Monocyte Chemotactic Protein-2 Activates CCR5 and Blocks CD4/CCR5-mediated HIV-1 Entry/Replication

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Human immunodeficiency virus, type I (HIV-1) cell-type tropism is dictated by chemokine receptor usage: T-cell line tropic viruses use CXCR4, whereas monocyte tropic viruses primarily use CCR5 as fusion coreceptors. CC chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β, and RANTES (regulated on activation normal T cell expressed and secreted) inhibit CD4/CCR5-mediated HIV-1 entry/replication. MCP-2 is also a member of the CC chemokine subfamily and has the capacity to interact with at least two receptors including CCR-1 and CCR2B. In an effort to further characterize the binding properties of MCP-2 on leukocytes, we observed that MCP-2, but not MCP-1, effectively competed with MIP-1β for binding to monocytes, suggesting that MCP-2 may interact with CCR5. As predicted, MCP-2 competitively inhibited MIP-1β binding to IHEK293 cells stably transfected with CCR5 (CCR5/293 cells). MCP-2 also bound to and induced chemotaxis of CCR5/293 cells with a potency comparable with that of MIP-1β. Confocal microscopy indicates that MCP-2 caused remarkable and dose-dependent internalization of CCR5 in CCR5/293 cells. Furthermore, MCP-2 inhibited the entry/replication of HIV-1ΔΔA in CCR5/293 cells coexpressing C4D. These results indicated that MCP-2 uses CCR5 as one of its functional receptors and is an additional potent natural inhibitor of HIV-1.

Members of the seven-transmembrane chemokine receptor superfamily have been identified as co-receptors for HIV-1 infection (1–8). HIV-1 cell type tropism seems to be dependent on chemokine receptor usage, and T-cell line tropic viruses use CXCR4, whereas monocyte tropic viruses primarily use CCR5 as fusion coreceptors. A minority of HIV-1 strains may also use other CC chemokine receptors as their fusion co-factors. Dual tropic HIV-1 strains presumably interact with more than one type of chemokine receptor (5, 8). The CC chemokines MIP-1α, MIP-1β, and RANTES were able to inhibit the entry of monocyte tropic viruses (9), whereas the CXC chemokine SDF-1 abrogates CXCR4-mediated fusion by T lymphotropic HIV-1 strains (6, 7).

Monocyte chemotactic protein (MCP)-2 is a CC chemokine co-purified with MCP-1 and MCP-3 from human osteosarcoma cells (10–12). It shares over 60% amino acid identity with MCP-1 and MCP-3 and has about 30% identity with the CC chemokines MIP-1α, RANTES, and MIP-1β (10–12). MCP-2, similar to MCP-3, is chemotactic for and activates a wide variety of inflammatory cells, including monocytes, T lymphocytes, NK cells, basophils, mast cells, and eosinophils (12), but differs from MCP-1, which is not active on eosinophils (13). We recently reported that MCP-2 uses CCR1 and CCR2B as its functional receptors (14), which may account for its action on a greater variety of target cells. In the course of studies on leukocyte activation by MCP-2, we observed that MCP-2 could competitively inhibit the binding to monocytes of 125I-MIP-1β, a CC chemokine that is believed thus far to exclusively use CCR5 as a functional receptor on human leukocytes (15–17). This prompted us to further investigate the effect of MCP-2 on cloned CCR5. We report that MCP-2 is also an efficient ligand for CCR5 and a potent inhibitor of CD4/CCR5-mediated HIV-1 entry/replication.

EXPERIMENTAL PROCEDURES

Chemokines—Recombinant human (rh) MCP-2 and other chemokines were purchased from PeproTech Inc. (Rocky Hill, NJ). Radiiodinated MCP-2 was a kind gift from Dr. G. Brown (NEN Life Science Products). Other radioiodinated chemokines were purchased from NEN. All radioiodinated chemokines have a specific activity of 2200 Ci/mmol.

