Expression of CCR2 by Endothelial Cells
Implications for MCP-1 Mediated Wound Injury Repair and In Vivo Inflammatory Activation of Endothelium


Abstract—Endothelial cell proliferation and migration may play a central role in angiogenesis, wound healing, and atherosclerosis. Although CXC chemokines can act on endothelial cells by influencing proliferation, an involvement of CC chemokines and endothelial expression of chemokine receptors remains to be elucidated. Reverse transcription-polymerase chain reaction, RNase protection, Western blot, and flow cytometric analysis showed that human umbilical vein endothelial cells express mRNA and surface protein of the monocyte chemotactic protein-1 (MCP-1) receptor CCR2, which was upregulated by inflammatory cytokines. MCP-1 induced migration of endothelial cells in a transwell assay, which was inhibited by the 9-76 MCP-1 receptor antagonist. Increased secretion of MCP-1 or interleukin-8, but not RANTES, on endothelial injury suggested a functional role of CCR2 in wound repair as measured by ELISA. After mechanical injury to endothelial monolayers, which spontaneously closed within 24 hours, wound repair was delayed by the 9-76 antagonist and by a blocking monoclonal antibody to MCP-1, but not to interleukin-8, and was improved by exogenous MCP-1. This was confirmed by quantification of cell migration into the wound area, whereas proliferation and viability were unaltered by MCP-1 or its analogue. Notably, immunohistochemistry of inflamed tissue revealed CCR2 staining on arterial, venous, and venular endothelium affected by cellular infiltration. This is the first demonstration of endothelial CCR2 expression ex vivo, inferring its involvement in inflammatory conditions. Thus endothelial cells express functional CCR2 that may have important implications for endothelial wound repair and inflammatory reactions. (Arterioscler Thromb Vasc Biol. 1999;19:2085-2093.)

Key Words: chemokine receptors ■ migration ■ chemokines ■ endothelial cells
tion to MCP-1, may contribute to the repair of endothelial wounds, and may be upregulated during inflammatory activation.

Methods

Cell Culture and Reagents

Human umbilical vein endothelial cells (HUVEC) were maintained in low serum PromoCell endothelial cell growth medium, as described. Monoclonal antibodies (mAb) to CCR2 were kindly provided by Dr G. LaRosa (clone 5A11, IgG1) (Leukosite, Cambridge, Mass) or purchased from R&D Systems (clone No. 48607.121, IgG2b). Neutralizing MCP-1 mAb (clone No. 24822.111, IgG1), IL-8 mAb (clone No. 6217.11, IgG1), or isotype control mAb were from R&D Systems. Recombinant human MCP-1 was from PeproTech. The 9-76 peptide analogue of MCP-1 was a kind gift from Dr I. Clark-Lewis (University of British Columbia, Vancouver). All other reagents were purchased from Sigma Chemical Co, unless otherwise stated.

Reverse Transcription-Polymerase Chain Reaction

Analysis of mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR) was performed as described. Briefly, total RNA was isolated from 10^6 HUVEC by phenol/chloroform/isooamylic alcohol extraction, and cDNA was reverse transcribed from 1 μg RNA. Primers were synthesized according to sequences with minimal homology to yield products of 766 bp for CCR2. The CCR2B cDNA was kindly provided by Dr I. Charo (University of California, San Francisco) was used as a control.

RNase Protection Assay

Total RNA was isolated from HUVEC left unstimulated or treated with IL-1β (5 ng/mL) for 24 hours as described. Twenty μg of RNA was analyzed according to the manufacturer’s instructions (PharMingen 1996 RiboQuant Multi-Probe RNase Protection Assay System). The probe standard template hCR-5 allowed determination of mRNA levels encoding the receptors: CCR1, CCR2, CCR3, CCR4, CCR8, and as control mRNA for glycerol aldehyde phosphate dehydrogenase (GAPDH). The protected mRNA bands were quantitated using a Molecular Dynamics phosphorimager. Data are expressed as relative expression of CCR2 mRNA normalized to GAPDH mRNA levels.

