IL-8 activates endothelial cell CXCR1 and CXCR2 through Rho and Rac signaling pathways

INGRID U. SCHRAUFSTATTER, JANICE CHUNG, AND MEIKE BURGER
La Jolla Institute for Molecular Medicine, San Diego, California 92121

Received 6 November 2000; accepted in final form 14 December 2000

Stimulation of microvascular endothelial cells with interleukin (IL)-8 leads to cytoskeletal reorganization, which is mediated by combined activation of the CXCR1 and the CXCR2. In the early phase actin stress fibers appear, followed by cortical actin accumulation and cell retraction leading to gap formation between cells. The early response (between 1 and 5 min) is inhibited by an antibody that blocks the CXCR1. The later phase (from about 5 to 60 min), which is associated with cell retraction, is prevented by anti-CXCR2 antibody. Furthermore, anti-CXCR2, but not anti-CXCR1, antibody blocked IL-8-mediated haptotaxis of endothelial cells on collagen. The later phase of the IL-8-mediated actin response is inhibited by pertussis toxin, indicating that the CXCR2 couples to Gi. In contrast, the early phase is blocked by C3 botulinum toxin, which inactivates Rho, and by Y-27632, which inhibits Rho kinase, but not pertussis toxin. Furthermore, the early CXCR1-mediated formation of stress fibers was prevented by dominant negative Rho. Dominant negative Rac on the other hand initially translocated to actin-rich filopodia after stimulation with IL-8 and later prevented cell retraction by blocking the CXCR2-mediated cytoskeletal response. These results indicate that IL-8 activates both the CXCR1 and the CXCR2 on microvascular endothelial cells, using different signal transduction cascades. The retraction of endothelial cells due to activation of the CXCR2 may contribute to the increased vascular permeability observed in acute inflammation and during the angiogenic response.

C-X-C chemokines; endothelial cells; interleukin-8 receptors; inflammation

INTERLEUKIN (IL)-8 is a member of the C-X-C family of chemokines that shows high-affinity binding to the CXCR1 (IL-8 receptor type 1) and the CXCR2 (IL-8 receptor type 2). Although the CXCR1 is selectively activated by IL-8 only, the CXCR2 responds to several additional chemokines including growth-related protein-α (GROα), neutrophil-activating peptide-2, and epithelial-derived neutrophil attractant-78. The common denominator shared by all chemokines that activate the CXCR2 is a Glu-Leu-Arg (ELR) sequence in the amino terminus, which appears to serve as a recognition sequence for receptor binding and activation (17).

Early investigations concentrated on the effect of IL-8 on neutrophils, which respond to IL-8 with calcium mobilization (3), actin polymerization (35), enzyme release, chemotaxis, and a weak respiratory burst. Despite similar affinities for IL-8 and similar receptor numbers of the CXCR1 and CXCR2, neutrophil chemotaxis is primarily mediated by the CXCR1 (6, 39). Pertussis toxin blocks all aspects of IL-8-mediated leukocyte activation, indicating that both the CXCR1 and CXCR2 are coupled to Gi in neutrophils (3) where Goi-2 is very abundant. It has, however, been shown that IL-8-receptor coupling is not restricted to Gi. At least under conditions where Goi-14 and Goi-16, were overexpressed, these G proteins were able to serve as alternate signal-transducing elements of IL-8-mediated cellular responses (54).

Apart from neutrophils and monocytes (13), numerous sessile cell types have been shown to express IL-8 receptors. These cell types include neurons (19), various cancer cells (30, 32, 36, 51), and endothelial cells (33). Although activation of the CXCR2 can enhance cell proliferation in cancer cells, the physiological role of IL-8 receptors on nonhematopoietic cells is far from clear but is expected to include cell locomotion.

