The chemokine receptor CCR8 mediates human endothelial cell chemotaxis induced by I-309 and Kaposi sarcoma herpesvirus-encoded vMIP-I and by lipoprotein(a)-stimulated endothelial cell conditioned medium

Nasreen S. Haque, John T. Fallon, Mark B. Taubman, and Peter C. Harpel

The CC chemokine receptor 8 (CCR8) is preferentially expressed on type 2 T lymphocytes. CCR8 is also expressed on monocytes and type 2 T lymphocytes and is a potent chemoattractant for both these cell types. The CC chemokine receptor 8 (CCR8) has recently been characterized.

Introduction

The CC chemokine receptor-8 (CCR8) has recently been characterized and is preferentially expressed on type 2 T lymphocytes. CCR8 is expressed on monocytes and T lymphocytes and is a potent chemoattractant for both these cell types. Our prior studies have shown that the apolipoprotein(a) (apo[a]) portion of the apolipoprotein Lp(a) stimulates human vascular endothelial cells to produce a monocyte chemotactic activity. We have recently identified I-309 as the principal monocyte chemottractant secreted by human umbilical vein endothelial cells (HUVECs) when incubated with apo(a). I-309 was found to inhibit HIV-1 envelope-mediated cell-cell fusion and virus infection.

I-309 is secreted by activated monocytes and lymphocytes and is a potent chemoattractant for both these cell types. Our prior studies have shown that the apolipoprotein(a) (apo[a]) portion of the apolipoprotein Lp(a) stimulates human vascular endothelial cells to produce a monocyte chemotactic activity. We have recently identified I-309 as the principal monocyte chemotactant secreted by human umbilical vein endothelial cells (HUVECs) when incubated with apo(a). I-309 was found to inhibit HIV-1 envelope-mediated cell-cell fusion and virus infection.

We now report that I-309, vMIP-I, and the endothelial conditioned medium (CM) produced by incubating Lp(a) or a recombinant apo(a) derivative with HUVECs induce endothelial chemotaxis. That CCR8 is the receptor responsible for mediating endothelial chemotaxis was supported by the demonstration that both a polyclonal and monoclonal antibody directed against CCR8 inhibited endothelial chemotaxis and that HUVECs expressed CCR8 messenger RNA as shown by RNA blot analysis as well by direct sequence analysis. Immunohistochemical studies identified CCR8 and I-309 on the endothelium of human atherosclerotic plaques and in endothelial-derived spindle cells of Kaposi sarcoma. These results indicate that CCR8 is an endothelial receptor that may modulate endothelial function.

Materials and methods

Proteins and antibodies

Recombinant I-309, monocyte chemotactic protein-1 (MCP-1), vMIP-I, goat polyclonal antibody against I-309 and CCR2, as well as a murine monoclonal antibody against CCR2 and the isotype controls were obtained from R&D Systems (Minneapolis, MN). Goat antibody against a 26-amino acid-containing peptide from the N-terminal portion of CCR8 (VTIYQNTNGKLC)8,15 (single-letter amino acid code) was from Alexis Biochemicals (San Diego, CA). We have produced a murine monoclonal antibody against this peptide using standard hybridoma methodology. Additional antibodies used in immunohistochemical studies included rabbit antihuman von Willebrand factor (WF; A0082, Dako, Carpentrya, CA), monoclonal antihuman CD-68 panmacrophage antibody (KP-1, M814, Dako), and monoclonal antibody to smooth muscle α-actin (1A4, Dako).

Purification of Lp(a) and a recombinant apo(a) derivative

Lipoprotein(a) was prepared as detailed previously. A recombinant apo(a) derivative comprised of kringle 4 types 5-10, kringle V, and the protease domain was produced as detailed previously.
domain, representing the carboxy-terminal portion of apo(a) [6K apo(a)]\textsuperscript{16,17}
was kindly provided by Dr Maryls Koschinsky.