Western Blot

HUVEC or Mono Mac 6 cells (10^6) were lysed in sample buffer and whole cell lysates separated by 15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membrane. Membranes were blocked overnight at 4°C, incubated with a CCR2 mAb (clone No. 48607.121) and reacted with a peroxidase-labeled sheep anti-mouse Ig mAb. Blots were developed with chemiluminescence (ECL, Amersham) and exposed to X-Omat AR film (Amersham).

Flow Cytometry

Flow cytometry was performed as described. Briefly, confluent HUVEC were detached and reacted with isotype control or CCR2 mAb 5A11 (10 μg/mL) in Hanks’ buffered salt solution with 10 mM Hepes, 1 mM Mg²⁺, 1 mM Ca²⁺, and 0.5% human serum albumin for 30 minutes on ice. Cells were stained with fluorescein isothiocyanate goat anti-mouse IgG mAb for 30 minutes on ice and analyzed in a FACScan (Becton Dickinson) with appropriate light scatter gates. After correction for unspecific binding, the specific mean fluorescence intensity was expressed in channels.

Endothelial Transmigration Assay

HUVEC transmigration assays were performed as described. Briefly, transwell inserts (Costar, 5 μm pores) were coated with 0.2% gelatin for 30 minutes at 37°C. MCP-1 in assay medium (RPMI-1640/M199, 0.5% human serum albumin) was added at indicated concentrations to 24-well plates. HUVEC (100 μL at 10^6 cells/mL) were added to the top chamber and allowed to transmigrate for 4 hours at 37°C. Some experiments were also performed with MCP-1 (100 nmol/L) present in the top and in the bottom chamber. Cells remaining in the upper chamber were removed with a cotton swab. Migrated cells that had adhered to the bottom surface of the filter were fixed and stained with 0.1% crystal violet in 0.1 mol/L borate, pH 9.0, and 2% ethanol. Stained cells were extracted with 10% acetic acid and absorbance (optical density) at 600 nmol/L was determined as a measure of transmigration. For inhibition assays, the 9-76 MCP-1 peptide analogue (125 nmol/L) was added to the top and bottom chamber. Data were expressed as mean±SD of 3 experiments.

Multiple Injury and Quantification of Chemokine Secretion

For multiple wound injury, HUVEC were grown to confluence in 100 mm petri dishes and injured by dragging a 1 mm-toothed comb across monolayers in a circular motion, removing 50% of HUVEC and resulting in concentric injuries to most of the remaining cells. Cells were washed and fed fresh medium, and after 6 hours supernatants were collected and sterile filtered. The concentration of MCP-1, IL-8, and RANTES in the supernatants were quantitated by sandwich ELISA (all R&D Systems) according to the manufacturer’s protocols with the protein standards provided and were adjusted for the number of cells remaining.

Endothelial Wound Injury Repair and Proliferation Assays

Single-path wound repair experiments were performed as described. Briefly, HUVEC monolayers were grown to confluence in 6-well dishes, and wounds were inflicted by dragging a sterile pipette tip across the monolayers to create a 1-mm cell-free path. The 9-76 MCP-1 peptide analogue (1.25 or 125 nmol/L), MCP-1 (50 nmol/L), blocking MCP-1 mAb (clone No. 24822.111), or IL-8 mAb (clone No. 6217.11, 10 μg/mL each) were added after injury, and cells were incubated for at least 24 hours. The wound areas were examined and photographed, and 24 hours after injury by phase contrast microscopy with a 10× objective. The number of cells that had migrated into 1 mm² of wound area after 12 hours was quantitated by counting within defined areas using light microscopy with a 10× objective and 10× ocular equipped with a 1 mm×1 mm grid. Treatment with the 9-76 peptide (100 nmol/L) for 24 hours did not affect HUVEC viability, proliferation, or trypan blue exclusion (93±2% viable cells versus 94±4% in controls).

