Brief Definitive Report

HCC-1, a Novel Chemokine from Human Plasma

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Summary

A novel CC chemokine, HCC-1, was isolated from the hemofiltrate of patients with chronic renal failure. HCC-1 has a relative molecular mass of 8,673 and consists of 74 amino acids including four cysteines linked to disulfide bonds. HCC-1 cDNA was cloned from human bone marrow and shown to code for the mature protein plus a putative 19-residue leader sequence. Mature HCC-1 has sequence identity of 46% with macrophage inflammatory protein (MIP)-1α and MIP-1β, and 29–37% with the other human CC chemokines. Unlike MIP-1α and the other CC chemokines, HCC-1 is expressed constitutively in several normal tissues (spleen, liver, skeletal and heart muscle, gut, and bone marrow), and is present at high concentrations (1–80 nM) in plasma. HCC-1 has weak activities on human monocytes and acts via receptors that also recognize MIP-1α. It induced intracellular Ca^{2+} changes and enzyme release, but no chemotaxis, at concentrations of 100–1,000 nM, and was inactive on T lymphocytes, neutrophils, and eosinophil leukocytes. In addition, HCC-1 enhanced the proliferation of $CD34^+$ myeloid progenitor cells. It was as effective as MIP-1α, but about 100-fold less potent.

Hemofiltration is a routine treatment for patients with chronic renal failure to remove substances that are normally cleared by the kidney. A filter membrane with a molecular mass cut-off of 20 kD is used, which virtually excludes plasma proteins (1, 2). Being available in large quantities, the hemofiltrate is an excellent source of biologically active human peptides that circulate in the blood. During the last 5 yr, a peptide bank was established from >200,000 liters of hemofiltrate, and several peptide hormones were purified (1). During the course of a systematic search for novel bioactive factors (3), we have identified a new member of the recently recognized family of chemotactic cytokines (chemokines). HCC-1 is structurally related to macrophage inflammatory protein (MIP)-1α. Unlike MIP-1α and the other CC chemokines, HCC-1 is highly expressed in normal tissues and is present at high concentrations in human plasma.

Materials and Methods

Purification. Peptides were extracted from batches of 2,000 liters of hemofiltrate by precipitation with 660 g/liter ammonium sulfate as described previously (2). The precipitate (~250 g) was dissolved in water (125 mg/ml). The peptides were precipitated again by addition of 4.5 vol of 2-propanol, redissolved in 10 mM phosphate buffer, pH 3.0, and fractionated by cation exchange chromatography (Fractogel TSK SP-650 M, 6 × 20 cm; Merck, Darmstadt, Germany) with a 0–1.0-M NaCl gradient in the same buffer. Fractions eluting at high salt concentrations were collected and further purified by preparative reverse-phase (RP) HPLC (Parcosil RP C4, 300 Å, 20–45 μm, 3 × 12.5 cm; Biotek, Oestringen, Germany) using a gradient generated from 0.01 M HCl and 50% 2-propanol/30% methanol in 0.01 M HCl. Analytical RP HPLC (Parcosil RP C4, 300 Å, 5 μm, 1 × 12.5 cm, Biotek, Oestringen, Germany) was then performed using an acetonitrile/TFA gradient (0–80% acetonitrile in 0.1% TFA).

Structure Analysis. The amino acid sequence of the native peptide and of peptide fragments obtained after cleavage with endoproteinase Lys C (EC 3.4.21.50) and chymotrypsin (EC 3.4.21.1) was determined by Edman degradation analysis. The position of the cysteines was determined by amino-terminal sequencing of the fragments obtained after carboxamidomethylation and Lys C digestion.

Mass spectrometry. Molecular masses were determined using a Sciex API III triple stage quadrupole mass spectrometer (Perkin-Elmer Cetus Corp., Norwalk, CT) equipped with an electrospray

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interface. Samples were dissolved in 50% acetonitrile/0.2% acetic acid (vol/vol) and introduced via a syringe pump (Harvard Apparatus Ltd., Kent, UK) at a flow rate of 5 μl/min. Measurements were performed in positive ion mode, and molecular masses were calculated from multiple protonated ions using the HyperMass program (Perkin-Elmer).