**Culture of HUVECs**

The HUVECs were harvested and cultured as described.\textsuperscript{13} For generating
endothelial cell CM, cells from passages 2 through 5 were grown in 6-well plates. Either Lp(a) (100 µg/mL; 100 nM) or 6K apo(a) (20 µg/mL; 100 nM) were incubated with HUVECs for 6 hours at 37°C, and the CM frozen
at −80°C until tested. Cell incubation mixtures contained 20 µg/mL polymyxin B (Sigma, St Louis, MO) to inhibit endotoxin.

**Endothelial cell transmigration activity**

Cell transmigration was measured as described for mononuclear cells.\textsuperscript{13,18} Using a Neuroprobe chamber (Nucleopore, Pleasanton, CA) containing a polycarbonate filter with 5-µm pores. The filter was washed with 0.2% methanol and stained with Diff-Quik (Baxter Healthcare, Miami, FL). The number of HUVECs on the lower surface of the filter was determined by counting 3 microscopic fields per well under oil immersion (400×). To establish the effect of antibodies against CCR8, the purified antibodies or isotype controls were added to confluent HUVECs (1 µg IgG/mL) in 6-well tissue culture plates and incubated for 30 minutes at 37°C before the assay. After washing, the treated endothelial cells were harvested. To study the effects of pertussis and cholera toxins on endothelial cell transmigration induced by I-309, vMIP-I, and the CM from HUVECs incubated with Lp(a), the cells were cultured in the presence of 0.1 µg/mL pertussis toxin or 0.4 µg/mL cholera toxin for 16 hours before the assays.\textsuperscript{2} All samples were tested for endothelial cell transmigration in triplicate wells in the Neuroprobe chamber.

**Sequence analysis of HUVEC CCR8 complementary DNA**

Total RNA was isolated using the RNeasy kit (Qiagen, Chatsworth, CA). Oligonucleotide primers (Operon Technologies, Alameda, CA) for CCR8 complementary DNA (cDNA) were designed as described.\textsuperscript{2} The sense
5′-TTATGTTCTCTGGCTGACCAG and the antisense 5′-TAGTCTTCA TT-9
were used to induce endothelial transmigration. The response to
the CC chemokines was chemotactic (directed), rather than chemokinetic (nondirected), a checkerboard analysis was performed (Figure 1). I-309 (100 ng/mL), vMIP-I (100 ng/mL), or the CM from 6K apo(a)-stimulated endothelial cells (KCM) was placed in the bottom wells, in the top wells with the indicator endothelial cells, or in both top and bottom wells of the chemotaxis chamber. All 3 agonists increased endothelial cell transmigration approximately 5-fold when placed in the bottom wells of the chemotaxis chamber, as compared to the medium 199 control. In contrast, the migration of endothelial cells was 3- to 4-fold less when the agonists were added either to the top or to both top and bottom wells. This checkerboard analysis indicates that the transmigration of HUVECs in response to the agonists tested was primarily chemotactic. This study shows for the first time that I-309, vMIP-I, and apo(a) CM are chemotactic for HUVECs.

**Polyclonal antibody against CCR8 inhibits chemotaxis of HUVECs induced by I-309 and vMIP-I**

To examine the effect of blocking polyclonal antibodies against CCR8, I-309, and vMIP-I receptor, HUVECs were pretreated for 30 minutes at 37°C with 1 µg/mL normal goat IgG, goat
induce endothelial chemotaxis. Lp(a) or the 6K apo(a) in the absence of incubation with HUVECs did not have significant endothelial chemotactic activity. LCM and KCM induced a 3-fold increase in endothelial transmigration as compared to the CM from endothelial cells incubated with medium 199 (P < .001; Figure 3). Blocking polyclonal antibodies directed against I-309 or CCR8 significantly inhibited chemotaxis induced by LCM or KCM, whereas the control goat IgG had no inhibitory effect.