For HUVEC proliferation assay, cells were seeded at a density of 10^5/cm². For 3H thymidine incorporation assays, cells were seeded in 96-well plates and were treated for 24 hours with or without indicated concentrations of MCP-1 or the 9-76 MCP-1 peptide analogue. Proliferation of cells grown in 24-well plates was quantitated over 72 hours by counting the number of cells in marked areas by light microscopy using a 10× objective and 10× ocular equipped with a 1 mm×1 mm grid. For multiple injury experiments, cells were wounded at 96 hours and incubated for 48 hours with MCP-1 peptide analogue (125 nmol/L) present in the top and bottom chamber. Cells remaining in the upper chamber were removed with a cotton swab, and migrated cells were stained with 0.1% crystal violet in 0.1 mol/L borate. Supernatants were collected and sterile filtered. The concentration of MCP-1, IL-8, and RANTES in the supernatants were quantitated by sandwich ELISA (all R&D Systems) according to the manufacturer’s protocols with the protein standards provided and were adjusted for the number of cells remaining.

Immunohistochemistry

Adult kidneys were received after surgical removal due to reflux nephropathy or renal cell carcinoma. Tumor-free parts were excised, frozen in isopentane, cooled in liquid nitrogen, and stored at −18°C. Immunohistochemistry was performed on 5-μm frozen tissue sections fixed in acetone at −18°C for 10 minutes. Sections were incubated with 5% goat serum in a Tris buffer (pH 7.6) containing 0.4% Triton-X100, then with a CCR2 mAb (clone No. 48607.121, 1.2 dilution) for 2 hours at 22°C, and finally with a rabbit anti-mouse IgG antibody (Dako, 1:40 dilution) for 1 hour at 22°C. Alkaline phosphatase–complexed mouse antialkaline phosphatase mAb (APAAP, Dako) diluted at 1:40 was then incubated at 22°C for 1 hour. All dilutions were in phosphate-buffered saline (pH 7.6). For
staining, slides were exposed to a solution of sodium nitrite (28 mmol/L), new fuchsin (basic fuchsin, 21 mmol/L), naphtol AS-BI phosphate (0.5 mmol/L), dimethylformamide (64 mmol/L), and levamisole (5 mmol/L) in a Tris/HCl buffer (50 mmol/L, pH 8.4) containing 146 mmol/L NaCl for 15 minutes. Control experiments included immunohistology 1) with nonimmune mouse IgG, 2) without primary mAb, and 3) without secondary mAb. Sections were evaluated with a Zeiss Axioplan microscope, and micrographs were recorded with a Hasselblad camera.

Results

Expression of CCR2 mRNA in Endothelial Cells
To investigate the expression of the MCP-1 receptor CCR2 on endothelium, we performed RT-PCR to amplify RNA isolated from HUVEC using specific primers for CCR2. The PCR products were analyzed by agarose gel electrophoresis (Figure 1). Using the CCR2 plasmid cDNA as a positive control template, a band migrating at an expected molecular weight of 766 kb was observed (Figure 1, lane 2), whereas analysis of the PCR products generated from HUVEC cDNA revealed a band migrating at an apparently identical molecular weight (Figure 1, lane 1). To confirm that this band corresponds to CCR2 mRNA, PCR products were analyzed by digestion with the restriction enzymes Apa1 and Dra1. Digestion of PCR products from control CCR2 plasmid cDNA and from HUVEC cDNA with Apa1 resulted in 2 fragments with expected molecular weights of 188 and 608 kb, whereas Dra1 digestion yielded fragments of 285 and 481 kb (Figure 1, lanes 4 to 7).