Cloning of the HCC-1 cDNA. From 5 μg of human adrenal gland RNA, cDNA first strand was synthesized using MMLV RTase and an oligo(dT) primer (UNIP-2) complementary to the poly(A) tail of eukaryotic mRNA. Using this primer and two degenerate sense primers (CC 1-1, CC 1-2) derived from a partial HCC-1 amino acid sequence (see Fig. 2 *B*), a homogenous adrenal gland cDNA fragment was amplified by a two-step PCR (4) consisting of a 40-cycle amplification with CC 1-1/UNIP-2 followed by a 20-cycle amplification of 1/100th of the first mixture with CC 1-2/UNIP-2. The fragment was then cloned in pBluescript (Stratagene, La Jolla, CA) and sequenced (5, 6). A 234-bp DNA fragment was amplified from the recombinant plasmid and used as a probe for screening a human bone marrow cDNA library in λ DR 2 (Clontech, Palo Alto, CA). From positive clones, the recombinant pDR 2 plasmids were excised and sequenced as described above.

Bone Marrow Cells. Human bone marrow mononuclear cells were enriched by Ficoll-Hypaque centrifugation, and $CD34^+$ cells were sorted using a FACStar® instrument (Becton Dickinson & Co., Mountainview, CA), defining the cells of interest within the mononuclear population in the forward versus side scatter gate exhibiting FITC (<CD34>), but not PE fluorescence (<CD14>). Cultures were performed in 96-well plates (50 cells per well) with IMEM medium containing 20% human plasma, penicillin/streptomycin, and several cytokines (rhSCF 20 ng/ml, rhIL-6 10 ng/ml, rhGM-CSF 100 ng/ml, and rhEPO 5 ng/ml). Cell numbers were assessed after 5 and 8 d using an image analyzer (Quantimet Q 570; Leica, Bensheim, Germany) and after 12 d by counting in a Neubauer chamber. Half of the medium was replaced after 6 d.

Blood Cells. Neutrophils, eosinophils and monocytes were prepared according to established methods (7–9). Cloned T lymphocytes obtained from Dr. A. Lanzavecchia (Basel Institute of Immunology, Basel, Switzerland) were assayed according to Loetscher et al. (10). Monocyte chemotaxis and enzyme release were assessed exactly as described by Uguccioni et al. (9), and changes of cytosolic free calcium were determined according to von Tscharner et al. (11).

Antisera. A linear peptide corresponding to the amino-terminal domain of HCC-1 (Thr^1-Cys^{16}) and a multiple antigenic peptide consisting of a branched heptalysine core with eight copies of the carboxyl-terminal domain of HCC-1 (Asp^{62}-Asn^{74}) covalently bound were synthesized (12, 13), and antisera were raised in New Zealand white rabbits.

RNA Expression. Northern blot analysis was performed with total RNA from different human tissues using a 234-bp fragment of HCC-1 cDNA (CC 1-3 to CC 1-4 in Fig. 2 *B*) as hybridization probe. For each tissue, 50 μg RNA was glyoxylated, separated in an agarose gel, transferred to nylon membrane, and hybridized (14) with the ^{32}P-labeled 234-bp fragment. For reverse transcription PCR analysis (4), a pair of highly specific primers (CC 1-3 and CC 1-4) was constructed from HCC-1 cDNA, and the reaction was performed with 1/30th of each cDNA synthesized from 5 μg total RNA. 10-μl aliquots of the PCR reaction mixtures were taken after 25, 30, 35, and 40 amplification cycles, and were analyzed in a 1.5% ethidium bromide agarose gel. The fragments obtained were of the expected size of ~230 bp.