A murine monoclonal antibody against the N-terminal extracellular domain of CCR8 inhibits HUVECs chemotaxis induced by I-309, vMIP-I, Lp(a) CM, but not by stromal cell-derived factor

Pertussis toxin inhibits HUVEC chemotaxis induced by I-309, vMIP-I, and LCM

To provide further evidence that the induction of endothelial chemotaxis by I-309, vMIP-I, and LCM was associated with a G-coupled chemokine receptor, the HUVECs were treated with the protein inhibitor pertussis toxin. Pertussis toxin inhibited endothelial transmigration induced by I-309, vMIP-I, and LCM (P < .001; Figure 5). Cholera toxin, in contrast, had no inhibitory effect on endothelial chemotaxis.
CCR8 mRNA is expressed in HUVECs

RNA blot analysis with CCR8 cDNA demonstrated a single band in Jurkat cells as well as in confluent HUVECs (Figure 6). The identity of the DNA transcript as CCR8 was confirmed by sequence analysis as detailed in the “Materials and methods” (GenBank accession number U62556).

The endothelium in human atherosclerotic plaques contains CCR8 and I-309

The luminal endothelium of coronary endarterectomy specimens stained positively for CCR8 with both the polyclonal anti-CCR8 (Figure 7A) and the murine monoclonal anti-CCR8 (Figure 7C).

Kaposi sarcoma contains CCR8 and I-309

Ten human biopsy specimens of Kaposi sarcoma were immuno-stained for multiple antigens including CCR8, I-309, macrophage antigen CD-68 (KP-1), smooth muscle cell α-actin, and vWF antigen. Sections from a representative biopsy specimen are shown in Figure 8. The tissue section shown is comprised mainly of endothelial-derived spindle cells that stain intensely and diffusely for CCR8 with both the polyclonal antibody (Figure 8A) and the murine monoclonal anti-CCR8 (Figure 8D). Similar positive staining was documented for I-309 (Figure 8B). vWF antigen stained diffusely indicating endothelial cell origin for the Kaposi tumor cells (Figure 8C). The Kaposi spindle cells also stained for CD-68 antigen (Figure 8E). Microvessels as identified by pericytes staining positively for α-actin are also scattered throughout the biopsy specimen (Figure 8F). Neither the secondary antibody (control, Figure 8G) nor the murine IgG1 (Figure 8H) significantly stained the Kaposi tissue. In addition to blocking the tissue sections with ovalbumin, the inclusion of horse serum or rabbit serum did not inhibit immunostaining for CCR8 or I-309. Absorption of the CCR8 with the N-terminal peptide of CCR8 blocked the immunostaining (data not shown).

Discussion

Endothelial cell migration in response to chemotactic signals appears to be required for angiogenesis, wound repair, and...
a variety of factors including vascular endothelial growth factor and basic fibroblast growth factor can stimulate endothelial migration. Chemokines, which are low-molecular-weight proteins (8-10 kd), that have the capacity to direct leukocyte trafficking into areas of inflammation may also participate in endothelial cell migration. Chemokines are classified into 2 major families that are distinguished by the position of the first of 4 conserved cysteines (CXC and CC). A CXC chemokine, SDF-1, is a potent inducer of endothelial chemotaxis, and its receptor CXCR4 has been reported as essential for vascularization of the gastrointestinal tract. The CC chemokine receptor CCR2 was recently identified on human endothelial cells, and its agonist, the CC chemokine MCP-1, was found to stimulate endothelial chemotaxis.

In the present study we document for the first time that the CC chemokine I-309 and the HHV-8 encoded CC chemokine vMIP-I stimulate the transmigration of HUVECs. The results of checkerboard analysis indicate that the endothelial response to these agonists was due to chemotaxis rather than to chemokinesis. The CM from Lp(a)- or from 6K apo(a)-stimulated HUVECs, previously shown to contain I-309 and to be chemotactic for monocytes, was also chemotactic for endothelial cells. That the endothelial agonist was I-309 in the CM was supported by the observation that anti-I-309 antibody inhibited endothelial migration.