To confirm these results, RNase protection assays were used to study whether mRNA for CCR2 or other CC chemokine receptors were transcribed in HUVEC. Analysis of total RNA isolated from untreated HUVEC in comparison with the probe standard template hCR-5 revealed a band corresponding to the CCR2 A and B subtypes (Figure 2A). A more intense band of identical size was found when analyzing the RNA from monocytic Mono Mac 6 cells (not shown). HUVEC isolates also expressed a weakly detectable band corresponding to CCR1 but not mRNA transcripts encoding CCR3, CCR4, CCR5, and CCR8 (not shown). Treatment of HUVEC with IL-1β (5 ng/mL) for 24 hours markedly increased the intensity of the band corresponding to CCR2 mRNA (Figure 2A). Quantification confirmed upregulation of CCR2 mRNA by IL-1β relative to GAPDH mRNA expression (Figure 2B). Differences in the amount of RNA loaded may account for the relatively less dramatic induction of CCR2 mRNA expression after normalization in Figure 2B. Because CCR2 mRNA in monocytes is downregulated by inflammatory cytokines,18,19 this excludes potential contaminations with monocyte mRNA. Hence this demonstrates that human endothelial cells express inducible mRNA for the MCP-1 receptor CCR2.

Surface Expression of CCR2 Protein as a Functional Receptor on HUVEC
We next studied whether CCR2 protein was present in endothelial cells. Immunoblotting of HUVEC lysates revealed a band migrating at 38kDa consistent with the expression of CCR2 protein in endothelial cells, whereas Mono Mac 6 cell lysates were used as a positive and Chinese hamster ovary cell lysates as a negative control (Figure 3A). The findings are consistent with similar bands observed for immunoblotting of CCR2 in B cells or Mono Mac 6 cells.30,31 To test whether CCR2 is detectable on the endothelial surface, we performed flow cytometric analysis. HUVEC

Figure 1. HUVEC express mRNA for the MCP-1 receptor CCR2. PCR was performed using reverse transcribed cDNA isolated from HUVEC (lanes 1, 4, and 6) and cDNA encoding CCR2B as a positive control (lanes 2, 5, and 7). Using specific primers for CCR2B, PCR products from HUVEC (lane 1) and the CCR2 plasmid (lane 2) were analyzed by agarose gel electrophoresis. Lane 3 represents a water control without cDNA. To confirm specificity, restriction enzyme analysis of the PCR products from HUVEC (lanes 4 and 6) and the CCR2 plasmid (lanes 5 and 7) was performed with Apa1 (lanes 4 and 5) or Dra1 (lanes 6 and 7). Shown is a representative experiment.

Figure 2. Upregulation of CCR2 mRNA expression in HUVEC by IL-1β. A. RNA was isolated from resting (lane 1) and IL-1β-stimulated (lane 2) HUVEC, and 20 μg from each sample was analyzed by RNase protection assay using a probe for CCR2. For comparison, the standard template hCR-5 is shown in lane 3. Shown is a representative experiment. B. Quantification of the amount of CCR2 mRNA in resting and IL-1β-stimulated HUVEC was made in comparison to GAPDH mRNA levels using a Molecular Dynamics phosphoimager and expressed as the relative amounts of CCR2 RNA.

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showed a distinct surface expression of CCR2 (Figure 3B), which was lower than that found on freshly isolated blood monocytes (not shown). Consistent with mRNA analysis, CCR2 protein and surface expression was upregulated by activation of HUVEC with inflammatory cytokines, eg, IL-1$\beta$ (5 ng/mL) or TNF-$\alpha$ (50 U/mL) for 24 hours, as indicated by immunoblotting (Figure 3A) or flow cytometry (Table 1). These data show that CCR2 protein is expressed and regulated on the endothelial surface.