The angiogenic property of IL-8 and related chemokines in vivo has been known for several years (25). Chemokines that do not possess the NH2-terminal ELR sequence or in which this sequence was mutated are devoid of angiogenic activity (47). This ligand usage implies that the CXCR2 is the endothelial cell receptor that mediates the angiogenic response, which has, however, not been shown experimentally. Although an early report demonstrated that IL-8 is a chemotactic factor for endothelial cells (25), there have been difficulties in detecting IL-8 receptors on human umbilical vein endothelial cells (HUVECs) consistently. This led to the conclusion that IL-8 receptors are lost in culture (38), a concept that only recently has been rechallenged by Murdoch et al. (33), who showed expression of both the CXCR1 and CXCR2 on HUVECs. These cells responded with similar chemotactic responses to IL-8 and stroma-derived factor (SDF)-1, a C-X-C chemokine

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
that lacks the NH₂-terminal ELR sequence, but only SDF-1 was capable of inducing intracellular calcium mobilization (33). This was a first indication that IL-8 receptors exist on endothelial cells in vitro and that they evoke behaviors that differ from those observed in leukocytes.

Because chemotaxis depends on a cytoskeletal response, we analyzed filamentous actin (F-actin) filament formation in cultured endothelial cells exposed to IL-8. As shown below, IL-8 caused cytoskeletal rearrangement due to activation of both the CXCR1 and the CXCR2, but both temporal and qualitative differences existed between the behavior of the two receptors. The results suggested that sequential receptor activation through two signaling pathways may regulate endothelial cell cytoskeletal responses to IL-8.

The small G proteins Rac, Rho and Cdc42 regulate the formation of polymerized actin. In fibroblasts, activation of Rac causes the formation of lamellipodia, sheet-like structures consisting of a cross-linked meshwork of actin filaments at the leading edge of migrating cells (41). Activation of Cdc42 induces fingerlike structures called filopodia, and Rho activation leads to the formation of stress fibers, which insert into focal adhesion complexes. In fibroblasts, the activities of these three proteins are arranged in a hierarchical fashion: Cdc42 activates Rac, which in turn activates Rho (34). Depending on cell type and possibly the activation pathway, the relationship between the different small G proteins varies, however, and activated Rac may even block Rho-induced stress fiber formation (50). In their GTP-bound active state, Rho family proteins couple to effector proteins, e.g., Ser/Thr kinases such as Rho kinase or p21-activated kinase (PAK) for downstream signaling, resulting in cytoskeletal responses. It has been shown that in endothelial cells, activation of Rac by thrombin leads to cell retraction (52). Activated PAK, a downstream target of activated Rac and Cdc42, also has been shown to cause endothelial cell contraction (23). Lysophosphatidic acid on the other hand, which activates Rho and stress fiber formation (15), caused actin polymerization in endothelial cells but not cell retraction (52). Here we report that IL-8 initially activates Rho and actin stress fiber formation in endothelial cells due to activation of the CXCR1. At later time points, Rac is activated in a CCR2-dependent fashion, leading to cell retraction and gap formation between neighboring cells.

METHODS AND MATERIALS

Reagents. Blocking antibodies against the CXCR1 and CXCR2 were purchased from R&D Systems (Minneapolis, MN). Anti-Myc tag 9E10 antibody and anti-rabbit and anti-Rho monoclonal antibodies were from Upstate Biotechnology (Lake Placid, NY), FITC-IgG was from BioSource (Camarillo, CA), and pertussis toxin and C3 Clostridium botulinum toxin were purchased from List Biological Laboratories (Campbell, CA). Y-27632 was a gift from Yoshitomi Pharmaceuticals (Osaka, Japan).

IL-8 and GROα were either obtained from R&D Systems or purified from Escherichia coli DH5α expressing the chemo-kine as a thrombin-cleavable fusion protein with glutathione transferase as described previously (43). The purified protein was treated with 0.1 mM phenylmethylsulfonyl fluoride to block any residual thrombin. No difference in cellular response was observed between the commercial and the laboratory-purified source of protein.

The Myc-tagged DNA constructs for T17NRac, T19NRho, and Q63LRho cloned into the pRK5 mammalian expression vector (27) were kindly provided by Dr. G. Bokoch (The Scripps Research Institute).

Cell culture. Human lung microvascular endothelial cells (HMVECs) were obtained from Clonetics (San Diego, CA) and used between passages 6 and 8. Immortalized dermal human microvascular endothelial cells (HMECs) (1) were obtained from the Centers for Disease Control (Atlanta, GA). Both cells were grown in EGM-2-MV (Clonetics).

