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# The CXC Chemokine Receptor 2, CXCR2, Is the Putative Receptor for ELR<sup>+</sup> CXC Chemokine-Induced Angiogenic Activity<sup>1</sup>

Christina L. Addison,\* Thomas O. Daniel,<sup>†‡</sup> Marie D. Burdick,<sup>§</sup> Hua Liu,<sup>†‡</sup> Jan E. Ehlert,<sup>§</sup> Ying Ying Xue,<sup>§</sup> Linda Buechi,<sup>¶</sup> Alfred Walz,<sup>¶</sup> Ann Richmond,<sup>‡||</sup> and Robert M. Strieter<sup>2§</sup>

We have previously shown that members of the ELR<sup>+</sup> CXC chemokine family, including IL-8; growth-related oncogenes  $\alpha$ ,  $\beta$ , and  $\gamma$ ; granulocyte chemotactic protein 2; and epithelial neutrophil-activating protein-78, can mediate angiogenesis in the absence of preceding inflammation. To date, the receptor on endothelial cells responsible for chemotaxis and neovascularization mediated by these ELR<sup>+</sup> CXC chemokines has not been determined. Because all ELR<sup>+</sup> CXC chemokines bind to CXC chemokine receptor 2 (CXCR2), we hypothesized that CXCR2 is the putative receptor for ELR<sup>+</sup> CXC chemokine-mediated angiogenesis. To test this postulate, we first determined whether cultured human microvascular endothelial cells expressed CXCR2. CXCR2 was detected in human microvascular endothelial cells at the protein level by both Western blot analysis and immunohistochemistry using polyclonal Abs specific for human CXCR2. To determine whether CXCR2 played a functional role in angiogenesis, we determined whether this receptor was involved in endothelial cell chemotaxis. We found that microvascular endothelial cell chemotaxis in response to ELR<sup>+</sup> CXC chemokines was inhibited by anti-CXCR2 Abs. In addition, endothelial cell chemotaxis in response to ELR<sup>+</sup> CXC chemokines was sensitive to pertussis toxin, suggesting a role for G protein-linked receptor mechanisms in this biological response. The importance of CXCR2 in mediating ELR<sup>+</sup> CXC chemokine-induced angiogenesis in vivo was also demonstrated by the lack of angiogenic activity induced by ELR<sup>+</sup> CXC chemokines in the presence of neutralizing Abs to CXCR2 in the rat corneal micropocket assay, or in the corneas of CXCR2<sup>-/-</sup> mice. We thus conclude that CXCR2 is the receptor responsible for ELR<sup>+</sup> CXC chemokine-mediated angiogenesis. *The Journal of Immunology*, 2000, 165: 5269–5277.

The CXC family of chemokines is a chemotactic group of cytokines, defined by the presence of four conserved cysteine amino acid residues in the amino terminus of the protein, in which the first two conserved cysteine amino acid residues are separated by one nonconserved amino acid residue (hence the CXC designation). This family of molecules can be further subdivided based on the presence or absence of the amino acid sequence Glu-Leu-Arg (ELR) preceding the first conserved cysteine amino acid residue in the primary structure of these proteins. The ELR motif has been shown to play a role in ligand/receptor interactions on neutrophils (1, 2). We have previously shown that the ELR motif is also the structural/functional domain

important in the regulation of CXC chemokine-induced angiogenesis (3). It has been demonstrated that CXC chemokines that contain the ELR motif (ELR<sup>+</sup>), such as IL-8; growth-related oncogenes (GRO)<sup>3</sup>  $\alpha$ ,  $\beta$ , and  $\gamma$ ; granulocyte chemotactic protein-2 (GCP-2); and epithelial neutrophil-activating protein 78 (ENA-78), are potent inducers of angiogenesis in vivo (3). In contrast, CXC chemokines that lack the ELR motif (ELR<sup>-</sup>), such as platelet factor 4, IFN-inducible protein-10, and monokine induced by IFN- $\gamma$ , are potent angiostatic factors, and can inhibit neovascularization mediated by ELR<sup>+</sup> CXC chemokines (3, 4). Furthermore, the ELR<sup>-</sup> CXC chemokines have also been shown to inhibit neovascularization induced by the classical angiogenic factors, basic fibroblast growth factor (bFGF) and vascular endothelial cell growth factor (VEGF) (3). The receptor responsible for the induction of angiogenesis by ELR<sup>+</sup> CXC chemokines has not yet been elucidated.

The CXC chemokines have been shown to interact with the CXC chemokine receptor (CXCR) family of molecules. These receptors are members of the rhodopsin-like seven-transmembrane G protein-coupled receptor family (5–8). The chemokine receptors range in size from 339 to 373 aa, and possess 25–80% homology to one another on the amino acid level (6, 7, 9, 10). To date, five CXC receptors have been identified in various human cell lines. CXCR1, which is also known as IL-8RA, binds IL-8 with high

\*Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI 48109; <sup>†</sup>Center for Vascular Biology and <sup>‡</sup>Departments of Medicine and Cell Biology, Vanderbilt University Medical Center, Nashville, TN 37232; <sup>§</sup>Division of Pulmonary and Critical Care Medicine, Department of Medicine, University of California, Los Angeles School of Medicine, Los Angeles, CA 90095; <sup>¶</sup>Theodor Kocher Institute, Universitat Bern, Bern, Switzerland; and <sup>||</sup>Department of Veteran's Affairs, Vanderbilt University Medical Center, Nashville, TN 37232

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<sup>2</sup> Address correspondence and reprint requests to Dr. Robert M. Strieter, Division of Pulmonary and Critical Care Medicine, University of California, Los Angeles School of Medicine, 900 Veteran Avenue, 14-154 Warren Hall, Box 711922, Los Angeles, CA 90095. E-mail address: rstrieter@mednet.ucla.edu

<sup>3</sup> Abbreviations used in this paper: GRO, growth-related oncogene; bFGF, basic fibroblast growth factor; CXCR, CXC chemokine receptor; DARC, Duffy Ag receptor for chemokines; ENA, epithelial neutrophil-activating protein; GCP, granulocyte chemotactic protein; HMVEC, human microvascular endothelial cell; HMVEC-D, human dermal microvascular endothelial cell; HMVEC-L, human lung microvascular endothelial cell; HPF, high power field; KLH, keyhole limpet hemocyanin; MIP, macrophage-inflammatory protein; NAP, neutrophil-activating protein; PTx, pertussis toxin; TFA, trifluoroacetic acid; VEGF, vascular endothelial cell growth factor.