To study whether CCR2 surface expression was associated with functional responses of endothelial cells to MCP-1, we analyzed transmigration of HUVEC in a gelatin-coated transwell filter assays. CCR2 mediates MCP-1-induced signaling and transmigration in leukocytes, eg, in mononuclear cells, which characteristically occurs in a bell-shaped dose-response curve and for monocytes, is optimal at 1 nmol/L of MCP-1. Addition of MCP-1 to the bottom chamber only (ie, presence of a MCP-1 gradient) elicited HUVEC transmigration with a maximum index (ratio of stimulated migration/spontaneous migration) of 2.84±0.68 (mean±SD, n=3) at 100 nmol/L; however, a bell-shaped dose-response curve was difficult to demonstrate (Figure 3C). Transmigration induced by MCP-1 was blocked by the 9-76 MCP-1 analogue receptor antagonist, inferring a dependence on CCR2 (Figure 3C). The presence of MCP-1 (100 nmol/L) in both top and bottom chamber (ie, absence of a MCP-1 gradient) induced transmigration of HUVEC with an index (1.97±0.46, mean±SD) that was lower than that in the presence of a gradient (ie, MCP-1 in the bottom chamber only). This indicates that migration induced by MCP-1 was both chemotactic (directed) and chemokinetic (nondirected). Thus endothelial CCR2 is a functional receptor for MCP-1.

MCP-1 Facilitates Endothelial Cell Migration in Wound Injury Repair Without Affecting Proliferation

Because endothelial migration is crucial for angiogenesis and wound injury repair and MCP-1 has been found at the wound edge in areas of endothelial denudation, it was intriguing to speculate whether MCP-1 was involved in this process by inducing endothelial migration via CCR2. To determine whether endothelial monolayers produce more MCP-1 or other chemokines in response to injury, soluble MCP-1 protein in HUVEC supernatants was quantitated by ELISA. A comb constructed to create multiple 1 mm-wide, concentrical wounds was dragged across confluent HUVEC monolayers so that most of the remaining cells were affected by the injuries. After 6 hours in culture, endothelial secretion of MCP-1 and IL-8 was increased after multiple injuries to the HUVEC monolayers (Table 2), whereas that of RANTES was hardly detectable and not altered by wound injury (data not shown). Induction of IL-8 secretion was less marked, possibly due to immobilization. Thus endothelial cells respond to mechanical injury by secreting MCP-1 and IL-8.

To study whether MCP-1 may contribute to endothelial wound injury repair via CCR2 expressed on endothelial cells,
we created a single path wound by dragging a sterile pipette tip across HUVEC monolayers (Figure 4A). After 6 or 12 hours in culture, cells at the wound edge were found to form cytoplasmic extensions protruding into the wound area, a process resulting in the spreading and migration of cells to close the injury (Figure 4B). After 24 hours, wound closure was complete in almost all cases (not shown). The presence of the 9-76 peptide (125 nmol/L) after the wound injury markedly inhibited the ability of HUVEC to migrate into wound areas and clearly delayed the wound closure at 12 hours in comparison with untreated cells (Figure 4C). Nevertheless, at 12 hours, some protrusions at the edge were apparent, and the wounds eventually closed at 36 hours or later. In contrast, the addition of MCP-1 (50 nmol/L) at the time of wound injury induced a more disorganized appearance of cells that had migrated to close the wound and resulted in a more efficient closure that was almost complete at 12 hours (Figure 4D). This was confirmed by quantitating the number of HUVEC that had migrated into the wound area 12 hours after the injury (Figure 5A). In untreated monolayers, 171±32 cells/mm² had migrated into the wound area. The presence of the 9-76 MCP-1 receptor antagonist dose-dependently and substantially reduced, whereas addition of MCP-1 markedly increased the number of cells present in the wound area (Figure 5A). Notably, the number of cells in the wound area was also reduced by a neutralizing MCP-1 but not IL-8 mAb (Figure 5A). Isotype control mAb had no effect (not shown). Our data indicate that MCP-1 and endothelial receptors, such as CCR2, are involved in directing endothelial migration required to accomplish efficient endothelial wound injury repair.