HMVECs or HMVECs were seeded at low density on fibronectin-coated coverslips and grown in EGM-2-MV (Clonetics) containing 10% fetal calf serum (FCS). On day 7, when the cells had reached confluence, they were serum starved for 2 h (HMVECs) or 3–4 h (HMECs) and stimulated with IL-8 or GROα for the times indicated for each experiment. All experiments were performed at 37°C in a tissue culture incubator.

For transient expression of dominant negative Rac1 (T17NRac), dominant negative RhoA (T19NRho), or constitutively active RhoA (Q63L), HMECs were grown to 70% confluence and transfected with DNA construct using LipofectAMINE as described by Ellis et al. (11). Transfection efficiency was around 40%.

To block Gα, HMVECs were incubated for 16 h with 100 ng/ml of pertussis toxin. Rho was inhibited with 3 μM of C3 botulinum toxin (10 μg/ml) for 24 h, which leads to inactivation of Rho in about 90% of endothelial cells (40). Rho kinase, a downstream target of Rho, was inhibited with 10 μM Y-27632 added 30 min before the addition of the stimulus (20).

Haptotaxis of HMECs on collagen. Transwell filters (8-μm² pore size, Costar) were coated overnight with 230 μl of bovine collagen (10 μg/ml, Cohesion) in PBS and blocked for 30 min with 1% bovine serum albumin (BSA). EGM-2-MV (50 μl/well, Clonetics) containing 0.1% BSA, 0.5 μg/ml hydrocortisone, and 50 μg/ml gentamicin were pipetted into the bottom well, and 5 × 10⁴ HMECs in the same medium were added to the inserts. For antibody inhibition studies, the cells were incubated with 10 μg/ml of anti-CXCR1 or anti-CXCR2 antibody for 30 min. After the addition of IL-8 to the bottom wells, the cells were incubated for 4 h at 37°C in a tissue culture incubator and stained for 10 min with 1 μM calcein acetoxyethyl ester (Molecular Probes). Cells in the upper well were carefully removed with a cotton swab, and transmigrated cells were counted at ×10 magnification on a Leitz Fluovert FS microscope using FITC excitation and emission. Results are expressed as percent of cells transmigrated in the absence of IL-8 (means ± SE of 4 experiments in triplicate).

Fluorescence microscopy. For F-actin localization, cells were fixed for 20 min in 3% paraformaldehyde in PBS, put on ice, and permeabilized for 5 min in 0.2% Triton X-100, incubated with 5 μM/ml of rhodamine-phalloidin or FITC-phalloidin (Molecular Probes) for 30 min, washed three times with PBS, and mounted with Antifade (Molecular Probes). For immune detection of Myc-tagged T17NRac and T19NRho, the same fixed and permeabilized cells were incubated with a 1:400 dilution of anti-Myc (9E10 monoclonal antibody from Upstate Biotechnology) for 30 min, washed three times with PBS containing 0.5% FCS, followed by incubation with a 1:200 dilution of FITC-labeled goat anti-mouse IgG (Biosource) for 30 min and three more washes in...
PBS. The same procedure was followed for staining with anti-Rac or anti-Rho antibody, except that a 1:200 dilution was used for the first antibody.

Fluorescence microscopy was performed on a Zeiss Axiocamert 100 microscope with the Spot 32 program to obtain digital images. Images were processed with Adobe Photoshop and Scion Image software was used for the quantification of F-actin in the fluorescent images.

RESULTS

Cytoskeletal effects of IL-8 on microvascular endothelial cells. The addition of IL-8 to HMVECs or HMECs caused increased F-actin formation in both cell types (Fig. 1). This cytoskeletal response was a consistent finding in over 20 experiments with microvascular endothelial cells. HUVECs showed a less reproducible response (results not shown). In unstimulated serum-starved HMVECs, F-actin staining of low intensity was concentrated in the cell periphery where adjacent cells touched each other. After the addition of IL-8, prominent stress fibers appeared within 1 min of activation.