Cells—Human peripheral blood monocytes were isolated from normal donors (National Institutes of Health Clinical Center, Transfusion Department, Bethesda, MD) with an iso-osmotic Percoll (Pharmacia Biotech Inc.) gradient as described elsewhere (18). The monocyte preparations were >90% pure. The 293 human embryonic kidney epithelial cell line stably transfected with FLAG-tagged CCR5 (CCR5/293) was generated and grown in monolayers as described (17).

Binding Assays with Radiolabeled Chemokines—Binding assays were performed by using a single concentration of radiolabeled chemokines in the presence of increasing concentrations of unlabeled ligands as described previously (14, 18). Cells (2 × 10^6/sample for monocytes and 1 × 10^6/sample for CCR5/293 cells) were suspended in 200 μl of modified binding medium composed of RPMI 1640, 1 mg/ml bovine serum albumin, 25 mM HEPES, and 0.05% sodium azide and incubated in duplicates at room temperature for 60 min. After incubation, the cells were pelleted through a 10% sucrose/phosphate-buffered saline cushion, and the radioactivity associated with cell pellets was determined in a γ-counter (Clicimagamma-Pharmacia, Gaithersburg, MD). Experiments were also performed at 4 °C in the absence of sodium azide and yielded similar binding and competition curves as obtained at room temperature. The binding data were then analyzed with a Macintosh computer program LIGAND (P. Munson, Division of Computer Research and Technology, NIH, Bethesda, MD). The degree of competition for binding by unlabeled chemokines was calculated as follows: % competition = 100 × (ligand bound in presence of competitor/ligand bound in absence of competitor).
compared with binding in the absence of unlabeled ligand, Student’s t test was performed yielding similar results. Unlabeled MCP-1 at 10 nM and more showed partial but significant competition of 125I-MIP-1 binding (Fig. 1A) and at 4 °C (Fig. 1B). The uni-directional inhibition of MIP-1β binding to monocytes by MCP-2 prompted us to investigate whether MCP-2, in addition to using CCR1 and CCR2B as functional receptors, also uses CCR5. HEK293 cells stably expressing CCR5 (CCR5/293) showed a high level of specific binding (∼ 10,000 cpm), bound/total; B/F; Scatchard analysis. Results from one experiment out of five performed are shown. 

RESULTS AND DISCUSSION

Recombinant human MCP-2 is a potent monocyte chemotactant and has been shown to activate at least two promiscuous CC chemokines receptors, CCR1 and CCR2B (14). The binding of 125I-labeled MCP-2 to human monocytes was of high affinity and was efficiently competed by MCP-1, which is a ligand for CCR2B. MCP-3, which uses both CCR1 and CCR2B, also efficiently competed with MCP-2 for binding to monocytes. MIP-1α and RANTES, two ligands for both CCR1 and CCR5, partially competed with MCP-2 for binding to monocytes, whereas MIP-1β, which only uses CCR5, had no effect (14). We further observed that although unlabeled MIP-1α did not competitively inhibit MCP-2 binding to monocytes, the binding of 125I-MIP-1β was completely inhibitable by unlabeled MCP-2 (IC50 = 2.9 nM versus 1.7 nM for unlabeled MIP-1β itself, Fig. 1). In contrast, the binding of 125I-MIP-1β to monocytes was only partially but significantly displaced by unlabeled MCP-1 at high concentrations (IC50 = 425 nM), in agreement with our earlier observations (22). There was no significant difference between the competition curves yielded at room temperature (Fig. 1A) and at 4 °C (Fig. 1B). The uni-directional inhibition of MIP-1β binding to monocytes by MCP-2 prompted us to investigate whether MCP-2, in addition to using CCR1 and CCR2B as functional receptors, also uses CCR5. HEK293 cells stably expressing CCR5 (CCR5/293) showed a high level of specific binding (∼ 10,000 cpm), bound/total; B/F; Scatchard analysis. Results from one experiment out of five performed are shown.

