Brief Definitive Report

Identification of C-C Chemokine Receptor 1 (CCR1) as the Monocyte Hemofiltrate C-C Chemokine (HCC)-1 Receptor

By Chia-Lin Tsou,* Ron P. Gladue,§ Laurie A. Carroll,§ Tim Paradis,§ James G. Boyd,§ Robin T. Nelson,§ Kuldeep Neote,§ and Israel F. Charo*‡

*From the *Gladstone Institute of Cardiovascular Disease, the Cardiovascular Research Institute, the ‡Daiichi Research Center, and the Department of Medicine, University of California, San Francisco, California 94141; and §Pfizer Inc., Groton, Connecticut 06340*

Summary

Hemofiltrate C-C chemokine (HCC)-1 is a recently cloned C-C chemokine that is structurally similar to macrophage inflammatory protein (MIP)-1α. Unlike most chemokines, it is constitutively secreted by tissues and is present at high concentrations in normal human plasma. Also atypical for chemokines, HCC-1 is reported not to be chemotactic for leukocytes. In this paper, we have investigated the chemokine receptor usage and downstream signaling pathways of HCC-1. Cross-desensitization experiments using THP-1 cells suggested that HCC-1 and MIP-1α activated the same receptor. Experiments using a panel of cloned chemokine receptors revealed that HCC-1 specifically activated C-C chemokine receptor (CCR)1, but not closely related receptors, including CCR5. HCC-1 competed with MIP-1α for binding to CCR1-transfected cells, but with a markedly reduced affinity (IC_{50} = 93 nM versus 1.3 nM for MIP-1α). Similarly, HCC-1 was less potent than MIP-1α in inducing inhibition of adenylyl cyclase in CCR1-transfected cells. HCC-1 induced chemotaxis of freshly isolated human monocytes, THP-1 cells, and CCR1-transfected cells, and the optimal concentration for cell migration (100 nM) was ~100-fold lower than that of MIP-1α (1 nM). These data demonstrate that HCC-1 is a chemoattractant and identify CCR1 as a functional HCC-1 receptor on human monocytes.

Key words: hemofiltrate C-C chemokine • C-C chemokine receptor 1 • chemokine • chemotaxis • macrophage inflammatory protein 1α

Chemokines are small (8–10 kD), secreted basic peptides that are involved in the directed migration and activation of leukocytes (for review see references 1, 2). The majority of chemokines can be divided into two groups, based on the arrangement of the first two of four conserved cysteines. The α-, or "C-X-C", branch includes IL-8, GRO (α, β, γ), neutrophil-activating peptide (NAP)-2, and platelet factor 4, and is characterized by the presence of a single amino acid between the first two cysteines. In the β-, or "C-C" branch, the first two cysteines are adjacent. Members of the β-branch include the monocyte chemoattractant protein (MCP)-1, RANTES (regulated on activation, normal T cell expressed and secreted), macrophage inflammatory protein (MIP)-1α, MIP-1β, and eotaxin. In general, C-X-C chemokines are chemotactic for neutrophils, whereas the C-C chemokines are chemotactic for monocytes and lymphocytes. In addition to their conserved structural motifs, the chemokines share certain functional properties, including a lack of constitutive secretion by cells, extremely low to nondetectable circulating levels in the plasma of healthy persons, and the ability to induce chemotaxis of leukocytes.

Hemofiltrate C-C chemokine (HCC)-1 is a recently described monocyte chemoattractant, originally isolated from the hemofiltrate of patients with chronic renal failure (3). Determination of the amino acid sequence of HCC-1 revealed four cysteine residues in positions characteristic of the C-C chemokine family, and comparison with the sequences of other chemokines revealed that HCC-1 was most homologous to MIP-1α. However, several functional properties of HCC-1 were atypical of chemokines. Unlike other chemokines, HCC-1 was expressed constitutively in a number of tissues and was present at high concentrations in normal human plasma. In addition, HCC-1 was reported not to be chemotactic for leukocytes (3). We have examined the chemokine receptor usage of HCC-1 and its functional properties on both primary leukocytes and cell lines transfected with cloned chemokine receptors. Here,

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we report that the endogenous receptor for HCC-1 is the C-C chemokine receptor 1 (CCR1) and that binding of HCC-1 leads to the generation of classical second messengers and a robust chemotactic response.