In contrast to the transient actin polymerization observed in leukocytes, which lasts for about 2 min (35), endothelial cells responded to IL-8 with prolonged cytoskeletal activation, which was still maximal between 15 and 30 min (Fig. 1) and had not returned to baseline at 1 h. Starting between 5 and 10 min, the cells retracted, leaving denuded surface areas between adjacent cells, as indicated by arrows in Fig. 1D.

These changes were observed within the physiologically relevant range of concentrations of IL-8 or GROα (Fig. 2), with a threshold dose of 0.3 nM for either ligand. It was noted that the magnitude and the sensitivity of the early response to GROα were low but that GROα almost equaled the effect of IL-8 at later time points.

Because GROα, which only interacts with the CXCR2, caused actin polymerization, involvement of the CXCR2 in endothelial cell activation was apparent, but simultaneous interaction with the CXCR1 could not be ruled out. To differentiate between activation of the two IL-8 receptors, specific blocking antibody to the CXCR1, the CXCR2, or a combination of the two antibodies was added before stimulation with IL-8. In combination the two antibodies completely inhibited IL-8-induced actin polymerization, indicating that endothelial cells express the two known IL-8 receptors (Fig. 3). When inhibition with each antibody was followed individually, it was noted that anti-CXCR1 antibody blocked the initial phase of actin responsiveness but that at later time points, the activation was mediated solely by the CXCR2 (Fig. 3). Indeed, at 15 min, blockade of the CXCR1 appeared to increase F-actin content in HMECs exposed to a concentration of IL-8 100-fold higher than the dissociation constant (Kd) for IL-8 binding. This concentration was chosen to ensure that the increased response was not a consequence of increased ligand binding to the CXCR2 when CXCR1 interaction could not proceed. The effect was, however, the same at 10 nM IL-8. Blocking anti-CXCR2 antibody also prevented the F-actin response in cells stimulated with GROα (Table 1). Because GROα shows low-affinity binding to the CXCR1 (Kd of 200 nM), a concentration of 10 nM GROα was used in this case to rule out activation of the CXCR1.

At later time points, the IL-8- or GROα-induced activation of endothelial cells caused cell retraction. Cell retraction was completely prevented in the presence of anti-CXCR2 antibody, whereas anti-CXCR1 antibody showed a trend toward enhanced cell retraction when cells were stimulated with IL-8 (Fig. 4). Pertussis toxin, which blocks IL-8 functions in leukocytes, also prevented cell retraction in HMECs (Fig. 4). The small increase of retracted cells observed when pertussis toxin-treated cells were stimulated with IL-8 or GROα was present even in the absence of added stimulus (21 ± 4 retracted cells per low-power field). It appeared that pertussis toxin itself caused some activation in endothelial cells, as previously observed by others (13).
Fig. 2. Dose response of filamentous actin (F-actin) content in HMECs stimulated with IL-8 or GROα for 1 or 15 min. Top: FITC-phalloidin-stained HMECs are shown after a 15-min exposure to IL-8. A: no stimulus. B: 1 nM IL-8. C: 3 nM IL-8. D: 10 nM IL-8. E: 30 nM IL-8. F: 100 nM IL-8. Bottom: dose response to IL-8 (open bars) or GROα (solid bars) after 1 and 15 min of exposure. conc, Concentration. Values are means ± SE of at least 9 images obtained from 3 different experiments.

Fig. 3. Effect of anti-IL-8 receptor antibodies on cytoskeletal changes induced in HMECs by stimulation with 100 nM IL-8. HMEC monolayers were incubated with 20 μg/ml anti-receptor antibody (AB) for 30 min before addition of IL-8. Top, top row: IL-8 response at 0, 1, 5, 15, and 30 min in the absence of antibody; second row: effect of anti-CXCR1 antibody on the IL-8-mediated cytoskeletal response; third row: effect of anti-CXCR2 antibody; bottom row: effect of combined anti-CXCR1/2 antibody. Bottom: F-actin content is quantified: open bars, no antibody; hatched bars, anti-CXCR1 antibody; solid bars, anti-CXCR2; horizontally striped bars, anti-CXCR1 and anti-CXCR2 combo. Values are means ± SE of at least 9 images obtained from 3 different experiments.
Table 1. Effect of inhibitors on IL-8- and GROα-induced F-actin content in HMECs