FIG. 3. Competition of 125I-MCP-2 binding to CCR5/293 cells and competition by CC chemokines. A, Scatchard analysis. B, Cross-competition. CCR5/293 cells were incubated with 0.12 nM of 125I-MCP-2 in the presence of different concentrations of unlabeled MCP-2. The binding assays were performed at room temperature. The data are from one representative experiments out of three performed and were analyzed using the Macintosh computer program LIGAND. MIP-1 and MIP-3 at 10 nM and more showed partial but significant competition of 125I-MIP-1β binding (p < 0.05 compared with binding in the absence of unlabeled ligand, Student’s t test).
binding for $^{125}$I-MIP-1 that was competitively inhibited by MCP-2 either at room temperature (Fig. 2A) or at 4 °C (Fig. 2B). Unlabeled MIP-1$\alpha$ also completely inhibited $^{125}$I-MIP-1 binding (Fig. 2). These results suggest that MCP-2 has the capacity to interact with CCR5 on HEK293 cells with an efficiency similar to known CCR5 ligands such as MIP-1$\beta$ and MIP-1$\alpha$.

Further support for the ability of MCP-2 to interact with CCR5 was obtained with binding studies using $^{125}$I-MCP-2. As shown in Fig. 3, $^{125}$I-MCP-2 specifically bound to CCR5/293 cells with an estimated $K_d$ in the 5 nM range (Fig. 3A). The binding of $^{125}$I-MCP-2 to CCR5/293 cells was efficiently inhibited by unlabeled MIP-1$\beta$ and RANTES (Fig. 3B) but less effectively by MCP-1 and MCP-3, which in contrast were able to completely displace MCP-2 binding to CCR1 and CCR2B (14). Thus, MCP-2 possesses binding domains for interaction with CCR5 in addition to CCR1 and CCR2B. This was supported by the observations that although MCP-1, MCP-2 and MCP-3 are all functional ligands for CCR2B, the binding of MCP-1 on CCR2B was poorly competed for by MCP-2 or MCP-3 (14, 23) suggesting differential utilization of certain binding domains on a receptor by multiple ligands. Because promiscuity is a common feature of chemokines and their receptors (24, 25) and receptor activation appears to be dependent on the relative affinity to interact with a ligand, structure analyses and mutagenesis studies are required to more precisely determine the functional epitopes on both ligands and receptors.

We next determined the functional role of MCP-2 on CCR5. Both rhMCP-2 and rhMIP-1$\beta$ are poor Ca$^{2+}$ mobilizers in monocytes (18, 26), and we were not able to observe significant Ca$^{2+}$ flux in CCR5/293 cells with these two ligands, although a RANTES (120 nM)-induced signal was obtained (data not shown). We therefore utilized chemotaxis assays, which in our previous studies were demonstrated to be very sensitive and reproducible in assessing the activity of a chemokine on a given receptor (14, 19). CCR5/293 cells showed a significant chemotactic response to MCP-2 and MIP-1$\beta$ (Fig. 4), yielding a typical bell-shaped dose response. MCP-2 was similar in potency to MIP-1$\beta$ with an almost 6-fold increase over medium control at 1.2–6 nM concentration range, but it was less efficacious (EC50: MCP-2, 0.12 nM; MIP-1$\beta$, 0.04 nM, respectively) in inducing CCR5/HEK293 cell migration. Other chemokines known to activate CCR5, such as MIP-1$\alpha$ and RANTES also bound to and induced considerable chemotactic migration of CCR5/293 cells. Although MCP-1 partially displaced MCP-2 binding to CCR5/293 cells, it only bound to and induced migration of CCR2B/293 but not CCR5/293 cells (data not shown). Likewise, MCP-3 also partially displaced MCP-2 binding to CCR5/293 cells; we did not detect significant binding of $^{125}$I-MCP-3 to the same cells, nor did it induce migration of CCR5/293 cells at a wide range of concentrations (data not shown). Further proof of the activation of CCR5 by MCP-2 is provided by confocal microscopy (Fig. 5) in which MCP-2 markedly and dose-dependently induced CCR5 internalization in CCR5/293 cells. Another CCR5 ligand RANTES and PMA, a potent protein kinase C activator, similarly caused internalization of CCR5. In contrast, CXC chemokine IL-8, which uses CXCR1 and CXCR2 as functional receptors, did not induce CCR5 internalization (Fig. 5).