Materials and Methods

Reagents. Chemokines were obtained from R&D Systems (Minneapolis, MN) and PeproTech, Inc. (Rocky Hill, NJ). HCC-1 was synthesized at Pfizer as described below. Lipofectamine, RPMI 1640, and MEM with Earle's balanced salt were from GIBCO BRL (Gaithersburg, MD). M1 antibody was obtained from Kodak (Eastman Kodak Co., Rochester, NY). FCS was from Hyclone Laboratories (Logan, UT). [γ-^{32}P]ATP and [^{3}H]adenine were purchased from DuPont-NEN (Boston, MA). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO), unless otherwise noted.

Cells. PBMCs were prepared from healthy adult donors. After the addition of heparin (20 U/ml; Elkin-Sinn, Cherry Hill, NJ), venous blood was subjected to centrifugation on Ficoll-Hypaque (1,000 *g*, 45 min, 25°C). The buffy coat of PBMCs at the interface was harvested, and contaminating erythrocytes were removed by hypotonic lysis. The PBMCs were washed and resuspended in cell culture medium. Monocytes were prepared as previously described (4). THP-1 cells were obtained from the American Type Culture Collection (Clone TIB-202, ATTC 30-2001, Rockville, MD) and grown in RPMI 1640 supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM glutamine, 10 mM Hepes, 100 U/ml penicillin/streptomycin, and 0.1 mM nonessential amino acids (BioWhittaker, Walkersville, MD). The 300-19 lymphocyte-like cell line (5) was a gift from Dr. Greg LaRosa (Leuko-Site, Inc., Cambridge, MA).

Transfected Cells. HEK-293 cells stably expressing CCR1, CCR2, CCR5, and CXCR1 were prepared as previously described (6). 300-19 cells stably expressing CCR4 and CCR3 were prepared as previously described for CCR2 (7) and CCR5 (8). HEK-293 cells transiently expressing human CCR6, CCR7, and CCR8 were prepared as previously described (9). The cDNAs for CCR6, CCR7, and CCR8 were obtained using oligonucleotides corresponding to published DNA sequences (10–13) and standard PCR protocols. All PCR products were completely sequenced.

Assays. Calcium fluorometry and adenylyl cyclase measurements were performed as previously described (7). HCC-1 was labeled with the Bolton-Hunter reagent (diiodide; DuPont-NEN) as previously described (9). Unconjugated iodide was separated from labeled protein by elution through a PD-10 column (Pharmacia Biotech, Piscataway, NJ) equilibrated with PBS and BSA (1% wt/vol). Competition binding was performed as described (9), and IC_{50} values were determined using the program "Prism" (GraphPad, Inc., San Diego, CA).

Chemotaxis. Human monocyte chemotaxis studies were performed using a 48-well Boyden chamber (Neuro Probe, Inc., Cabin John, MD). PBMCs (2×10^5) were added to the upper chamber, and the indicated concentrations of the agonists to the lower chamber. The upper and lower chambers were separated by a 5-μm PVP-coated filter. Chambers were incubated for 90 min at 37°C in a humidified incubator in the presence of 5% CO_2. The filter was removed and the upper surface was scraped to remove nonadherent cells and stained with DiffQuik (Fisher Scientific Co., Pittsburgh, PA). The number of migrating cells in six randomly chosen high-power fields was counted. Chemotaxis studies of THP-1 cells were performed using a 96-well neuroprobe apparatus (Neuro Probe, Inc.). Agonists were placed in the bottom wells, and THP-1 cells (800,000 cells/well) were added to the upper wells. After a 3-h incubation (37°C, 5% CO_2, 95% humidity), the upper chambers were washed free of cells, the top of the filter was scraped, and EDTA was added. After 20 min, the plate and filter were centrifuged, and the number of cells that migrated was determined by colorimetry using fluorescein diacetate. Chemotaxis studies of 300-19 cells stably expressing CCR1 were performed using a transwell apparatus, as previously described (7).