affinity (8), and more recently has been shown to also bind GCP-2 with a somewhat reduced affinity (11). CXCR2, also known as IL-8RB, possesses 78% identity with CXCR1 on the amino acid level with the majority of divergence between the two proteins being located in the amino terminus, the carboxyl terminus, and within the second extracellular loop (6). This divergence in the extracellular regions (the amino terminus and the second extracellular loop) may partly explain why CXCR2 shows less selectivity in chemokine binding than does CXCR1. CXCR2 has been shown to bind all of the ELR<sup>+</sup> CXC chemokines, including IL-8; ENA-78; GRO- $\alpha$ , - $\beta$ , and - $\gamma$ ; neutrophil-activating protein-2 (NAP-2); and GCP-2 with high affinity (6, 8, 9, 12). CXCR3 has been shown to bind the ELR<sup>-</sup> CXC chemokines IFN-inducible protein-10 and monokine induced by IFN- $\gamma$  (5). CXCR4 has recently been the focus of a number of studies, as it has been shown to be a cofactor for HIV infection of T lymphocytes by T cell tropic viruses (13). To date, stromal cell-derived factor-1 is the only known ligand for CXCR4, and this ELR<sup>-</sup> CXC chemokine can inhibit HIV infection by competing with lymphotropic HIV virus for binding of CXCR4 (14). CXCR5 is found mostly on B cells and is responsible for B cell chemotaxis mediated by B cell-attracting chemokine 1 (15). There is a sixth receptor that has been shown to bind CXC chemokines, DARC or the Duffy Ag receptor for chemokines (16, 17). IL-8, GRO- $\alpha$ , and NAP-2 have all been shown to bind to DARC; however, ligand binding of this receptor is not restricted to the CXC chemokines, and various CC chemokines, such as RANTES and monocyte chemoattractant protein-1, also bind to DARC with high affinity (18). Moreover, DARC does not appear to demonstrate ligand-receptor signal coupling (19). Another receptor whose ligand binding is restricted to the CXC chemokines is encoded by an open reading frame from Herpesvirus saimiri (20). The ECRF3 open reading frame has been shown to encode a seven-transmembrane receptor, and this receptor has been shown to bind CXC chemokines, in particular the ELR<sup>+</sup> CXC chemokines IL-8, GRO- $\alpha$ , and NAP-2 (20). This receptor is most homologous to CXCR2; however, this homology is only 30% at the amino acid level, even though the CXC chemokine-binding repertoire is essentially identical (20).

In this study, we investigated the identity of the chemokine receptor responsible for mediating angiogenesis induced by the ELR<sup>+</sup> CXC chemokines. As mentioned, the ELR<sup>+</sup> CXC chemokine IL-8 can bind both CXCR1 and CXCR2 with high affinity, while the ELR<sup>+</sup> CXC chemokines GRO- $\alpha$ , - $\beta$ , - $\gamma$ , and ENA-78 have been shown to bind only CXCR2 with high affinity (8, 21). Because all of these ELR<sup>+</sup> CXC chemokines only bind CXCR2 in common, and all induce angiogenesis *in vivo*, we hypothesized that CXCR2 is the putative receptor present on endothelial cells that mediates angiogenesis induced by ELR<sup>+</sup> CXC chemokines *in vivo*. In this study, we demonstrate that CXCR2 is expressed by cultured human microvascular endothelial cells (HMVEC), and that not only can neutralizing Abs to CXCR2 inhibit endothelial cell chemotaxis *in vitro*, but also inhibit neovascularization induced by the ELR<sup>+</sup> CXC chemokines in the rat CMP assay *in vivo*. Furthermore, corneal neovascularization induced by ELR<sup>+</sup> CXC chemokines in CXCR2<sup>-/-</sup> mice is impaired as compared with that induced in wild-type mice.

## Materials and Methods

### Cell lines and isolation

Human neutrophils were isolated from heparinized venous blood collected from healthy volunteers, by mixing 1:1 with 0.9% saline and separation from mononuclear cells by Ficoll-Hypaque density-gradient centrifugation. Human neutrophils were then isolated by sedimentation in 5% dextran in 0.9% saline (Sigma, St. Louis, MO) and separated from erythrocytes by

hypotonic lysis. After washing twice, neutrophils were suspended in HBSS with calcium/magnesium (Life Technologies, Grand Island, NY) at a concentration of  $2 \times 10^6$  cells/ml. Neutrophils were >99% viable, as determined by trypan blue exclusion. Human dermal microvascular endothelial cells (HMVEC-D) and human lung microvascular endothelial cells (HMVEC-L) were both obtained from Cell Systems (Kirkland, WA), and were propagated in CS-C complete media on attachment factor-coated tissue culture flasks according to the manufacturer's directions. All endothelial cell lines were stained for factor VIII-related Ag to confirm their identity as being of endothelial origin. Both the HMVEC-L- and HMVEC-D-cultured cells were positive for the expression of factor VIII-related Ag, while human neutrophils were negative following immunohistochemical staining.

To generate 293 cells that expressed human CXCR2, the cDNA for human CXCR2 was amplified by RT-PCR from total RNA isolated from human neutrophils using the Access PCR kit (Promega, Madison, WI). The primers used for amplification were: forward, 5'-GTC AGG ATC CAA GTT TAC CTC AAA AAT GG-3', and reverse, 5'-CTT AGG TCG ACG GTC TTA GAG AGT AGT GG-3'. The reverse-transcription reaction was performed at 42°C for 45 min, followed by denaturation at 94°C for 2 min. PCR was then performed for 40 cycles as follows: 94°C denaturation for 1 min, 55°C annealing for 1 min, and 68°C elongation for 2 min. The resulting PCR was subjected to electrophoresis on an agarose gel, and a band of ~1.1 kb was removed and purified using the Wizard PCR DNA purification kit (Promega). The PCR product was ligated into the T-overhang plasmid pTARGET according to the manufacturer's directions kit (Promega). This resulted in human CXCR2 expression under control of the human CMV promoter and allowed for generation of stably transfected mammalian cells by the presence of a neomycin resistance gene. The 293 cells were transfected with 5  $\mu$ g of CXCR2 plasmid DNA or control pTARGET DNA by calcium phosphate transfection, as previously described (22). G418-resistant colonies were expanded, and expression of CXCR2 was confirmed by FACS analysis using a mAb against human CXCR2 (R&D Systems, Minneapolis MN).

### Abs and Ab generation

A rabbit polyclonal Ab to the carboxyl terminus of human CXCR2 was purchased from Santa Cruz Biotechnologies (Santa Cruz CA). Anti-rabbit IgG Abs conjugated to HRP were purchased from Bio-Rad (Hercules CA). For Ab generation, peptides specific to the amino-terminal region of human or murine CXCR2 were generated by solid-phase peptide synthesis performed on a Millipore 9050 continuous flow peptide synthesizer (Millipore, Milford, MA) using Fmoc chemistry. Briefly, 1 g of polyethylene glycol polystyrene-graft copolymer peptide synthesis support (PEG-PS resin) and 0.8 mM of each Fmoc-amino acid active ester were used. Cleavage and deprotection were conducted in 88% trifluoroacetic acid (TFA), 5% liquefied phenol, 2% triisopropylsilane, and 5% water for 2–12 h at room temperature. The free peptide was then precipitated and washed repeatedly with ice-cold ether and dried under vacuum. The peptide was resuspended in water, acidified with TFA, and purified by preparative reversed-phase HPLC in a C4 column (25  $\times$  100 mm, 15 mm, 300A,  $\Delta$ -Pak; Waters, Millipore, Bedford, MA). The column was eluted at 5 ml/min with a gradient of 0–60% acetonitrile in 0.1% TFA at an increment of 1.3% per mm. Fractions were lyophilized and analyzed by analytical reversed-phase HPLC and mass spectroscopy.