Results and Discussion

A protein was purified from the crude peptide extract of 2,000 liters of hemofiltrate by cation exchange chromatography followed by preparative and analytical RP-HPLC. As shown in Fig. 1, the final product was homogenous, as judged by capillary zone electrophoresis, and had a relative molecular mass of 8,673. The complete sequence of 74 amino acids including four cysteines was determined by Edman degradation analysis of the native protein and of peptide fragments obtained by proteolytic cleavage (Fig. 2 *A*). Analysis by mass spectrometry indicated that the cysteines form two disulfide bonds. A search for homology, which was performed in the Swiss-Prot, EMBL, and gene bank data bases, showed that the sequence is new. On the basis of sequence homology, molecular size, and the position of the four cysteines, it was concluded that the novel protein represents a CC chemokine (15). Since it was found in the hemofiltrate, it was termed HCC-1.

HCC-1 shares 46% sequence identity with MIP-1α and MIP-1β. The overall identity is 29–37% with MCP-1, MCP-2, MCP-3, RANTES, and I-309, but only 16% with IL-8, a CXC chemokine. The amino acid sequences of HCC-1 and MIP-1α are aligned in Fig. 2 *A*. Considerable identity (61%) is observed in the region between the

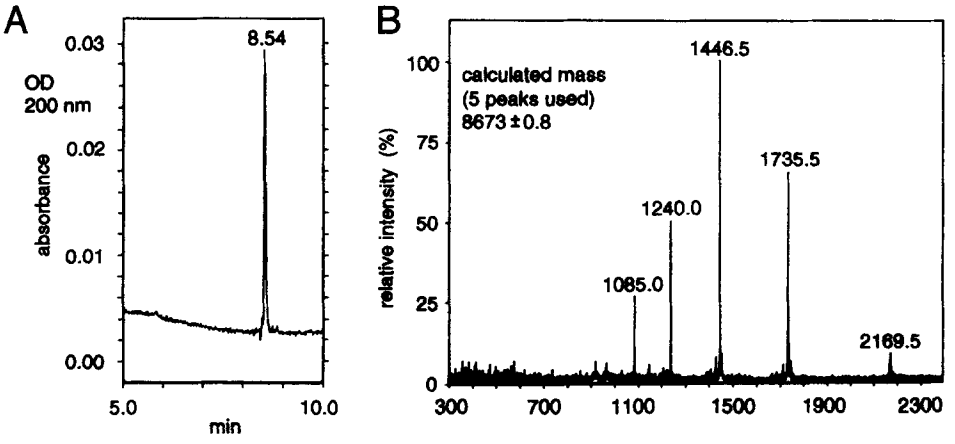

Figure 1. Physicochemical characterization of purified HCC-1. (*A*) Capillary zone electrophoresis. (*B*) Electrospray mass spectrometry performed in positive ion mode. The mass of HCC-1 was calculated from the five multiple charged ions $[M + 4\ H]^{4+}$ to $[M + 8\ H]^{8+}$.