Chemical Synthesis of HCC-1. Automated Fmoc-based solid-phase peptide syntheses were performed on solid-phase peptide synthesizer (model 431 A; software version Synthassist 2.0; PE Applied Biosystems, Foster City, CA) retrofitted with deprotection monitoring. HBTU (benzotriazoleyl tetramethyluronium hexafluophosphate) activation and single amino acid coupling cycles were used, except following sluggish deprotection steps, in which case the following residue was double coupled. Syntheses were started using 0.25 mmol preloaded Fmoc amino acid (Wang) resin, 50% of the resin cake was removed midway through the synthesis, and the synthesis was completed. The final synthesis resin was cleaved and deprotected by treatment with a solution of 83% TFA (trifluoroacetic acid), 5% phenol, 5% water, and 2.5% ethandithiol for 1 h, 23°C. The mixture was filtered, the TFA filtrate was diluted into 50 ml of diethylether, and the precipitated crude peptide salt was collected by centrifugation. The peptide was purified by preparative reverse-phase HPLC (20 × 250 mm Waters C18 column; Waters Corp., Milford, MA) using a water/acetonitrile (0.1% TFA) gradient. Fractions were assayed by analytical HPLC (4.5 × 250 mm Vydac C18 column; Western Analytical, Murrieta, CA), appropriate fractions were pooled (>85% purity), and the resulting solution was adjusted with redox additives (final concentrations: 0.5 mM cysteine, 0.5 mM cystine, 10 mM methionine, 75 mM Hepes, pH 8.0, 0.05–0.5 mg/ml peptide). The reaction was essentially complete after 15 h at 4°C, as indicated by a shift to shorter retention time on analytical HPLC. The oxidized product was purified as above to >98% purity. Concentration was estimated by UV absorbance at 280 nm and confirmed by amino acid analysis (Michigan Protein Structure Facility, Ann Arbor, MI). The product was aliquoted, lyophilized, and stored at −80°C. Electrospray mass (PE-Sciex API100) of folded HCC-1 1–74 = 8673.3 daltons (calculated: 8673.8 daltons), 4–74 = 8342.8 daltons (calculated: 8343.4 daltons), 6–74 = 8126.7 daltons (calculated: 8127.2 daltons).

Results

The cDNA for HCC-1 encodes a predicted protein of 93 amino acids (Fig. 1). We synthesized three variants of HCC-1, based upon the method of Neilsen et al. (14) for predicting signal sequence cleavage points. Thus, threonine 20, glutamic acid 23, and serine 25 were all considered potential NH_2 termini of the mature protein.

To identify its receptor, we tested HCC-1's ability to activate cloned C-C or C-X-C chemokine receptors ex-

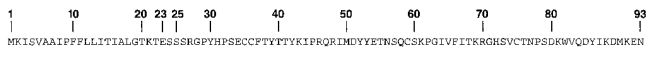

Figure 1. Amino acid sequence of HCC-1. The sequence of the full-length protein is shown on the top line. Three NH_2-terminally truncated variants of HCC-1 (20–93, 23–93, and 25–93), based on alternative sites for cleavage of the putative signal sequence, are indicated.

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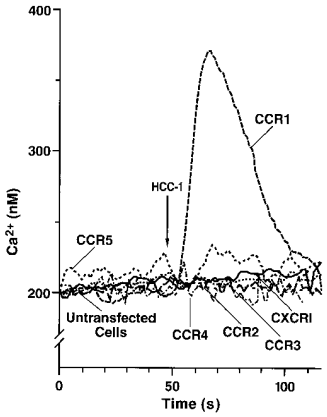

Figure 2. HCC-1 induces calcium mobilization in cells expressing CCR1. Each of the indicated chemokine receptors, except CCR3 and CCR4, was stably expressed in HEK-293 cells. CCR3 and CCR4 were expressed in 300-19 cells. All cells were loaded with Indo-1 AM and then challenged with HCC-1 (100 nM). Intracellular calcium levels were measured as described in Materials and Methods. Data shown are typical of at least three independent experiments.