A 17-mer peptide constituting the amino terminus of the mouse CXCR2 (MGEFKVDKFNIEDFFSG) and a 21-mer peptide constituting the amino terminus of human CXCR2 (CMEDFNMESDSFEDFWKGEDL) were synthesized as described above. The peptides contained an additional alanine residue at the carboxyl terminus, and a cysteine residue at the amino terminus for conjugation with keyhole limpet hemocyanin (KLH). Conjugation with Imject maleimide-activated KLH was conducted according to the manufacturer (Pierce, Rockford, IL). Polyclonal antiserum against either murine CXCR2 or human CXCR2 was generated following s.c. or i.m. injections of 100  $\mu$ g of the KLH-conjugated peptide in CFA, followed by at least three boosters of 100  $\mu$ g of KLH-conjugated peptide in IFA. Direct ELISA was used to evaluate antisera titers, and sera were drawn when titers had reached greater than 1:1,000,000.

The specificity of the Ab to human CXCR2 was confirmed following receptor neutralization studies on stably transfected cells. This Ab specifically recognized human CXCR2 and prevented binding of IL-8 to 293 cells transfected to overexpress human CXCR2. This Ab did not cross-react with CXCR1, nor did it prevent binding of IL-8 to human CXCR1, when used to detect receptor expression in 293 cells overexpressing human CXCR1. To determine the specificity of the anti-mouse CXCR2 Abs, we tested the ability of the Ab to inhibit neutrophil recruitment *in vivo*. CBA/J mice or Fischer rats were injected i.p. with 80 ng of recombinant KC (K

and C coordinates on the autorad from initial cloning (23)) in combination with either 0.5 ml of the anti-murine CXCR2 antisera or 0.5 ml of normal rabbit serum. Four hours later, animals were sacrificed and peritoneal lavage was performed using 3 ml of PBS with 5 mM EDTA. The concentration of cells within the lavage fluid was counted using a hemacytometer; equal numbers of cells were subjected to cytocentrifugation; and the slides were fixed and stained using the Diff-Quik kit (Baxter Diagnostics, McGaw Park, IL), followed by cell differential determination. It was found that the presence of anti-murine CXCR2 Abs specifically inhibited neutrophil migration in response to KC, while having no effect on monocyte infiltration in both rodent systems.

#### RT-PCR for CXCR2 gene expression

Cells were isolated as described above. Total RNA was extracted from cells using Trizol reagent (Life Technologies, Grand Island, NY), according to manufacturer's instructions. RT-PCR was performed using an Access RT-PCR kit (Promega, Madison, WI).  $\beta$ -actin was used as a house-keeping gene. For  $\beta$ -actin, the sense primer used was 5'-GTGGGGCGC CCCAGGACCA; the antisense primer was 5'-GCTCGGCCGTGGTG GTGAAGC (550 bp). For human CXCR2, the sense primer used was 5'-CCGGGCGTGGTGGTGAG; the antisense primer was 5'-TCTGCCTTT TGGGTCTTGTGAATA (385-bp product). PCR products were visualized by agarose gel electrophoresis. To exclude genomic DNA contamination, PCR was performed in the presence or absence of a preceding step that included reverse-transcriptase reaction with the isolated RNA.

#### Western blot analysis

Total protein extracts were made by scraping endothelial or 293 cell monolayers into TNE lysis buffer (20 mM Tris-HCl, pH 8, 150 mM NaCl, 1% Nonidet P-40, 2.5 mM EDTA) supplemented with 2 ng/ml aprotinin and 35 ng/ml PMSF or by resuspending isolated neutrophils into supplemented TNE lysis buffer. Cell extracts were incubated on ice for 30 min, followed by centrifugation at 4°C for 30 min. Supernatants were then removed and assayed for total protein content using bicinchoninic acid protein assay reagents (Pierce, Rockford, IL) and comparison with known amounts of BSA. A total of 40  $\mu$ g of total protein was loaded in each well of a 12% polyacrylamide gel, and extracts were subjected to SDS-PAGE. The separated proteins were transferred to polyvinylidene fluoride membrane (Pierce) by electrophoretic transfer overnight in Tris-glycine buffer (20 mM Tris, 150 mM glycine, pH 8, methanol added to a final concentration of 20% (v/v)). Blots were blocked in 5% skim milk in TBST buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.05% Tween 20) for 2 h at room temperature, followed by incubation in rabbit primary Ab sera against human CXCR2 diluted 1/1000 in blocking solution for 2 h at room temperature. Blots were washed for three 10-min washes in TBST and were incubated for 1 h at room temperature in goat anti-rabbit HRP-conjugated secondary Ab (Bio-Rad, Hercules, CA) at a 1/20,000 dilution. Blots were again washed for four 10-min washes in TBST, and proteins were visualized following incubation of the blots in SuperSignal chemiluminescent substrate solution according to the manufacturer's protocol (Pierce) and exposure to XAR-5 film (Kodak, Rochester, NY).

#### Immunohistochemistry

Immunolocalization of CXCR2 was performed as previously described (24). Briefly, cytopins of human peripheral blood neutrophils, or Tissue-Teks (Fisher, Pittsburgh, PA) of 90% confluent unstimulated endothelial cell monolayers were fixed in 4% paraformaldehyde in PBS for 10 min, then rinsed twice with PBS. Before staining, the slides were again fixed for 30 min in the presence or absence of 1:1 absolute methanol and 3% H<sub>2</sub>O<sub>2</sub>, and rinsed in PBS, and then nonspecific binding sites were blocked with a 1/50 dilution of normal goat serum by incubation at room temperature for 30 min. Following the blocking step, a 1/500 dilution of either control (rabbit) or rabbit anti-human CXCR2 serum was added as a primary Ab, and slides were incubated for 30 min at room temperature. Slides were then rinsed with PBS, overlaid with biotinylated goat anti-rabbit IgG (Vector ABC Elite Kit; Vector Laboratories, Burlingame, CA), and incubated for an additional 30 min. Slides were again rinsed two times with PBS, and were then treated with streptavidin-conjugated peroxidase (Vector ABC Elite Kit; Vector Laboratories) for 30 min at room temperature. Following three washes with PBS, the slides were subjected to colorimetric detection using the substrate chromogen 3,3'-diaminobenzidine (Vector Laboratories). Slides were incubated for 5–10 min in 3,3'-diaminobenzidine solution at room temperature to allow color development, and rinsed with distilled water to quench the reaction. Mayer's hematoxylin was used as a counterstain.

#### Endothelial cell chemotaxis

Endothelial cell chemotaxis assays were performed essentially as previously described (3). HMVEC-L cells were harvested by trypsinization, resuspended in CS-C medium without growth factors with 2% FBS added (Cell Systems), and preincubated with either anti-human CXCR2 antiserum or normal rabbit serum at a final dilution of 1/250 at 37°C for 1 h. Alternatively, endothelial cells were preincubated in 2.5 mM pertussis toxin (PTx; Sigma) at 37°C for 1 h. Following preincubation, an aliquot of 160  $\mu$ l containing  $5 \times 10^5$  cells/ml was added to each of the lower wells of a 12-well chemotaxis chamber (Neuro Probe, Cabin John, MD). The chambers were assembled (by placing 0.1% gelatin-coated 5- $\mu$ m pore-size filters over the lower wells, followed by a gasket and the upper chamber) and incubated at 37°C in a CO<sub>2</sub> incubator for 2 h in an inverted position. The chambers were then turned upright, and 100- $\mu$ l aliquots of various chemotactic agents in solution were added to the upper wells of the chamber in the following concentrations: 80 ng/ml IL-8, ENA-78, or GRO- $\alpha$ ; 40 ng/ml bFGF or VEGF. The chambers were placed in a 37°C CO<sub>2</sub> incubator for 2 h, at which time the filters were removed, the bottom of the filters were scraped to remove cells that did not undergo chemotaxis, and then the filters were subjected to Diff-Quik (Baxter) staining. The number of endothelial cells that had migrated through the filters into the upper chambers was calculated by counting the total number of cells in 15 separate fields of view under  $\times 40$  power. Results were expressed as the number of endothelial cells that migrated per high power field (HPF) after subtracting the background (unstimulated control) to demonstrate specific migration. Experiments were repeated independently at least three times.