Endothelial wound injury repair within the first 24 hours is mediated by migration, whereas cell proliferation occurs after 36 hours. Hence we investigated whether MCP-1 affects HUVEC proliferation. HUVEC were seeded at 10⁴ cells/cm² and the number of cells was determined over 72 hours in the presence of various concentrations of MCP-1 and/or the 9-76 peptide analogue. Although the proliferation rate increased after 48 hours, it was unaltered by MCP-1 or 9-76 peptide

### Table 2. Increased Endothelial Chemokine Secretion in Response to Injury

<table>
<thead>
<tr>
<th>Protein (ng/mL)</th>
<th>MCP-1</th>
<th>IL-8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.49±0.07</td>
<td>1.94±0.35</td>
</tr>
<tr>
<td>Injured</td>
<td>2.50±0.38</td>
<td>2.86±0.48</td>
</tr>
</tbody>
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Confluent HUVEC monolayers were left intact (control) or were injured, as described in Materials and Methods. Cells were washed and fed fresh medium, and after 6 hours in culture, the concentration of MCP-1 or IL-8 in the supernatants was quantitated by ELISA and adjusted for the number of cells. Data represent mean±SD of 3 separate experiments.

**A (10 min, control)**

**B (12 hours, control)**

**C (12 hours, 9-76 peptide)**

**D (12 hours, MCP-1)**

**Figure 4.** Involvement of MCP-1 and its receptor in endothelial wound injury repair. HUVEC monolayers were injured as described in Materials and Methods and photographed 10 minutes after injury with a 200× magnification (A). Cells were cultured without treatment (B), in the presence of the 9-76 MCP-1 peptide analogue, (125 nmol/L, C), or after addition of MCP-1 (50 nmol/L, D) and were photographed 12 hours after injury with a 100× magnification (B to D). Shown are photographs of representative fields.
Expression of CCR2 in Activated Endothelium In Vivo

Immunohistochemistry of heavily inflamed renal peripelvic tissue with CCR2 mAb revealed a distinct cytoplasmic staining of activated endothelial cells in irritated venules (Figure 6A), whereas negative control samples showed no specific red staining (Figure 6B). In addition, positive staining for CCR2 was found on arterial and vein endothelial cells (Figure 6C and data not shown). A robust CCR2 staining on endothelial cells appeared to be associated with subendothelial infiltrates of mononuclear cells in their vicinity (Figure 6A). Particularly intense CCR2 staining was detected on luminal mononuclear cells (Figure 6A), whereas less marked staining was detectable in the cytoplasmic periphery of extravasated mononuclear cells and in directly subintimal cells (Figures 6A and 6C). Moreover, some sections showed docking of strongly stained mononuclear cells to CCR2-positive endothelial cells in the initial stages of attachment and extravasation (Figure 6A). No staining for CCR2 could be detected on endothelial cells of arteries or venules in kidneys without signs of inflammation, whereas positively stained mononuclear cells served as an internal positive control (Figure 6D and data not shown). Thus our data clearly indicate that CCR2 is expressed on activated endothelium in tissue affected by chronic inflammatory reactions with mononuclear cell infiltrates, suggesting important implications for inflammatory conditions in vivo.

Discussion

We found that endothelial cells express mRNA and surface protein for the MCP-1 receptor CCR2. This was associated with a migratory response to MCP-1 that may play a role in directing cell migration during endothelial wound injury. To our knowledge, this is also the first demonstration of CCR2 expression on inflamed endothelium in vivo. Although effects of CC chemokines and their receptors on endothelial cells have not yet been elucidated, various CXC chemokines have been shown to modulate endothelial cell proliferation.11,12 For example, PF-4 and IP-10 can suppress angiogenesis, possibly by competing with known growth factors, such as bFGF and TGF-β, for heparan sulfate proteoglycan binding sites and inhibiting the proliferative effects of these growth factors. Endothelial cells express CXCR1 and CXCR4, which can mediate effects of the CXC chemokines IL-8 and stromal cell-derived factor-1 (SDF-1), respectively.13,38–40 The ability of IL-8 to induce angiogenesis in association with the presence of CXCR1 on endothelial cells was paralleled and expanded by our findings that MCP-1 and endothelial CCR2 participate in direct receptor-mediated functions.