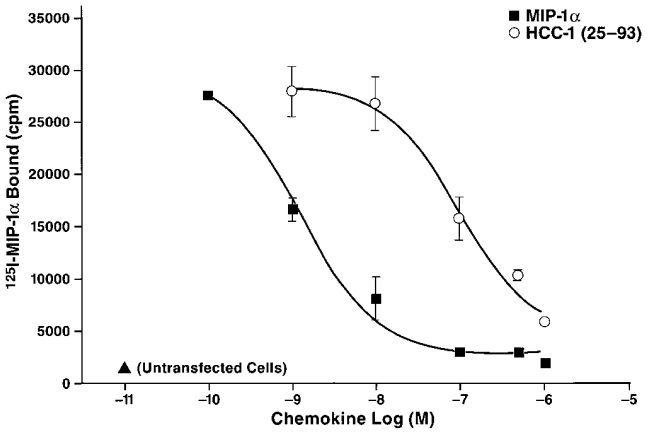

Figure 4. Competition binding of HCC-1 to CCR1. Radiolabeled MIP-1α (1.5 nM) was added to HEK-293 cells stably expressing CCR1 in the presence of the indicated concentrations of unlabeled MIP-1α or HCC-1. Binding to untransfected cells was negligible (▲). The IC_{50} for HCC-1, determined as described in Materials and Methods, was 1.3 nM for MIP-1α and 93 nM for HCC-1.

pressed in stably transfected cells. As seen in Fig. 2, HCC-1 (20–93) induced a robust and prompt mobilization of intracellular calcium in cells expressing CCR1, but not in cells expressing CCR2, CCR3, CCR4, CCR5, or CXCR1. In control experiments, the CCR2-transfected cells gave a robust response to MCP-1, the CCR3 cells to eotaxin, the CCR4 cells to thymus and activation–regulated chemokine (TARC), the CCR5 cells to MIP-1α, and the CXCR1 cells to IL-8 (data not shown). In addition, although HEK-293 cells transiently transfected with CCR6, CCR7, or CCR8 failed to respond to HCC-1, they signaled well to liver and activation–regulated chemokine (LARC), secondary lymphoid tissue chemokine (SLC), and I-309, respectively (data not shown). These data provided the first evidence that CCR1 is a functional receptor for HCC-1.

Ligation of CCR1 by MIP-1α or RANTES causes activation of Gαi, inhibition of adenylyl cyclase, and a fall in cAMP levels (6). Similarly, addition of HCC-1 to CCR1-transfected cells caused a dose-dependent decrease in cAMP (Fig. 3). Comparison of the three NH_2-terminally truncated variants of HCC-1 revealed that the rank order of potency was inversely correlated with the length of the protein. Thus, the shortest version of HCC-1 (25–93) was the most potent (IC_{50} = 8 nM), and the longest version of HCC-1 (20–93) was the least potent (IC_{50} = 49 nM). However, even the most potent form of HCC-1 was at least 10-fold less active than either MIP-1α or RANTES in inducing activation of CCR1.

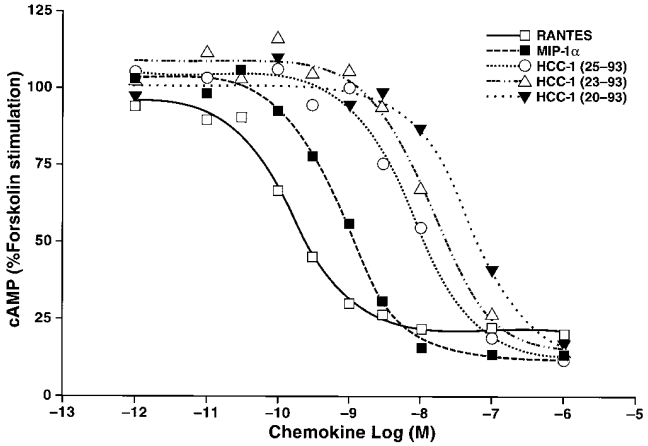

Figure 3. HCC-1 induces inhibition of adenylyl cyclase in cells expressing CCR1. HEK-293 cells stably expressing CCR1 were labeled with [^{3}H]adenine and incubated with 10 μM forskolin in the presence of increasing concentrations of RANTES, MIP-1α, or each of the three variants of HCC-1. [^{3}H]cAMP levels were determined as described in Materials and Methods. The IC_{50} values were as follows: RANTES, 0.15 nM; MIP-1α, 0.8 nM; HCC-1 (25–93), 8 nM; HCC-1 (23–93), 14 nM; HCC-1 (20–93), 49 nM. All data points were determined in duplicate. Shown is one of three similar experiments.

Competition binding studies confirmed that HCC-1 bound to CCR1 (Fig. 4), but with lower affinity than MIP-1α (IC_{50} for HCC-1 = 93 nM, as compared with 1.3 nM for MIP-1α). These data are consistent with the relative potencies determined in the cyclase assay above.