It has been reported recently that vMIP-I is a specific agonist for CCR8. In a study using 65 recombinant chemokines to characterize the recognition properties of CCR8, only 2, I-309 and vMIP-I, acted as agonists as they bound to CCR8 and induced calcium flux. In another study, cell lines transfected with known or suspected chemokine receptors were tested for calcium flux response to 40 different chemokines. Only cells transfected with CCR8 (CCR8-Y3 cells) responded to vMIP-I and I-309. Cell lines stably transfected with 12 other chemokine receptors were tested and were found not to respond to either vMIP-I or I-309 emphasizing the specificity of CCR8.

We have also documented by RNA blot analysis and more importantly by direct sequence analysis the presence of CCR8 cDNA in HUVECs. Our immunohistochemical studies of human coronary endarterectomy specimens demonstrate that CCR8 and I-309 are present on the luminal endothelium of atherosclerosis. A variety of factors including vascular endothelial growth factor and basic fibroblast growth factor can stimulate endothelial migration. Chemokines, which are low-molecular-weight proteins (8-10 kd), that have the capacity to direct leukocyte trafficking into areas of inflammation may also participate in endothelial cell migration. Chemokines are classified into 2 major families that are distinguished by the position of the first of 4 conserved cysteines (CXC and CC). A CXC chemokine, SDF-1, is a potent inducer of endothelial chemotaxis, and its receptor CXCR4 has been reported as essential for vascularization of the gastrointestinal tract. The CC chemokine receptor CCR2 was recently identified on human endothelial cells, and its agonist, the CC chemokine MCP-1, was found to stimulate endothelial chemotaxis.

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the atherosclerotic plaque. We have also observed CCR8, I-309, and vWF antigen in subendothelial areas of the atherosclerotic plaque. The cellular source of these antigens in the intima are unknown at present. These findings support the possibility that the I-309 and its receptor, CCR8, may contribute to the athero-
genous process.

Kaposi sarcoma HHV-8 is causally linked to hematologic malignancies including all types of Kaposi sarcoma, primary effusion lymphomas, and to multicentric Castleman disease. The mechanisms by which Lp(a) participates in atherogenesis are not defined. Our findings that Lp(a) stimulates endothelial chemotaxis by means of its capacity to induce endothe-

The findings in the present study indicate that vMIP-I is directly chemotactic for endothelial cells. Immunohistochemical studies of Kaposi sarcoma biopsy specimens document diffuse staining for CCR8 associated with Kaposi sarcoma spindle cells. We also found intense and diffuse staining for I-309 in the Kaposi sarcoma lesion in a similar distribution to that of CCR8. Spindle cells appear to represent a mixed population of activated endothelial cells and macrophages, and these cells have been shown to stain for CD-68 antigen, as observed in the present study. These findings raise questions as to the possible role of I-309 and CCR8 in the formation of the Kaposi sarcoma lesion. Endothelial CCR8 may be in part responsible for the vasculogenic activity of vMIP-I previously documented in the chorioallantoic assay. Lipoprotein(a) has been shown to be an independent risk factor for atherosclerosis (reviewed in reference 39). Lp(a) consists of a low-density lipoprotein particle disulfide linked to apo(a), a glycoprotein of variable size that shares partial homology with plasminogen. The mechanisms by which Lp(a) participates in atherogenesis are not defined. Our findings that Lp(a) stimulates endothelial chemotaxis by means of its capacity to induce endothe-

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The present demonstration that CCR8 is present on vascular endothelial cells and that its stimulation by I-309 leads to endothelial migration indicates that I-309 may also function as an autocrine modulator of endothelial function. Two different angiogenesis inhibitors were found to reduce both intimal neovasculariza-

The identification of CCR8 as an I-309 and vMIP-I receptor on vascular endothelial cells may be important to our understanding of the mechanisms underlying endothelial function in atherogenesis, in the formation of the lesions in Kaposi sarcoma, and as a novel target for the modulation of angiogenesis.


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