#### Rat corneal micropocket assay of angiogenesis

To address the ability of neutralizing CXCR2 Abs to inhibit angiogenesis *in vivo*, the corneal micropocket assay was performed in the rat eye, as previously described (3). Human IL-8, human ENA-78, murine KC, or murine macrophage-inflammatory protein (MIP)-2 was diluted in PBS plus 0.25% human serum albumin to a final concentration of 80 ng per pellet. VEGF or bFGF was diluted as above to a final concentration of 50 ng per pellet. Rabbit anti-murine CXCR2 antiserum or normal rabbit serum was added at a 1/1000 dilution to each of the above chemokines and cytokines, and mixed with an equal volume of Hydron casting solution (Hydro Med Sciences, New Brunswick, NJ). Five-microliter aliquots were pipetted onto the flat surface of a sterile polypropylene specimen container and were allowed to polymerize overnight under UV light in a laminar flow hood. Before implantation, the pellets were rehydrated with normal saline. Animals were given 150 mg/kg ketamine and 250  $\mu$ g/kg atropine as an anesthetic, and the rat corneas were anesthetized with 0.5% proparacaine hydrochloride ophthalmic solution, followed by implantation of the Hydron pellet into an intracorneal pocket (1–2 mm from the limbus). Six days after implantation, animals received heparin (1000 U) and ketamine (150 mg/kg) i.p. 30 min before sacrifice, followed by perfusion with 10 ml of colloidal carbon via the left ventricle. Corneas were then harvested and photographed. No inflammatory response was observed in any of the corneas treated with the above cytokines. Sustained directional ingrowth of capillary sprouts and hairpin loops toward the implant were considered positive neovascularization responses. Negative responses were characterized by either no vessel growth or by the presence of only an occasional hairpin loop or sprout that displayed no evidence of sustained growth.

#### Mouse corneal angiogenesis assay

Hydron pellets incorporating sucralfate with either vehicle alone, bFGF (3 pmol/pellet, gift from Scios, Sunnyvale, CA), murine rMIP-2 (20 pmol/pellet, gift from Elias Lolis at Yale University School of Medicine, New Haven, CT), or human rIL-8 (20 pmol/pellet, purchased from R&D Systems, Minneapolis, MN) were made as described (25). Pellets were surgically implanted into corneal stromal micropockets created 1 mm medial to the lateral corneal limbus of C57BL/6 CXCR2<sup>-/-</sup> and CXCR2<sup>+/+</sup> mice (9–10 wk old, derived from the strain developed at Genentech (South San Francisco, CA) by Calalano et al. (26), a gift from Dr. Robert Terkeltaub, and maintained by Ann Richmond). Five days postimplantation, corneas were photographed at an incipient angle of 35–50° from the polar axis in the meridian containing the pellet, using a Zeiss split lamp. Images were digitalized and processed by subtractive color filters (Adobe Photoshop 4.0; Adobe Systems, Mountain View, CA); the fraction of the total corneal image that was vascularized (vascular area), and the ratio of pixels marking neovascular capillaries, both within the vascularized region (regional vascular density) and within the total corneal image (total vascular density), were calculated using the Bioquant software (Nashville, TN).

### Statistical analysis

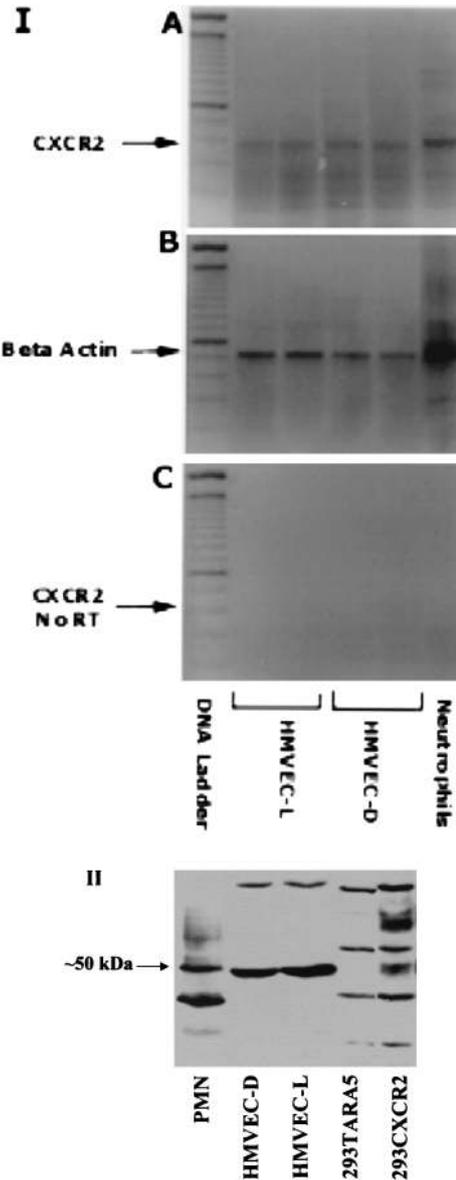
Data were analyzed on a PC computer using the Statview 5.0 statistical package (Abacus Concepts, Berkeley, CA). Comparisons were made using the unpaired *t* test or the Mann-Whitney test for nonparametric data. Data were considered statistically significant if *p* values were 0.05 or less.

## Results

### *CXCR2 is expressed by microvascular endothelial cells*

We sought to determine the mechanism by which the ELR<sup>+</sup> CXC chemokines mediate the angiogenic activity of endothelial cells, by examining the presence and the role of the chemokine receptor CXCR2, in angiogenesis. Because all ELR<sup>+</sup> CXC chemokines induce angiogenic activity, and CXCR2 on neutrophils appears to bind all of these chemokines, we first evaluated whether CXCR2 mRNA was expressed in HMVEC, as compared with neutrophils. Total RNA was extracted from HMVEC-L and HMVEC-D, as well as neutrophils. RT-PCR analysis demonstrated the presence of an appropriate band for CXCR2 mRNA by gel electrophoresis in both HMVEC lines, as well as in neutrophils (Fig. 1, *IA*).  $\beta$ -actin served as an internal control (Fig. 1, *IB*). No bands were visualized using the same primers for CXCR2 under conditions in which reverse transcriptase was excluded from the reaction before PCR (Fig. 1, *IC*). This finding supported the notion that the RT-PCR product/bands seen in Fig. 1, *IA*, was not due to genomic DNA contamination of the specimens before PCR. To further confirm that this mRNA was transcribed into protein, we used an Ab specific for human CXCR2 in Western blot analysis to determine whether this receptor was expressed in microvascular endothelial cells at the protein level. The molecular mass of CXCR2 has been reported to be a 44- to 46-kDa band in Western blot analysis by other investigators (27, 28). Total protein extracts of human neutrophils, HMVEC-D, HMVEC-L, control-transfected 293 cells, and CXCR2-transfected 293 cells were made in TNE lysis buffer. An aliquot containing 50  $\mu$ g of total protein was subjected to SDS-PAGE and transferred to polyvinylidene fluoride membrane for Western blot analysis using a rabbit polyclonal Ab specific to human CXCR2. A protein band of molecular mass of ~50 kDa was specifically recognized by the human CXCR2 Ab in lysates from human neutrophils and CXCR2-transfected 293 cells (positive controls), while this band was absent in control-transfected 293 cells (Fig. 1, *II*). A protein band of similar molecular mass was also recognized by the Ab in both HMVEC-D and HMVEC-L protein extracts (Fig. 1, *II*). The presence of this ~50-kDa protein species in endothelial cell extracts was also confirmed by Western blot analysis using a second commercially available Ab to human CXCR2 (data not shown).