Our results show that endothelial cells expressed low levels of CCR2 mRNA and to a lesser extent, CXCR1 mRNA. Previously, the expression of CXCR1 but not CCR2 mRNA has been described in HUVEC.13 In a recent report,39 generation of a cDNA plasmid library from HUVEC mRNA using consensus region PCR primers for chemokine receptors and sequencing of random clones revealed the presence of mRNA for CCR4 and CXCR3 as well as for CXCR1, CCR1, and CCR2. Northern blot analysis confirmed the abundance of CXCR4 mRNA; however, it failed to detect mRNA for CXCR1 or CCR2. A more recent study did not reveal CCR2 mRNA in HUVEC using only RT-PCR or MCP-1-induced chemotaxis of HUVEC.40 In contrast, our data clearly demonstrate the expression of CCR2 mRNA in endothelial cells. Immunoblotting, surface staining, and migration assays further confirmed the expression of functional CCR2 on HUVEC. Similarly, flow cytometric analysis and migration assays indicated expression of functional CCR2 on HUVEC. In contrast to this and other reports,11,12 Contrasting findings may be due to differences in the sensitivity of RT-PCR, as well as the migration assays used or due to the heterogeneity of chemokine receptor expression in distinct HUVEC preparations or under culture conditions with different media.41

The CCR2 mRNA and surface protein expression could be upregulated by activation of HUVEC with inflammatory cytokines, such as IL-1β or TNF-α. This is in accordance
with findings that IL-1β or TNF-α cause a biphasic regulation of endothelial CXCR4 mRNA expression with an initial inhibition and subsequent prolonged induction. In contrast, IL-1β or TNF-α downregulated CCR2 expression and MCP-1-mediated transmigration in monocytes, whereas IFN-γ decreased CXCR4 mRNA stability in HUVEC but did not affect CCR2 in monocytes. Moreover, IL-1α did not alter CXCR1 expression in HUVEC, and TNF-α did not regulate CCR1 and CCR2 in smooth muscle cells. The differential regulation of chemokine receptors by various cytokines in distinct cellular systems may indicate a cell-specific involvement in various pathophysiological conditions. Our results suggest that regulation of endothelial CCR2 by inflammatory cytokines, which induce endothelial secretion of MCP-1, may promote endothelial migration under conditions such as inflammatory reactions or wound injury repair after angioplasty.

We found that MCP-1 induced a maximum index of HUVEC migration at 100 nmol/L, whereas leukocyte chemotaxis to MCP-1 is optimal at 1 nmol/L. Inhibition with the 9-76 MCP-1 receptor antagonist confirmed that MCP-1–induced migration of endothelial cells was dependent on CCR2, as seen in monocytes. Endothelial cell migration and Ca2+ mobilization in response to the CXC chemokines IL-8 and SDF-1 also occurred more effectively at higher concentrations than those required for leukocyte responses. In addition, Ca2+ mobilization in smooth muscle cells was only induced by MCP-1 or MIP-1α at concentrations of 500 nmol/L, inferring cell type-specific differences in chemokine responsiveness. Flow cytometry revealed that surface expression of CCR2 on HUVEC was lower than on monocytes. Moreover, specific binding sites for IL-8 on HUVEC have been suggested to be of lower affinity and density than on neutrophils. Thus migratory responses may vary with differences in chemokine receptor characteristics. A bell-shaped dose-response curve was difficult to demonstrate for HUVEC migration to SDF-1. Moreover, transmigration of HUVEC was induced by a MCP-1 gradient and, to a lesser extent, in the presence of nongradient MCP-1, showing that it was both chemotactic (directed) and chemokinetic (nondirected), as has been shown with transferrin. This suggests that MCP-1 increases overall movement of HUVEC.