The identification of CCR5 as the principal co-receptor for cellular entry by monocyte tropic HIV-1 strains (2–5, 8) and the inhibition of viral fusion and replication by the CCR5 ligands MIP-1$\alpha$, MIP-1$\beta$, and RANTES (9) led us to question whether MCP-2 similarly could inhibit HIV-1 entry and replication. The susceptibility to HIV-1 infection of CCR5/CD4/293 cells was determined in the presence or the absence of MCP-2 (Fig. 6). Preincubation of CCR5/CD4/293 cells with either 800 ng/ml MCP-2 or RANTES completely inhibited HIV-1 entry and subsequent reverse transcription. No reverse transcription products were detected in CCR5/CD4/293 cells treated with HIV-1RF, a T lymphocyte tropic strain (data not shown). CCR5/293 cells without CD4 were not infected by HIV-1ADA. Therefore, the ability of MCP-2 to internalize CCR5 confers on it the
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Fig. 6. Inhibition of HIV-1Ada entry/replication in CCR5/C4-HEK293 cells by MCP-2. CCR5/293 cells coexpressing CD4 were preincubated in the presence or the absence of chemokines for 30 min at 37 °C followed by 24 h of incubation with HIV-1Ada. After washing, cellular genomic DNA was extracted, and HIV-1 reverse transcription products were monitored by PCR with primers corresponding to HIV-1 gag region. Cells, CCR5/C4/293 cells without virus; Cells + Virus, CCR5/C4/293 cells incubated with HIV-1Ada; RANTES, cells pretreated with 800 ng/ml RANTES then with HIV-1Ada; MCP-2, cells pretreated with 800 ng/ml MCP-2 and then with HIV-1Ada; PCR-control, CEM-SS cells without HIV-1 infection; PCR+control, CEM-SS cells infected with HIV-1. Results are from one experiment out of three performed.

ability to interrupt virus entry, with an antiviral activity equivalent to RANTES. MCP-2 also appears to be a natural inhibitor of CD4/C5R5-mediated HIV-1 entry/replication in host cells in addition to MIP-1α, MIP-1β, and RANTES (6, 7, 9).

MCP-2 is constitutively expressed in tumor cells and is inducible by pro-inflammatory cytokines in mononuclear cells and fibroblasts (10, 11). MCP-2 exhibits a broader spectrum of targeted cells, including cells of dendritic phenotype (27). In the present study, MCP-2 may play an important role in recruiting/activating immune cells at inflammatory and neoplastic foci. In the present study, we demonstrated for the first time using cloned CCR5 that MCP-2 is a highly efficacious ligand for this receptor in addition to CCR1 and CCR2 (14). In our preliminary study, HEK293 cells transfected with CCR3, which is a receptor for CC chemokines eotaxin and MCP-4 (28, 29), were induced to migrate significantly and reproducibly in response to MCP-2 in addition to eotaxin and MCP-4 (data not shown). Thus, MCP-2 appears to use CCR1, CCR2B, CCR5, and possibly CCR3, suggesting it has a more promiscuous functional pattern than other known CC chemokines.

Despite an apparent redundancy in chemokines and their receptor family (24, 25, 30, 31), CC chemokines have been implicated as important mediators of many pathological conditions such as chronic inflammation, immune diseases, neoplasia, and atherosclerosis (30, 31). CCR5 ligands MIP-1α, MIP-1β, and RANTES are also major HIV-1 inhibitors produced by activated mononuclear cells (9). Our current observations extend the functional scope of MCP-2 as a potent inhibitor of monocyte tropic HIV-1 infection in CD4+/CCR5+ cells. Investigation into the shared and unique functional domains on MCP-2 in comparison with other chemokine ligands will be important in the development of therapeutic approaches to chemokine- and chemokine receptor-mediated pathological states.

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