10 binding to CXCR3 transfectants, induces chemotaxis of such cells and that these biological activities are inhibited by anti-CXCR3 antibodies (18) indicating that CXCL13 may be regarded as a novel ligand for CXCR3.

In our studies we show that CXCR5 is expressed by HUVEC, therefore the biological activities we described for CXCL13 may potentially be exerted through either CXCR3, abundantly expressed by endothelial cells, or CXCR5. In conclusion, we have reported that CXCL13 inhibits FGF-2-induced chemotaxis, proliferation, and rescue from apoptosis possibly through binding of CXCL13 with both FGF-2 and FGF-2 binding sites on endothelial cells.

The identification and characterization of a novel modulator of FGF-2 activities may have important therapeutic implications due to the role of FGF-2 in processes such collateral blood vessel development in response to ischemia, tumor growth and wound repair.

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