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IL-8</th>
<th>GROα</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inhibitor</td>
<td>291 ± 44</td>
<td>179 ± 51</td>
</tr>
<tr>
<td>Anti-CXCR1</td>
<td>190 ± 14</td>
<td>167 ± 8</td>
</tr>
<tr>
<td>Anti-CXCR2</td>
<td>267 ± 39</td>
<td>128 ± 29</td>
</tr>
<tr>
<td>Anti-CXCR1/2 combo</td>
<td>141 ± 8</td>
<td>112 ± 17</td>
</tr>
<tr>
<td>Pertussis toxin</td>
<td>300 ± 43</td>
<td>139 ± 27</td>
</tr>
<tr>
<td>C3</td>
<td>131 ± 21</td>
<td>160 ± 29</td>
</tr>
<tr>
<td>Y-27632</td>
<td>156 ± 16</td>
<td>172 ± 56</td>
</tr>
</tbody>
</table>

15 min

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IL-8</th>
<th>GROα</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inhibitor</td>
<td>325 ± 44</td>
<td>237 ± 46</td>
</tr>
<tr>
<td>Anti-CXCR1</td>
<td>356 ± 54</td>
<td>260 ± 74</td>
</tr>
<tr>
<td>Anti-CXCR2</td>
<td>120 ± 14</td>
<td>129 ± 19</td>
</tr>
<tr>
<td>Anti-CXCR1/2 combo</td>
<td>129 ± 9</td>
<td>131 ± 16</td>
</tr>
<tr>
<td>Pertussis toxin</td>
<td>159 ± 14</td>
<td>134 ± 13</td>
</tr>
<tr>
<td>C3</td>
<td>281 ± 63</td>
<td>219 ± 43</td>
</tr>
<tr>
<td>Y-27632</td>
<td>358 ± 10</td>
<td>315 ± 33</td>
</tr>
</tbody>
</table>

Values are means ± SE expressed as percent fluorescence intensity of unstimulated human microvascular endothelial cells (HMECs); n = 9. IL-8, interleukin-8; F-actin, filamentous actin; C3, Clostridium botulinum toxin. F-actin content was determined as described in METHODS AND MATERIALS.

Effect of IL-8 and anti-CXCR antibodies on HMEC haptotaxis. Because formation of F-actin is a prerequisite for cell migration, HMEC haptotaxis on collagen was determined in response to IL-8 and GROα. As expected from the preceding F-actin staining studies, both IL-8 and GROα were chemotactic for HMECs (Fig. 5). Anti-CXCR2 antibody, but not anti-CXCR1 antibody, prevented this behavior (Fig. 5).

Effect of CXCR and G protein inhibitors on cytoskeletal response. Pertussis toxin caused moderate formation of stress fibers in unstimulated HMECs (Fig. 6). It prevented, however, the response to GROα overall and the response to IL-8 at the later time points (Table 1). In contrast, it showed little effect early on after IL-8 stimulation (Fig. 6 and Table 1) during the time points when anti-CXCR1 antibody was most effective. Because pertussis toxin blocks CXCR1- and CXCR2-mediated responses in neutrophils, this response in endothelial cells was unexpected, although it had been shown previously that the CXCR1 has the potential to couple to G proteins other than G₁ (54).

Because Rho activation is known to cause stress fiber formation, we tested the effect of two components of the Rho cascade, C3 botulinum toxin, which inactivates Rho, and Y-27632, an inhibitor of Rho kinase. Both these inhibitors blocked the early response to IL-8 but not the later phase or the response to GROα (Fig. 6 and Table 1), suggesting that the CXCR1, but not the CXCR2, led to activation of Rho. Furthermore, inhibition of the Rho cascade failed to protect from cell retraction. Under the conditions described in Fig. 4, 55 ± 9 and 64 ± 18 retracted cells were counted per low-power field when cells stimulated with IL-8 were pretreated with C3 toxin or Y-27632, respectively. No increase of retracted cells over background levels was seen when the cells were incubated with the inhibitor in the absence of IL-8.