Further evidence that CCR1 is a functional receptor for HCC-1 was obtained using chemotaxis assays. As seen in Fig. 5 *A*, HCC-1 induced robust migration of a lymphocyte cell line (300-19) stably transfected with CCR1. The maximal chemotactic response to HCC-1 was achieved at 100 nM, a concentration ~1,000-fold higher than that of MIP-1α. However, when present at their optimal concentrations HCC-1 and MIP-1α attracted similar numbers of cells. Consistent with the results of the cyclase assays, the shorter variant of HCC-1 was a more efficient chemoattractant than the longer version (Fig. 5 *B*).

Evidence that CCR1 is the endogenous HCC-1 receptor on human mononuclear cells was sought using cell lines and freshly isolated human monocytes. As seen in Fig. 6, HCC-1 was a chemoattractant for both purified monocytes (Fig. 6 *A*) and THP-1 cells (Fig. 6 *B*). Optimal migration required ~100–500 times more HCC-1 than MIP-1α, which is consistent with the results obtained using CCR1-transfected 300-19 cells (Fig. 5).

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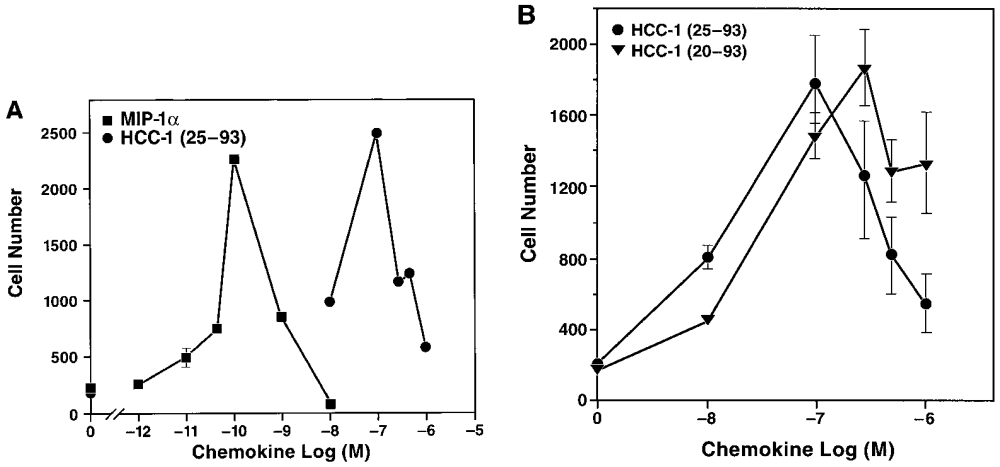

Figure 5. HCC-1 induces CCR1-dependent chemotaxis. (*A*) 300-19 cells stably expressing CCR1 migrated towards the indicated concentrations of HCC-1 and MIP-1α. (*B*) Comparison of the long (20–93) and short (25–93) variants of HCC-1 in inducing chemotaxis. Error bars represent standard deviation. Shown is one of at least three similar experiments.

Further evidence that CCR1 is the endogenous monocyte receptor for HCC-1 was obtained in cross-desensitization experiments. MIP-1α effectively desensitized monocytes to HCC-1, but not to MCP-1 (Fig. 7 *A*), and very similar results were seen using RANTES (Fig. 7 *B*). Conversely, HCC-1 desensitized monocytes to MIP-1α (Fig. 7 *C*). In contrast, incubation of monocytes with MIP-1β, which activates CCR5 but not CCR1, failed to induce a measurable intracellular calcium flux, but the subsequent addition of HCC-1 did (Fig. 7 *D*). Direct evidence of cross-desensitization was obtained in assays using HEK-293 cells expressing transfected CCR1. As shown in Fig. 7 *E*, MIP-1α desensitized the CCR1-expressing cells to HCC-1, and, conversely, HCC-1 desensitized the cells to MIP-1α (Fig. 7 *F*).