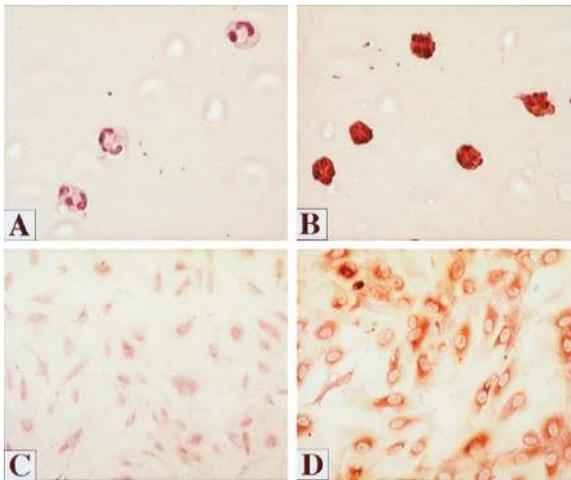
To further demonstrate the presence of CXCR2 on human endothelial cells, we determined the immunolocalization of CXCR2 by immunohistochemistry. Human neutrophils were used as a positive control for CXCR2 immunolocalization, and following immunohistochemistry, a distinct positive staining pattern was observed in human neutrophils in the presence of CXCR2-specific Ab (Fig. 2, *IIB*), which was absent when normal rabbit serum was used as a primary Ab control (Fig. 2, *IIA*). In a similar manner, HMVEC-L demonstrated specific staining for expression of CXCR2 in the presence of anti-human CXCR2-specific Ab (Fig. 2, *IID*), as compared with the absence of staining in the presence of control Ab (Fig. 2, *IIC*). CXCR2 protein was also detected in HMVEC-D monolayers following immunohistochemical staining (data not shown). These findings support the presence of an immunoreactive protein consistent with CXCR2 from HMVEC that appears to be similar to that which has been detected on human neutrophils.



**FIGURE 1.** CXCR2 is detected in HMVEC by both RT-PCR and Western blot analysis. *IA*, Demonstrates RT-PCR detection of CXCR2 mRNA, as compared with  $\beta$ -actin (*IB*) and PCR in the absence of prior reverse-transcriptase (RT) treatment (*IC*). *II*, Western blot detection of CXCR2 protein from PMN; HMVEC-D and HMVEC-L, respectively; and 293 cells transfected with CXCR2 mRNA (293CXCR2), as compared with control cells (293TARA5). Endothelial cells were found to possess a band (indicated by the arrow) that was recognized by the CXCR2 Ab at ~50 kDa. This protein comigrated with a similar protein in human neutrophils and CXCR2-transfected 293 cells.

### *Abs to CXCR2 inhibit chemotaxis of microvascular endothelial cells in response to ELR<sup>+</sup> CXC chemokines*

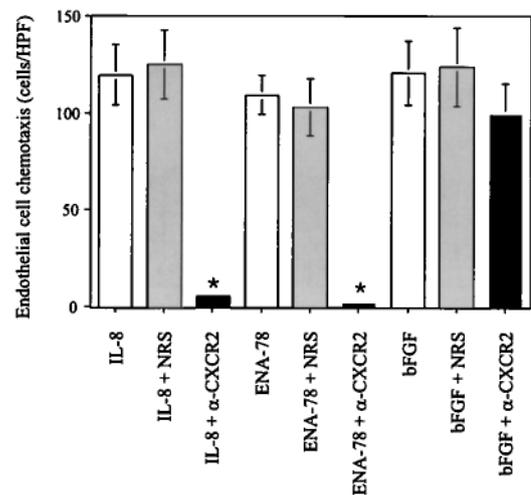
A function of an angiogenic factor is to induce the chemotaxis of endothelial cells. We have previously shown that the ELR<sup>+</sup> CXC chemokines are potent agonists of endothelial cell chemotaxis, while the ELR<sup>-</sup> CXC chemokines inhibit chemotaxis mediated by both ELR<sup>+</sup> CXC chemokines and bFGF (3). To determine whether this chemotaxis was mediated through ligand binding and subsequent signal transduction via CXCR2, we performed chemotaxis assays in the presence or absence of neutralizing Ab to human CXCR2, as compared with normal rabbit serum as a control. As shown in Fig. 3, addition of 10 nM of the ELR<sup>+</sup> CXC chemokines



**FIGURE 2.** Immunohistochemical staining in both human neutrophils and cultured microvascular endothelial cells. Cytopins of human peripheral neutrophils (A and B) or monolayers of human lung microvascular endothelial cells (C and D) were subjected to immunohistochemical staining using either normal rabbit serum (A and C) or a polyclonal Ab specific to human CXCR2 (B and D) as a primary Ab. Both human neutrophils and endothelial cells were found to stain positive for the presence of CXCR2.

IL-8 or ENA-78 induced specific HMVEC-L chemotaxis ( $118.4 \pm 15.6$  and  $108.1 \pm 9.6$  cells/HPF, respectively). bFGF (5 nM) was also shown to induce similar levels of endothelial cell chemotaxis in our assay system to those observed with the ELR<sup>+</sup> CXC chemokines ( $119.4 \pm 16.1$  cells/HPF). To determine whether CXCR2 was mediating the endothelial cell chemotactic effect of ELR<sup>+</sup> CXC chemokines, we addressed whether specific Abs to human CXCR2 inhibited HMVEC-L chemotaxis. HMVEC-L were preincubated in the presence of anti-human CXCR2 or normal control Abs, and then used in the chemotaxis assay. As compared with cells incubated with angiogenic stimuli alone, HMVEC-L chemotaxis in response to IL-8 or ENA-78 was attenuated by 97% or 99%, respectively ( $p < 0.001$ ), by the presence of neutralizing Ab to human CXCR2. In contrast, preincubation of HMVEC-L with control Abs yielded chemotaxis results similar to those obtained in the presence of the ELR<sup>+</sup> CXC chemokines alone ( $123.8 \pm 17.7$  and  $101.7 \pm 14.4$  cells/HPF for IL-8 and ENA-78, respectively). A similar inhibition of HMVEC-L chemotaxis in response to the ELR<sup>+</sup> CXC chemokine GRO- $\alpha$  was also observed in the presence of anti-CXCR2 Abs (data not shown). Moreover, preincubation of endothelial cells with Abs to human CXCR2 had no significant effect on HMVEC-L chemotaxis in response to bFGF (Fig. 3). A similar inhibition of chemotaxis by anti-human CXCR2 in response to ELR<sup>+</sup> CXC chemokines was also found when HMVEC-D were used (data not shown).