Role of Rac and Rho. To assess the role of the small G proteins in the cytoskeletal response directly, two approaches were taken: 1) HMECs were transiently transfected with dominant negative Rac (T17NRac) or dominant negative Rho (T19NRho) followed by stimulation with IL-8 and detection of the Myc-tagged Rac or Rho with anti-Myc antibody in parallel with F-actin staining and 2) cellular Rac and Rho were detected in untransfected cells by indirect immunofluorescence staining with the respective antibody, again in combination with F-actin detection.

T19NRho prevented the early CXCR1-mediated formation of stress fibers in the presence of IL-8 (Fig. 7, A–C). In contrast, cells transfected with constitutively active Rho (Q63LRho) demonstrated stress fibers in
Together with the inhibitor studies, these results strongly support the idea that the CXCR1-mediated cytoskeletal response in microvascular endothelial cells is mediated by Rho activation.

Dominant negative Rac also affected the morphology of the cytoskeleton following stimulation with IL-8. Initially, at 1–2 min after the addition of IL-8, translocation of T17NRac to the cell periphery and formation of filopodia characteristic of Cdc42 activation were observed (Fig. 7, D–F). Addition of GROα caused the same changes, indicating that this activation pathway was primarily mediated by the CXCR2. If cells transfected with T17NRac were preincubated with anti-CXCR2 antibody, addition of GROα produced no cytoskeletal response (results not shown). Addition of IL-8, on the other hand, was associated with translocation of the dominant negative Rac to focal adhesion complexes, presumably resulting from activation of the CXCR1 (Fig. 7, G–I). When untransfected HMECs were labeled with anti-Rac antibody, translocation of Rac to the cell periphery was observed, but now lamellipodia were formed instead of filopodia, which were particularly prominent when GROα was used as stimulant (Fig. 7, M–O). In IL-8-stimulated cells, cellular Rac also accumulated in focal complexes (Fig. 7, J–L), as described above for T17NRac-transfected cells. Staining with an antibody against vinculin (Sigma, St. Louis, MO) verified that these processes were focal adhesions.

At later time points, a cortical ring of F-actin accumulated in the cell periphery of cells transfected with T17NRac and stimulated with IL-8 (Fig. 7, P–R). This ring appeared to attach the cells to the substratum. T17NRac-transfected cells did not retract following the addition of IL-8 or GROα but appeared flattened out on the surface. Cellular Rac staining in the absence of...
dominant negative Rac remained translocated to the membrane region of the cell, but now the cells retracted from the surface as a consequence of Rac activation. Cells transfected with dominant negative Rho started to retract by about 5 min following stimulation with IL-8 as shown for the 15-min time point in Fig. 7, S–U. It appears that activation of the CXCR2 leads to activation of Rac, which translocates to the plasma membrane. Dominant negative Rac still translocates to the plasma membrane but fails to produce the downstream retractive cytoskeletal response.

DISCUSSION

Although it has been known for several years that IL-8 and other ELR-containing C-X-C chemokines are angiogenic factors in vivo (25), the mechanism involved has remained unresolved because the presence of IL-8 receptors on cultured endothelial cells has been disputed (38). Here we report that microvascular endothelial cells responded to IL-8 on all occasions in vitro and that this response resulted from combined activation of the CXCR1 and the CXCR2. These functional studies are in agreement with the recent findings of Murdoch et al. (33), who detected the CXCR1 and the CXCR2 on endothelial cells by RT-PCR and fluorescence-activated cell-sorter analysis with specific anti-receptor antibodies.

Activation of the CXCR2 on endothelial cells by IL-8 or GROα led to cell retraction and gap formation between adjacent cells. This behavior leads to increased
permeability of the endothelial cell monolayer as has been described for thrombin (52), which stimulates endothelial cells in a fashion similar to IL-8. In vivo endothelial cell retraction causes increased vascular permeability, a hallmark of acute inflammation, which is observed in disease states in which IL-8 is implicated to play a role (31) and where increased vascular permeability is partially independent of neutrophils (56). Future experiments will have to show the relevance of our in vitro observations to the in vivo situation.