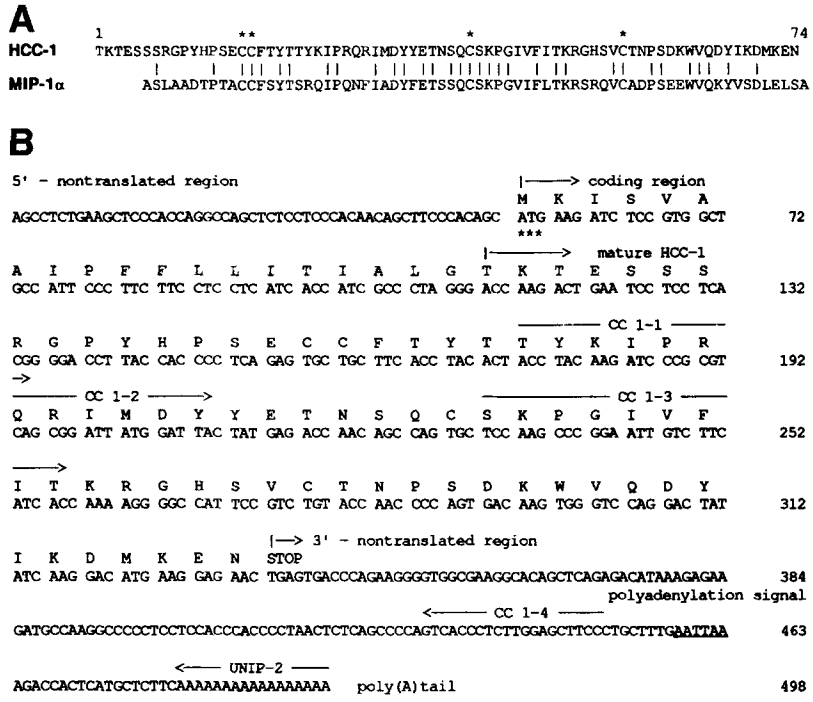

Figure 2. Amino acid and nucleotide sequences. (*A*) Purified HCC-1; amino acid sequence and alignment with MIP-1α. Vertical lines indicate identical amino acids. The four cysteine residues are marked with asterisks. (*B*) HCC-1 cDNA; nucleotide and deduced amino acid sequence. The 5′ nontranslated region consists of 54 bp, the coding region of 279 bp, and the 3′ nontranslated region of 148 bp. The positions and orientations of the PCR primers used are indicated by arrows. The ATG start codon is marked by asterisks and the polyadenylation signal is underlined. The primers used are indicated. The sequence of the HCC-1 cDNA has been communicated to the EMBL database (accession No. Z49270).

first and the last cysteine. The amino-terminal region preceding the first cysteine, by contrast, differs almost completely from MIP-1α, and is five residues longer than that of MIP-1α and all other known CC chemokines (15). This may be important with regard to the biological activity of HCC-1, since it has been shown that amino-terminal truncation drastically affects the activity of MCP-1 (16). The length of the amino-terminal region is also critical for the activity of CXC chemokines (17).

The HCC-1 cDNA was cloned from a human bone marrow cDNA library in λ DR 2 using a probe that was amplified from adrenal gland cDNA. Six independent clones were isolated and the insertions of three of them were completely sequenced. The 5′ termini of the clones differed slightly in length, but the nucleotide sequences of the overlapping regions were identical. The first ATG appearing in the 5′ to 3′ direction starts a 279-bp open reading frame that totally matches the amino acid sequence of HCC-1 plus an amino-terminal extension of 19 residues with the characteristics of a leader sequence. The largest