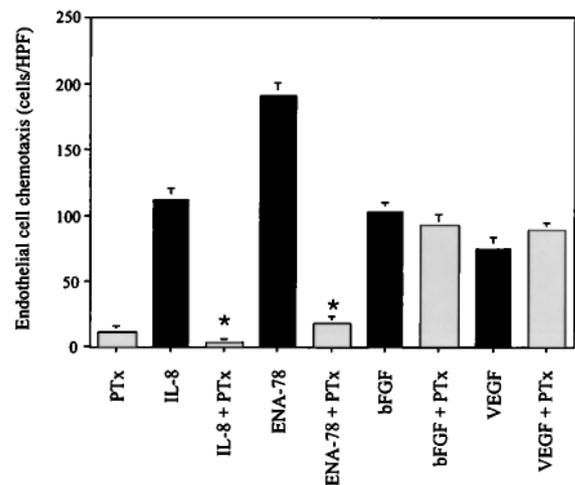
To exclude that CXCR1 might be contributing to the endothelial cell chemotactic response to IL-8, we investigated the role of CXCR1 in the chemotactic response of endothelial cells to the ELR<sup>+</sup> CXC chemokines. HMVEC-L were preincubated with either anti-human CXCR1 or control Abs, and the specific migration in response to IL-8, ENA-78, bFGF, or VEGF was determined. We observed no difference in endothelial cell-specific migration in response to any of the angiogenic factors tested in the presence of Abs to human CXCR1, as compared with the specific migration observed in the presence of control Abs (data not shown). These results support that CXCR2 is the primary CXCR responsible for endothelial cell migration induced by the ELR<sup>+</sup> CXC chemokines.



**FIGURE 3.** Endothelial cell chemotaxis induced by the ELR<sup>+</sup> CXC chemokines is inhibited by Ab to human CXCR2. Endothelial cell chemotaxis (cells/HPF;  $\times 400$ ) was determined by the average number of cells that had migrated in response to IL-8 (80 ng/ml) or ENA-78 (80 ng/ml), or of 50 ng/ml of bFGF in the presence or absence of anti-CXCR2, as compared with control Abs. Each bar is a representation of the mean and SEM determined from the average counts of three independent experiments. \*,  $p < 0.05$ .

#### Endothelial cell chemotaxis in response to ELR<sup>+</sup> CXC chemokines is inhibited by PTx

To confirm that signal transduction through CXCR2, a G protein-linked receptor, was responsible for the endothelial cell chemotactic response to the ELR<sup>+</sup> CXC chemokines, we tested the ability of PTx to inhibit this specific migration. The chemotactic response of HMVEC-L to either IL-8, ENA-78, bFGF, or VEGF in the presence or absence of PTx is shown in Fig. 4. Endothelial cell chemotaxis in response to IL-8 or ENA-78 was attenuated by 97% and 91%, respectively ( $p < 0.001$ ), in the presence of 2.5 mM PTx. In contrast, HMVEC-L chemotaxis in response to bFGF or VEGF was unaltered in the presence of PTx ( $p = 0.25$  and  $0.28$ ,



**FIGURE 4.** Endothelial cell chemotaxis induced by the ELR<sup>+</sup> CXC chemokines is PTx sensitive. Endothelial cell chemotaxis (cells/HPF;  $\times 400$ ) was determined by the average number of cells that had migrated in response to IL-8 (80 ng/ml) or ENA-78 (80 ng/ml), or of 50 ng/ml for bFGF and VEGF in the presence or absence of PTx. Each bar is a representation of the mean and SEM determined from the average counts of three independent experiments. \*,  $p < 0.05$ .

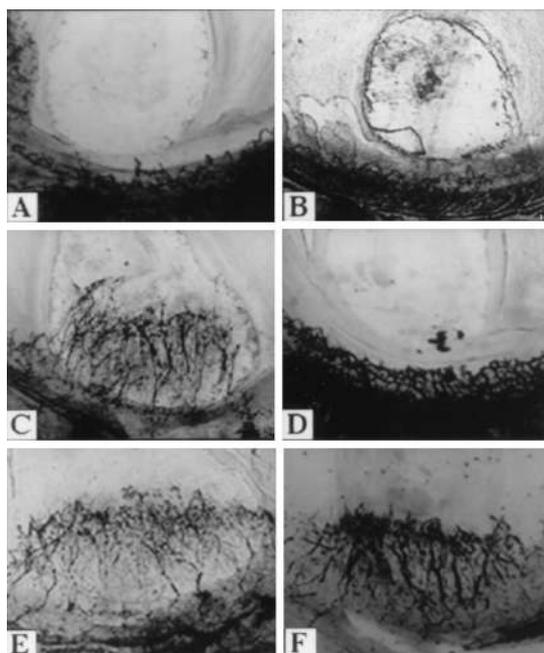
respectively). These data indicate that HMVEC-L chemotaxis in response to ELR<sup>+</sup> CXC chemokines, but not to bFGF or VEGF, is mediated by a signal transduction pathway associated with PTX-sensitive G proteins. These data further support CXCR2 as the candidate receptor mediating endothelial cell chemotaxis to ELR<sup>+</sup> CXC chemokines.

*The ability of ELR<sup>+</sup> CXC chemokines to induce angiogenesis in vivo is blocked by the presence of Abs to CXCR2*

To ascertain that CXCR2 was responsible for angiogenesis mediated by the ELR<sup>+</sup> CXC chemokines in vivo, we performed the corneal micropocket assay of neovascularization in the rat. Various angiogenic agents, including human IL-8, KC (the murine homologue of human GRO- $\alpha$ ), MIP-2 (the murine homologue of human GRO- $\beta$ ,  $\gamma$ ), human ENA-78, and the nonchemokine angiogenic factors bFGF and VEGF, were embedded in Hydron pellets in the presence of anti-murine CXCR2 or control Abs. All the ELR<sup>+</sup> CXC chemokines tested, IL-8, ENA-78, KC, and MIP-2, induced neovascularization in the presence of control Abs (Fig. 5C and Table I). However, in the presence of neutralizing anti-murine CXCR2 Abs, angiogenesis induced by ELR<sup>+</sup> CXC chemokines was markedly inhibited (Fig. 5D and Table I). In contrast, anti-murine CXCR2 Abs failed to inhibit angiogenesis induced by either bFGF or VEGF (Fig. 5, E and F, and Table I). These findings support the contention that ELR<sup>+</sup> CXC chemokine-induced angiogenesis in vivo is dependent upon CXCR2.