Discussion

The major finding in this study is that CCR1, a well-characterized receptor for the C-C chemokines MIP-1α (15), RANTES (15), MCP-3 (16), and MCP-2 (17), is also a functional receptor for HCC-1. Several lines of evidence support this conclusion. First, HCC-1 induced a rapid mobilization of intracellular calcium, as well as inhibition of adenylyl cyclase, in CCR1-transfected HEK-293 cells. Second, this response was specific for CCR1 in that HCC-1 failed to induce signaling in cells transfected with CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, or CXCR1. The failure to induce activation of CCR5 is particularly significant because MIP-1α, the chemokine to which HCC-1 is most homologous, is a potent ligand for CCR5. Third, the dose–response studies revealed that HCC-1 was ~100-fold less potent than MIP-1α in activating CCR1, in close agreement with the relative potency reported for inducing proliferation of $CD34^+$ myeloid progenitor cells (3). Fourth, HCC-1 competed with MIP-1α for binding to CCR1. Consistent with the signaling data, the affinity of HCC-1 for CCR1 was ~100-fold less than MIP-1α. Fifth, HCC-1 induced chemotaxis of CCR1-transfected 300-19 cells with a classical biphasic dose-dependency. The optimal HCC-1 concentration for chemotaxis was ~100 nM, as compared with 0.1–1.0 nM for MIP-1α. Finally, evidence that CCR1 is the endogenous monocyte receptor for HCC-1 was obtained in cross-desensitization studies. Pretreatment with either MIP-1α or RANTES, but not MIP-1β, completely blocked the response of monocytes to HCC-1, and the converse was also true when HCC-1 was added at high concentrations. Since HCC-1 does not activate CCR5, these results strongly suggest competition for binding to CCR1. Taken together, these data indicate that HCC-1 is a functional ligand for CCR1.

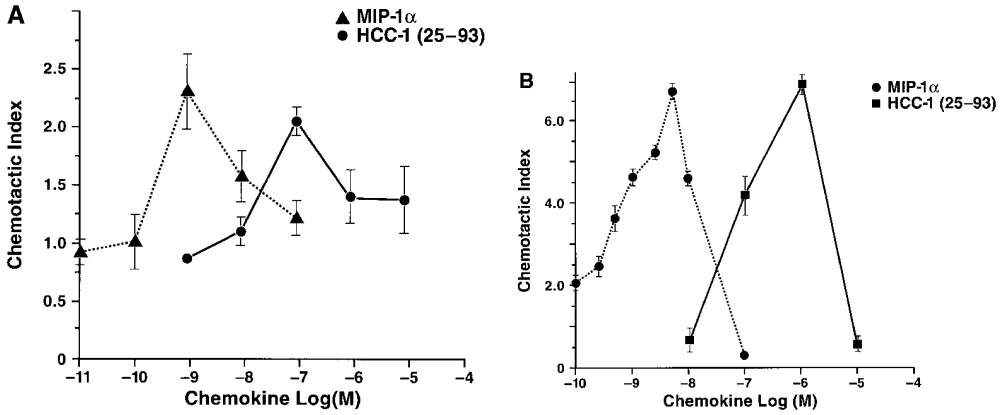

Figure 6. HCC-1 induces monocyte chemotaxis. (*A*) Freshly isolated primary human monocytes migrated towards the indicated concentrations of MIP-1α or HCC-1. (*B*) THP-1 cells. Chemotaxis for each cell type was performed as described in Materials and Methods. Error bars denote standard deviations. Shown is one of at least three similar experiments.

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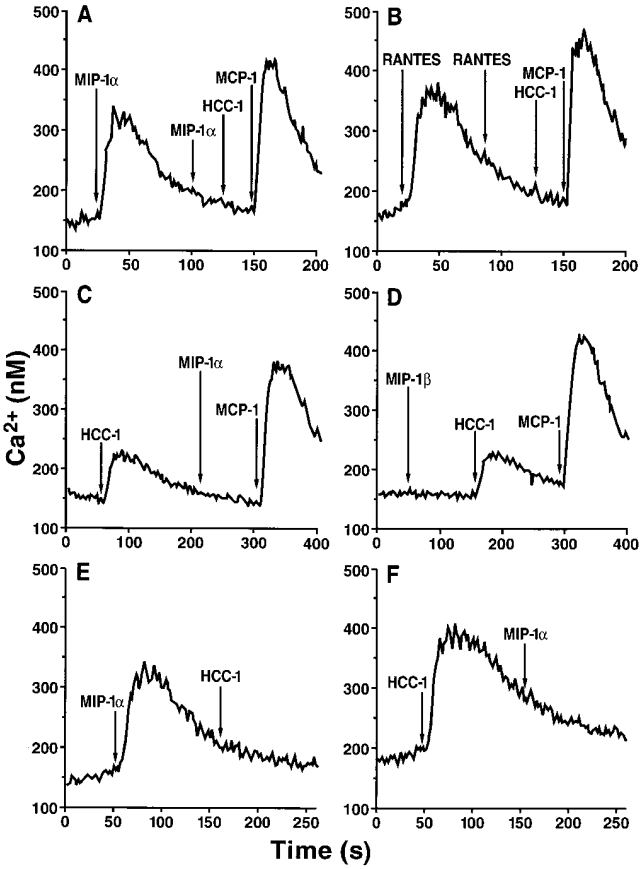