Angiogenesis, which can be induced by ELR chemokines, is similarly associated with a highly permeable vasculature (10). The role of endothelial cell IL-8 receptors in these in vivo situations deserves future exploration because interruption of the activation of endothelial cell IL-8 receptors appears to be a possible therapeutic intervention in acute inflammatory disease. Furthermore, the same approach also appears promising for the development of antiangiogenic therapies for cancers that produce high concentrations of ELR chemokines, which often have a poor prognosis and are characterized by high vascular density rather than neutrophil accumulation (45).

The CXCR1 and CXCR2 are G protein-coupled seven-transmembrane receptors coupled to Gi. Although it has been shown that the CXCR1 can also interact with Go14 and Go16 but not with Ga (54), IL-8-induced activation of leukocytes, which are rich in Ga, is abolished by pertussis toxin (3). In contrast, the early CXCR1-mediated response to IL-8 in endothelial cells was insensitive to pertussis toxin, indicating that the CXCR1 couples to another G protein family in these cells. Besides Ga, which does not interact with the CXCR1 (54), only Go12 and Go13 promote stress fiber formation in a pertussis toxin-insensitive way (15). The Go12- or Go13-mediated cytoskeletal response is blocked by C3 botulinum toxin (4) as was the case for the early IL-8-mediated response in microvascular endothelial cells described here. The response to IL-8 in HMECs preincubated with anti-CXCR2 antibody was short-lived as has been described previously for cytoskeletal changes caused by lysophosphaticid acid and endothelin, two mediators that couple to Go13 and Go12, respectively, and signal through Rho stimulation (14). Inhibition of Rho kinase (29), a downstream effector of Rho, similarly prevented the early response to IL-8. Activation of the Rho/Rho kinase pathway is associated with stimulation of phospholipase D (42). In accordance, it has been shown previously that phospholipase D is activated by stimulation of the CXCR1 but not of the CXCR2 (28). Thus it appears that the CXCR1 in endothelial cells couples to Go12 or Go13 followed by activation of the Rho/Rho kinase cascade.

It has been shown that activation of Rho in endothelial cells induces clustering of E-selectin and intercellular and vascular cell adhesion molecules, which leads to increased monocyte adhesion (53). By this mechanism, activation of the CXCR1 on endothelial cells may actively recruit leukocytes to an area of inflammation.

The relationship between Rac and Rho activation and cytoskeletal responses such as cell retraction and cell spreading is complex, not fully understood, and variable depending on cell type and specific experimental conditions (26). Furthermore, when small G proteins are stimulated due to receptor activation, additional signal transduction pathways, such as increases in intracellular free calcium, often contribute to the cytoskeletal response. Mobilization of calcium does not appear to have a role in the activation of endothelial cells by IL-8 (33). Because calcium/calmodulin-dependent myosin light chain kinase phosphorylates the myosin light chain (55), calcium flux as it is observed in endothelial cells stimulated with thrombin may contribute to the Rho-dependent cell retraction seen in these cells (12). In contrast, we could not detect Rho-mediated cell retraction in the presence of IL-8.

The later effect of IL-8 or the effect of GROα, which stimulates the CXCR2 only, was blocked by pertussis toxin, indicating that the CXCR2 in endothelial cells couples to Gi. In contrast to the transient response in leukocytes, which lasts for less than 5 min, activation of the CXCR2 on endothelial cells lasted for more than 1 h. This prolonged activation may be a consequence of integrin activation, which enhances various signaling pathways in adherent cells (44). In particular, adhesion to the extracellular matrix results in Rac translocation to the plasma membrane, followed by PAK activation (9). This translocation of Rac to the plasma membrane was characteristic of IL-8- or GROα-induced endothelial cell activation. Because GROα was capable of causing translocation, this Rac response is a function of activation of the CXCR2. Rac translocation to the plasma membrane is associated with activation of PAK, which has been shown to lead to cell retraction in endothelial cells (57). Similarly, activation of the NADPH oxidase pathway, which causes the respiratory burst, involves translocation of Rac to the plasma membrane (24). Although this pathway is most prominent in leukocytes, it is operative in endothelial cells and whether IL-8 can induce superoxide anion generation in endothelial cells deserves future investigation.