*Angiogenesis induced by ELR<sup>+</sup> CXC chemokines is attenuated in CXCR2<sup>-/-</sup> mice*

To further demonstrate the importance of CXCR2 in ELR<sup>+</sup> CXC chemokine-induced angiogenesis, we performed corneal micro-



**FIGURE 5.** Abs to rodent CXCR2 inhibit angiogenesis mediated by the ELR<sup>+</sup> CXC chemokines in the rat corneal micropocket assay. Representative corneas are shown for the neovascularization induced by vehicle alone, IL-8 (80 ng), or bFGF (50 ng) in the presence of normal rabbit serum (A, C, and E, respectively) or in the presence of neutralizing Ab to rodent CXCR2 (B, D, and F, respectively). Angiogenic responses in the presence or absence of Abs to CXCR2 for ENA-78, KC, or MIP-2 appeared identical with those observed for IL-8 (data not shown). In addition, the response to VEGF was similar to those observed for bFGF (data not shown).

Table I. *Ab to mCXCR2 inhibits neovascularization induced by the ELR<sup>+</sup> CXC chemokines in the rat cornea*

| Angiogenic Agent           | Percent Positive for Neovascularization |                                 |
|----------------------------|---|---------------------------------|
|                            | Normal rabbit serum <sup>a</sup>        | Rabbit anti-mCXCR2 <sup>a</sup> |
| Vehicle alone <sup>b</sup> | 0/6 (0%)                                | 0/6 (0%)                        |
| IL-8 <sup>c</sup>          | 4/4 (100%)                              | 1/5 (20%)                       |
| ENA-78 <sup>c</sup>        | 4/4 (100%)                              | 0/5 (0%)                        |
| MIP-2 <sup>c</sup>         | 4/4 (100%)                              | 1/5 (20%)                       |
| KC <sup>c</sup>            | 4/4 (100%)                              | 0/5 (0%)                        |
| bFGF <sup>d</sup>          | 4/4 (100%)                              | 4/4 (100%)                      |
| VEGF <sup>d</sup>          | 4/4 (100%)                              | 4/4 (100%)                      |

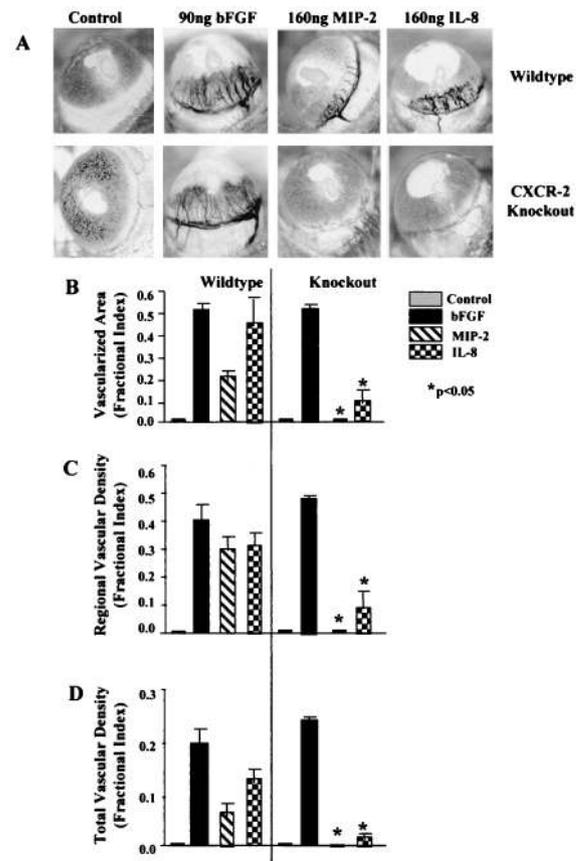
<sup>a</sup> Normal rabbit serum or rabbit polyclonal Abs specific to mCXCR2 were added to a final dilution of 1:1000.

<sup>b</sup> Vehicle used as a diluent was PBS with 0.25% human serum albumin.

<sup>c</sup> Concentration used was 80 ng/pellet.

<sup>d</sup> Concentration used was 50 ng/pellet.

pocket assays in CXCR2<sup>-/-</sup> mice and their wild-type counterparts. Vigorous neovascular responses toward Hydron pellets containing bFGF, murine MIP-2, or human IL-8 were observed in wild-type mice (Fig. 6A, top panels). In contrast, neovascular responses to the ELR<sup>+</sup> CXC chemokines were drastically reduced in



**FIGURE 6.** Angiogenic responses to rMIP-2 and rIL-8 are impaired in the corneas of CXCR2<sup>-/-</sup> as compared with CXCR2<sup>+/+</sup> mice. Hydron pellets impregnated with vehicle, bFGF (3 pmol), rMIP-2 (20 pmol), or rIL-8 (20 pmol) were implanted. Corneas were photographed at 5 days postimplantation (A). Digitalized images were analyzed to yield vascularized area (B), regional vascular density (C), and total vascular density (D). Data are expressed as mean values where indicated,  $\pm$  SEM. \*,  $p < 0.05$ . Three animals and six corneas were used in each group.

CXCR2<sup>-/-</sup> mice, while angiogenesis in CXCR2<sup>-/-</sup> mouse corneas that had been implanted with pellets containing bFGF remained vigorous (Fig. 6A, *bottom panel*). The degree of neovascularization of each cornea was estimated by analysis of digitized images, as described in *Materials and Methods*. A reduction in the vascularized area (Fig. 6B), the regional vascular density (Fig. 6C), and the total vascular density (Fig. 6C) was observed in response to IL-8 and MIP-2 in CXCR2<sup>-/-</sup> corneas as compared with their wild-type counterparts. In contrast, there was no significant difference in these parameters between CXCR2<sup>-/-</sup> mice and wild-type controls when bFGF was used as the angiogenic stimulus. These data further support the importance of CXCR2 in mediating angiogenesis induced by the ELR<sup>+</sup> CXC chemokines.

## Discussion

The ELR<sup>+</sup> CXC chemokines have been shown to be potent inducers of endothelial cell chemotaxis *in vitro* and of angiogenesis *in vivo* (3, 29, 30). The expression of these molecules has also been found to be associated with disease states that are known to involve neovascularization. For instance, IL-8, ENA-78, and GRO- $\alpha$  overexpression is associated with tumor progression and metastasis in a variety of tumor models, such as nonsmall cell lung carcinoma (31, 32), gastric carcinoma (33), and melanoma (34). Moreover, survival of nude mice bearing ovarian tumors was inversely correlated with the expression of IL-8 by these tumors (35). The overexpression of these ELR<sup>+</sup> CXC chemokines has also been shown to be associated with angiogenesis of psoriasis (36), idiopathic pulmonary fibrosis (24), and granulation tissue of burn wounds (37). To act as an angiogenic agent, the ELR<sup>+</sup> CXC chemokines should be able to induce the chemotaxis of endothelial cells following specific ligand-receptor interactions. We hypothesized that the chemokine receptor CXCR2 was present on microvascular endothelial cells and is responsible for the angiogenic effect mediated by ELR<sup>+</sup> CXC chemokines. This contention is supported by the fact that CXCR2 is known to bind all the ELR<sup>+</sup> CXC chemokines that induce angiogenesis, including IL-8; ENA-78; GRO- $\alpha$ , - $\beta$ , and - $\gamma$ ; NAP-2; and GCP-2 (8). The expression of microvascular endothelial cell CXCR2 has also been found in various disorders that are associated with a predominance of neovascularization. CXCR2 expression has been localized to malignant melanoma cells and microvascular endothelium within melanomas (34), as well as to microvessels in head and neck squamous cell carcinoma (38), and to both large and small vessel endothelial cells in human breast carcinoma (39). CXCR2 expression has also been localized to microvascular endothelial cells of granulation tissue associated with burn wounds (37).