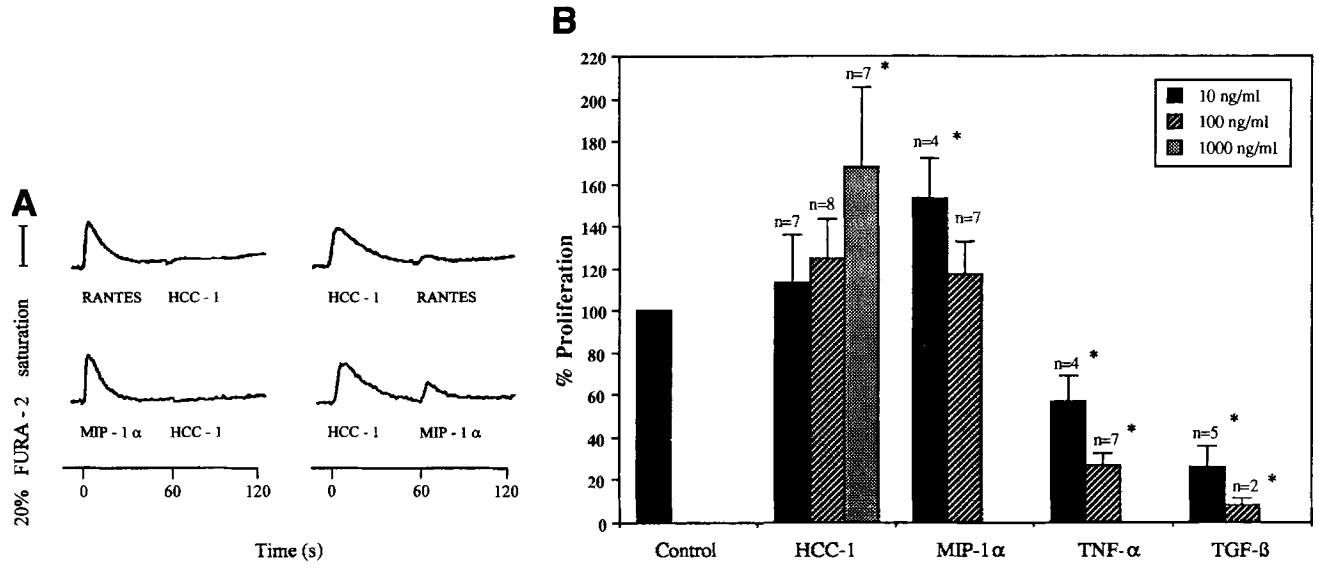

Figure 3. Biological activities. (*A*) Cytosolic free Ca^{2+} changes in human monocytes. Cross-desensitization of the responses induced by 1,000 nM HCC-1 and 30 nM RANTES or MIP-1α. Monocytes isolated from donor blood were loaded with Fura-2 acetoxymethyl ester, and intracellular calcium changes were recorded. (*B*) Effect of HCC-1 on $CD34^+$ human bone marrow cells. Comparison with MIP-1α, TNF-1α, and TGF-β. Cells numbers counted on day 12 are expressed in percent of control (mean ± SEM). Four to eight separate experiments were performed with cells from different bone marrow donors. Significant difference from control ($P \leq 0.05$, two-tailed Student's *t* test) is indicated with an asterisk.

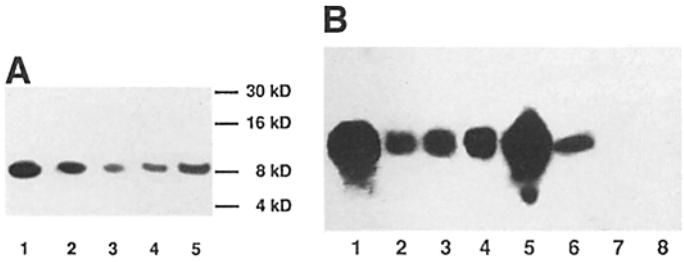

Figure 4. HCC-1 in plasma and tissues. (*A*) Detection of HCC-1 by Western blotting. (Lanes *1–3*) Purified HCC-1 from hemofiltrate (50, 10, and 2 ng, respectively). (Lanes *4* and *5*) 250 μl plasma from a healthy individual and a patient with chronic renal failure. (*B*) Detection of HCC-1 mRNA by Northern blotting. RNA was extracted from spleen (lane *1*), colon (lane *2*), small intestine (lane *3*), liver (lane *4*), heart muscle (lane *5*), skeletal muscle (lane *6*), kidney (lane *7*), and brain (lane *8*).

cDNA insert consisted of 481 bp, including a putative 5′ nontranslated region of 54 bp and a 3′ nontranslated region of 148 bp (Fig. 2 *B*). A typical polyadenylation signal (AATTAAA) occurs at the expected distance of ~20 bp from the poly(A)tail.