We have used a previously described HUVEC wound injury model, which reproducibly exhibited migration of a comparable number of endothelial cells into the wound area and closure of the wound path within 24 hours to 36 hours, thus representing a suitable model to study wound healing and repair of vascular endothelium in vitro. The presence of 9-76 MCP-1 receptor antagonist or neutralizing MCP-1 mAb impaired the ability of HUVEC to migrate and close the wound, whereas addition of MCP-1 appeared to facilitate the repair, suggesting a role for MCP-1–induced migration in endothelial wound repair. Moreover, endothelial MCP-1 secretion was increased by multiple wound injury. This clearly extends findings that MCP-1 mRNA expression was increased by endothelial denudation after balloon angioplasty, particularly evident at the migrating cell front, and that higher
levels of MCP-1 mRNA were found in migrating sub confluent endothelial cells, which was attributed to effects of endothelial bFGF.37 Secretion of chemokines and their immobilization to matrix proteins being established by the endothelial cell front may create a naturally occurring gradient to direct migration. Notably, exogenous MCP-1 resulted in a more efficient wound injury repair despite a more haphazard appearance of the monolayer. This may be due to a immobilization of MCP-1 to continuously generated matrix components at high concentrations (P.J.N. et al, unpublished data, 1999). Endothelial cells have been shown to respond by migrating and spreading within 24 hours after wound injury, whereas proliferation becomes prominent only after 36 hours.4 Unlike the CXC chemokines IL-8 and PF-4, we observed that MCP-1 or its receptor antagonist did not affect HUVEC proliferation. Moreover, inhibition by the MCP-1 receptor antagonist was obvious within the first 12 hours. This suggests that MCP-1 predominantly acts by inducing migration during endothelial wound injury. Our results expand findings that MCP-1 and MIP-1α are critical in wound repair by mediating macrophage recruitment43,44 and that MCP-1 may facilitate angiogenesis,23 implying that these processes are in part due to endothelial mechanisms.

Notably, immunohistochemistry revealed CCR2 expression on activated endothelium of venules, veins, and arteries in chronically inflamed tissue, demonstrating for the first time endothelial expression of a CC chemokine receptor in vivo. The most prominent CCR2 staining on endothelial cells appeared to occur in the proximity of mononuclear infiltration. This may suggest an upregulation of endothelial CCR2 in association with secretion of inflammatory cytokines by mononuclear cells, whereas CCR2 staining on subendothelial mononuclear cells appeared to be less prominent than in cells before extravasation, consistent with the differential regulation of CCR2 by cytokines in distinct cell types. A chronic inflammatory irritation may result in a disturbed endothelial integrity, which may require endothelial CCR2 expression to maintain vascular hemostasis under conditions of inflammatory activation. Conversely, excessive endothelial CCR2 levels may contribute to vascular damage. Vascular injury, eg, by angioplasty, can also trigger inflammatory reactions leading to dysregulated wound repair and ultimately restenosis. Our data may thus have important implications for many inflammatory reactions in vivo.

Our findings exemplify the diverse functions of chemokines that appear to play an increasingly evident role in vascular pathology. The identification of CCR2 in endothelial cells shown here and in vascular smooth muscle cells24 indicates that MCP-1 may not only mediate monocyte infiltration but may participate in endothelial or smooth muscle cell migration and proliferation during processes, such as atherosclerosis, restenosis, and chronic stages of inflammatory diseases. Intimal hyperplasia of vein grafts, which involves an exaggerated vascular wound healing response, has been associated with increased MCP-1 levels that may stimulate monocyte infiltration as well as promote endothelial migration.45 The advent of chemokine peptide antagonists, eg, the 9-76 MCP-1 analogue, which has already been tested in a murine arthritis model,46,47 may offer encouraging novel approaches in the treatment of inflammatory conditions, restenosis, or angiogenic tumors.

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