Our results indicate that the seven-transmembrane G protein-linked chemokine receptor CXCR2 can be found in microvascular endothelial cells of both dermal and lung origin. This result is in contrast to that reported by other researchers, who have indicated that CXCR1, but not CXCR2 expression could be detected on large vessel endothelial cells (40). It is unlikely that CXCR1 is responsible for the angiogenic activity attributable to the ELR<sup>+</sup> CXC chemokines, because only IL-8 and GCP-2 have the potential to bind to and signal via CXCR1 (11). Our data demonstrate that microvascular endothelial cell chemotaxis in response to the ELR<sup>+</sup> CXC chemokines IL-8 and ENA-78 was unaffected by the presence of Abs to human CXCR1, indicating that although this receptor may be expressed on HMVEC-L, it is not responsible for endothelial cell chemotaxis mediated by ELR<sup>+</sup> CXC chemokines. Additionally, there is no known rodent homologue of CXCR1; therefore, neovascularization induced by the ELR<sup>+</sup> CXC chemokines in the rodent corneal micropocket assay for angiogenesis may not be mediated through CXCR1 binding. This notion was

confirmed in the studies using CXCR2<sup>-/-</sup> mice, in which we found that murine MIP-2 failed to induce an angiogenic response, as compared with a positive angiogenic response in the wild-type mice. However, we also found that the angiogenic response to human IL-8 was not completely inhibited in the CXCR2<sup>-/-</sup> mice. We cannot exclude that this response was due to a nonspecific angiogenic effect related to inflammation/immunity in response to the use of a human Ag in the mouse cornea. In addition, we cannot exclude that this small angiogenic response may be due to another receptor in the mouse that binds IL-8, but not MIP-2. The Abs that recognize human CXCR2 used in our experiments were from two separate sources, and have been shown to be specific to CXCR2 and do not cross-react with CXCR1. Thus, the results of our Western blot and immunohistochemical staining cannot be due to cross-reaction with CXCR1 present on endothelial cells. Our results indicating the presence of CXCR2 on microvascular endothelial cells are further supported by the fact that this Ab can functionally inhibit endothelial cell chemotaxis in response to not only IL-8, but also ENA-78 and GRO- $\alpha$ , which do not bind CXCR1.

Our results also indicate that the chemotactic response to the ELR<sup>+</sup> CXC chemokines is inhibited by PTx, which is consistent with a mechanism whereby the ELR<sup>+</sup> CXC chemokines induce endothelial cell chemotaxis through a PTx-sensitive G protein signal transduction pathway. PTx, isolated from the bacteria *Bordetella pertussis*, is known to inhibit signal transduction mediated by certain G proteins, such as the G<sub>i</sub>/G<sub>o</sub> proteins, following ADP ribosylation of the G $\alpha$  subunit of the heterotrimeric G protein species (41, 42). Signal transduction mediated by ligand binding of CXCR2 has been shown to be dependent on the interaction of the chemokine receptor with the PTx-sensitive G<sub>12</sub> G protein in neutrophils and 293 cells stably transfected to express CXCR2 (43, 44). The failure of PTx to inhibit endothelial cell chemotaxis mediated by bFGF in our assay system is consistent with the specificity of PTx to inhibit G protein-mediated signal transduction pathways, and further supports the contention that ELR<sup>+</sup> CXC chemokines induce angiogenesis through interaction with and activation of CXCR2.

There have been many discrepancies found in the literature regarding CXCR2 expression by endothelial cells. It is possible that endothelial cells may express different chemokine receptor repertoires depending on the cell culture conditions, the degree of confluence (45), or matrix the cells are grown on at the time of analysis of receptor expression. In addition, it has been demonstrated that binding of IL-8 to the endothelium *in vivo* shows heterogeneity in different segments of the vascular tree and within similar types of vessels located in different organs (46). Therefore, the disparate results concerning the expression of CXCR1 or CXCR2 on endothelium could be a result of different sources of endothelial cells, or different isolation and culture techniques. Rot et al. (46) have also reported that dermal microvascular endothelial cells that have previously demonstrated the capacity to bind IL-8 *in vivo* lose their ability to bind IL-8 during isolation and subsequent cell culture, suggesting that some phenotypic changes may occur in endothelial cells as a result of these culture techniques. While these studies concluded that IL-8 binding to endothelial cells was a result of association of the ligand with glycosaminoglycan residues on the surface of the cells (46), our data would support the contention that CXCR2 is also present on microvascular endothelial cells and that this receptor is critically involved in mediating endothelial cell chemotaxis and angiogenesis in response to ELR<sup>+</sup> CXC chemokines.

DARC has also been shown to be expressed on postcapillary endothelial cells (47, 48), and therefore could potentially be the putative receptor mediating angiogenesis induced by the ELR<sup>+</sup>

CXC chemokines. This is unlikely, because although this receptor does bind all the ELR<sup>+</sup> CXC chemokines known to induce angiogenesis, it also binds other chemokines of the CC family, which to date have no known angiogenic properties. In addition, there have been no indications that DARC mediates signal transduction events following ligand binding of chemokines (19), and because of this observation, it has been proposed to act as a sponge that binds excess free chemokines during inflammatory responses. Moreover, our finding for the fundamental role of CXCR2 in mediating endothelial cell migration in vitro and angiogenesis in vivo would suggest that DARC is unlikely to play a direct role in endothelial cell chemotaxis to ELR<sup>+</sup> CXC chemokines. Similarly, although CXCR4 is readily expressed on endothelial cells (45, 49, 50), CXCR4 is not known to bind the ELR<sup>+</sup> CXC chemokines and can thus be excluded as the receptor responsible for ELR<sup>+</sup> CXC chemokine-induced angiogenesis.

The identification of CXCR2 as the receptor responsible for mediating angiogenesis induced by the ELR<sup>+</sup> CXC chemokines has important implications. As mentioned, CXCR2 expression has been shown to be associated with neovascularization in the context of certain diseases such as psoriasis and tumorigenesis. Antagonists specific for CXCR2 could be used therapeutically to inhibit ELR<sup>+</sup> CXC chemokine-dependent neovascularization, and improve the prognosis of patients with diseases that are associated with marked angiogenesis. Inhibition of neovascularization has been shown to drastically inhibit the progression and metastasis of various cancers; thus, inhibition of ELR<sup>+</sup> CXC chemokine-mediated angiogenesis could also be advantageous as adjuvant cancer therapy. We have shown that neutralization of IL-8 or ENA-78 using specific neutralizing Abs to these molecules can inhibit the growth of nonsmall cell lung carcinoma in SCID mice (31, 32). These treatments, however, only reduced tumor growth by 40–60%. This is most likely the result of the fact that these tumors produce multiple ELR<sup>+</sup> CXC chemokine ligands contributing to their tumorigenicity. Inhibition of ligand binding to CXCR2, or inhibition of the signal transduction events following activation of the CXCR2 receptor would in essence simultaneously inhibit the biological effect of multiple ELR<sup>+</sup> CXC chemokines. Future work from our laboratory will focus on the inhibition of tumor growth and metastasis by antagonizing the function of the CXCR2 receptor.

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