Figure 7. Cross-desensitization of cells to HCC-1 and MIP-1α/RANTES. (*A–D*) Human monocytes were loaded with Indo-1AM, and intracellular calcium levels were determined as described in Fig. 2. Chemokines (100 nM) were added at the indicated time points. HCC-1 was at 1 μM. Shown is one of three similar experiments. (*E* and *F*) HEK-293 cells stably expressing CCR1 were exposed to the indicated chemokines (100 nM), and calcium levels were measured as described above.

The cDNA for HCC-1 encodes a 93–amino acid protein, with a putative 19-residue leader sequence (3). Analysis of the NH_2-terminal domain by the method of Neilsen et al. (14) revealed at least three other possible sites for cleavage of the signal peptide, yielding proteins of 74, 71, and 69 amino acids. In patients with renal failure, the predominate form of HCC-1 appears to be the 74–amino acid variant (3). Interestingly, the shorter forms of HCC-1 were significantly more potent on a molar basis than the 74–amino acid form. Earlier studies reported that HCC-1 induced an elevation of intracellular calcium, but failed to induce chemotaxis of human monocytes (3). This was a surprising result, since chemotaxis is the prototypic function of this family of cytokines. The failure of HCC-1 to induce chemotaxis would thus have provided intriguing evidence for distinct and agonist-specific signal transduction pathways downstream of a chemokine receptor (i.e., induction of chemotaxis in response to activation of CCR1 by MIP-1α or RANTES, but not by HCC-1). Our results demonstrate that HCC-1 does indeed induce chemotaxis via activation of CCR1, albeit at a higher concentration than MIP-1α. HCC-1's chemotactic effect was observed with CCR1-transfected cells, THP-1 cells, and freshly isolated primary human monocytes.

The finding that HCC-1 is a ligand for CCR1 and has chemotactic activity raises the question of the physiological significance of the chronically high levels of HCC-1 in the plasma. Since the dissociation constant for binding to CCR1 and the plasma concentration of HCC-1 are approximately equal (~100 nM), about half of the CCR1 molecules at the cell surface would be expected to be occupied by HCC-1. The leukocyte might therefore be unable to respond to small changes in HCC-1 levels but could retain the ability to respond to other CCR1 agonists, such as MIP-1α and RANTES, since these chemokines bind to CCR1 with a much higher affinity than HCC-1. This scenario is very much in keeping with the model of combinatorial control of leukocyte chemotaxis recently proposed by Foxman et al. (18). In this case, however, multiple agonists of different affinity for the same receptor (i.e., CCR1), rather than sequential activation of different chemokine receptors, may be responsible for the navigation of monocytes through complex chemoattractant gradients. MIP-1γ, a murine chemokine with homology to HCC-1, is secreted constitutively in vivo, is present in the plasma of healthy mice at a concentration of ~90 nM, and competes with MIP-1α for the same receptor on murine neutrophils (19). MIP-1γ may thus be the murine equivalent of HCC-1.

Finally, these studies provide evidence for a fifth functional ligand for CCR1. CCR1 is expressed in neutrophils, monocytes, eosinophils, and lymphocytes, and plays important roles in pancreatitis (20) and hematopoiesis (21). CCR1 ligands that contribute to the pathophysiology of these states are not well defined. HCC-1 can now be added to the list of chemokines that may have functions in specific disease processes associated with CCR1 activation.

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Address correspondence to Israel F. Charo, Gladstone Institute of Cardiovascular Disease, PO Box 419100, San Francisco, CA 94141-9100. Phone: 415-826-7500; Fax: 415-285-5632; E-mail: izzy_charo@quickmail. ucsf.edu

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