In view of the structural relationship to CC chemokines, potential biological activities of HCC-1 were studied on circulating leukocytes and bone marrow cells. HCC-1 showed activity towards monocytes, as indicated by the rise of intracellular Ca^{2+} at 100 nM or higher, and a slight but significant release of *N*-acetyl-β-D-glucosaminidase at 1,000 nM. HCC-1, however, did not induce monocyte chemotaxis at 0.1–1,000 nM (data not shown). Sequential stimulation of monocytes with HCC-1 and related CC chemokines was used to assess cross-desensitization, as observed with agonists that share receptors. As shown in Fig. 3 *A*, MIP-1α and RANTES at 30 nM prevented responsiveness to 1,000 nM HCC-1, while 1,000 nM HCC-1 markedly decreased the response to 30 nM MIP-1α or RANTES. HCC-1 was also tested for stimulation of T lymphocytes and eosinophil and neutrophil leukocytes, but no cytosolic free Ca^{2+} changes were detected.

As shown in Fig. 3 *B*, HCC-1, like MIP-1α, enhanced the proliferation of $CD34^+$ cells. The maximum effects obtained with HCC-1 (168%) and MIP-1α (153%) were comparable, while proliferation was inhibited by TNF-α and TGF-β in agreement with previous reports (18). Despite similar efficacy, HCC-1 was markedly less potent than MIP-1α. Additional experiments using very early progenitor/stem cells ($CD34^+/CD38^-$ bone marrow cells) cultured in the presence of stem cell factor confirmed the proliferative effect of HCC-1 and MIP-1α at 1,000 and 100 ng/ml, respectively. It was reported earlier that MIP-1α inhibits proliferation (19, 20), but recent studies, using progenitor/stem cell assays, which are more suited to assess cytokine pleiotropy and concentration dependence, indicate that MIP-1α stimulates very early as well as late committed $CD34^+$ cells (18, 21–23).

Antisera against amino- and carboxyl-terminal regions of mature HCC-1 (see Materials and Methods) were used for ELISA and Western blotting. Determination in plasma and hemofiltrate was performed after solid-phase cation exchange extraction of the samples. The plasma concentrations ranged between 2.5 and 80 nM (16.5 ± 9.2, mean ± SEM) in patients with chronic renal failure ($n = 8$), and 1.6 and 10 nM (5.6 ± 1.3, mean ± SEM) in healthy subjects ($n = 10$). These concentrations are surprisingly high compared to those of other chemokines, which are normally undetectable in healthy individuals and rarely reach high levels even in disease (24, 25). In plasma samples from all patients and healthy controls tested so far, HCC-1 was readily detectable by Western blotting. A band of ~8 kD was found in every instance, and no degradation products were apparent (Fig. 4 *A*).

The expression was studied in several tissues by Northern analysis. Positive bands of the expected size were obtained with RNA extracted from the spleen, liver, skeletal and heart muscle, and gut, but not from the kidney or brain (Fig. 4 *B*). Analysis by reverse transcription PCR (data not shown) was positive for the spleen, liver, the hepatoma cell line HUH-7, skeletal and heart muscle, gut, and bone marrow. A faint band was obtained for the kidney, suggesting that some HCC-1 expression may occur in this tissue as well. Since the tissues were considered normal and were not subjected to stimulation, these results indicate that HCC-1 is expressed constitutively in a variety of different tissue cells. No expression was observed in the brain, blood leukocytes, the monocytic cell lines, THP-1, U937, and HL-60, or in Jurkat cells.

Chemokines have some highly characteristic properties: they are expressed in different tissues in response to inflammatory stimuli (e.g., cytokines and bacterial toxins), are believed to act locally rather than systemically, and have one major effect, the activation and attraction of leukocytes (15). HCC-1 shares marked sequence identity and some biological activities on monocytes and myeloid precursor cells with MIP-1α. Certain properties of HCC-1, however, are not common for chemokines, namely, nanomolar concentration in plasma, even in healthy individuals, and marked expression in normal tissues. These features indicate that HCC-1 is a CC chemokine with an unconventional bioactivity profile. Its presence in plasma suggests that it may exert systemic effects on myeloid and possibly yet unknown target cells.

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