Retraction of endothelial cells by IL-8 was mediated by activation of the CXCR2 in a Rac-dependent fashion. This Rac-mediated retraction of endothelial cells has been observed previously in thrombin-activated endothelial cells (52). Particularly when cells are plated on collagen, Rac has been shown to promote cell migration (21). In accordance, HMECs migrated toward a source of IL-8 or GROα in the presence of collagen (Fig. 5). Interestingly, this behavior was a function of the CXCR2 and could be inhibited by anti-CXCR2 antibody. This contrasts with the situation in neutrophils, where chemotaxis is primarily, but not exclusively, mediated by the CXCR1 (7, 8, 16, 39) despite similar affinities and receptor numbers for the CXCR1 and CXCR2. It therefore appears that chemotaxis is not determined simply by the receptor sequence but depends on the specific downstream effector interplay that differs in different cell types.

Activation of the CXCR2 through Rac may represent a mechanism for chemokine-dependent endothelial cell migration in vivo, which is necessary for new vessel
growth during angiogenesis. Because all ELR chemokines have an angiogenic potential (47), it appears that activation of the CXCR2 is involved in the angiogenic response. This does not exclude, however, that the CXCR1, which activates the Rho cascade, also has a role in this process because Rho blockade has been shown to inhibit angiogenesis (48).

Various inflammatory stimuli, including tumor necrosis factor-\( \alpha \) and IL-\( \beta \), induce the production of ELR chemokines in endothelial cells (22). Furthermore, IL-8 is prestored in Weibel-Palade bodies and released from endothelial cells on stimulation (49). Under our conditions in which the endothelial cells were serum starved for 2–4 h, concentrations of IL-8 and GRO\( \alpha \) were in the 10\(^{-11} \) to 10\(^{-10} \) M range, which is below the concentration range that activated the endothelial cells. Furthermore, the growth medium in which the HMECs were grown contains hydrocortisone that suppresses production of ELR chemokines by endothelial cells (37). It is, however, likely that activated endothelial cells produce ELR chemokines and stimulate their own IL-8 receptors in an autocrine fashion, which may lead to continuous activation of the endothelium. Activation of endothelial cell Rac and Rho as experienced when endothelial cell IL-8 receptors are stimulated induces nuclear factor-\( \kappa \)B-dependent gene expression including synthesis of IL-8 (18), thus enhancing this autocrine stimulation pathway. Under conditions of autocrine stimulation, functions of the CXCR2 are activated continuously, but surface expression of the receptor is minimal (5). In addition, surrounding cells including fibroblasts, normal epithelial cells, and cancer cells may provide the source of chemokine for the endothelium (2, 46). This paracrine supply of endothelial cells with chemokines may be particularly important for angiogenesis in the proximity of cancer cells that produce high concentrations of ELR chemokines.

In summary, IL-8 activates both the CXCR1 and the CXCR2 on endothelial cells. The two receptors use different signal transduction cascades that result in the activation of small G proteins and evoke responses that deserve to be further investigated. These chemokine-mediated endothelial cell responses may contribute to increased vascular permeability and leukocyte adhesiveness as observed during acute inflammation on the one hand and endothelial cell migration and proliferation during the angiogenic process on the other hand.

We thank G. Bokoch for expression vector constructs and Yoshitomi Pharmaceuticals for the gift of Y-27632. This work was supported by National Heart, Lung, and Blood Institute Grant HL-55657 to I. U. Schraufstatter and Deutsche Forschungsgemeinschaft Fellowship SA-632–2–1 to M. Burger.

Present address of M. Burger: Dept. of Nephrology, Univ. of Freiburg, 79106 Freiburg, Germany.

REFERENCES


33. Schrauffstatter IU, Barrett DS, Ma M, Oades ZG, and Cochrane CG. Multiple sites on IL-8 responsible for binding to α and β IL-8 receptors. *J Immunol* 151: 6418–6428, 1993.


