

Monocyte Chemotactic Protein 4 (MCP-4), a Novel Structural and Functional Analogue of MCP-3 and Eotaxin

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Summary

A novel human CC chemokine complementary DNA was identified in a library constructed from human fetal RNA, cloned into a baculovirus vector, and expressed in Sf9 insect cells. The mature recombinant protein that was released had the NH₂-terminal sequence *pyro*-QPDALNVPSTC...and consisted of 75 amino acids. Minor amounts of two variants of 77 and 82 residues (NH₂ termini: LAQPDA...and FNPQGGLAQPDA...) were released as well. The novel chemokine was designated monocyte chemotactic protein 4 (MCP-4) and the variants were designated (LA)MCP-4 and (FNPQGGLA)MCP-4. MCP-4 shares the pyroglutamic acid-proline NH₂-terminal motif and 56–61% sequence identity with the three known monocyte chemotactic proteins and is 60% identical to eotaxin. It has marked functional similarities to MCP-3 and eotaxin. Like MCP-3, MCP-4 is a chemoattractant of high efficacy for monocytes and T lymphocytes. On these cells, it binds to receptors that recognize MCP-1, MCP-3, and RANTES. On eosinophils, MCP-4 has similar efficacy and potency as MCP-3, RANTES, and eotaxin. It shares receptors with eotaxin and shows full cross-desensitization with this eosinophil-selective chemokine. Of the two variants, only (LA)MCP-4 could be purified in sufficient quantities for testing and was found to be at least 30-fold less potent than MCP-4 itself. This suggests that the 75-residue form with the characteristic NH₂ terminus of an MCP is the biologically relevant species.

The number of chemokines of the CC subfamily has grown considerably during the past few years, and important new information has been gathered about their activities on different types of leukocytes (1). Of particular interest were the findings that the monocyte chemotactic proteins (MCPs) are not only effective on monocytes (2–4), but also attract CD4⁺ and CD8⁺ T lymphocytes (5, 6) and basophil leukocytes (7–10). MCP-3 (10) and RANTES (11) were also shown to be powerful attractants of eosinophil leukocytes and, most recently, eotaxin, a CC chemokine with marked sequence similarity to MCP-3, was found to share this activity and to be unusually selective for eosinophils (12–14).

In a program aiming at the discovery of human genes by large-scale sequencing of partial cDNA clones, a novel chemokine cDNA was identified in a library constructed from human fetal mRNA. The full-length cDNA was cloned into a baculovirus expression vector, and the chemokine obtained was designated CKβ10. A screening for the

induction of changes of the cytosolic free Ca²⁺ concentration ([Ca²⁺]_i) in human blood monocytes showed that CKβ10 was as effective as MCP-1 and MCP-3. We compared its biological activity on human monocytes, neutrophils, eosinophils, and T lymphocytes with that of MCP-1, MCP-2, MCP-3, RANTES, MIP-1α, and eotaxin, and found that CKβ10 is functionally very similar to MCP-3. Since the novel CC chemokine also shares marked sequence identity to the monocyte chemotactic proteins, we adopted the term MCP-4. This designation is actually not new. A 153-bp PCR fragment was previously cloned from human bone marrow mRNA and shown to encode a fragment of a novel CC chemokine (15). On the basis of the nucleotide sequence similarity with MCP-1, MCP-2, and MCP-3, the putative chemokine was termed MCP-4, even though the protein was not expressed and no information about biological activities could be obtained (15). The sequence of the cloned fragment (Berger, M., personal communication) corresponds exactly to part of the CKβ10 gene.

Materials and Methods

Cloning and Expression. The coding sequence of MCP-4 was amplified by PCR from a human fetal cDNA library (Human Genome Sciences Inc., Rockville, MD) using the forward primer 5'-CGCGGGATCCTTAACCTTCAACATGAAA and the reverse primer 5'-CGCGGGTACCTTAACACATAGTACATTTT. The gene was dissected with BamHI and Asp718, inserted downstream of the polyhedrin promoter into the vector pRG1, a modification of pNR704 (16), and sequenced. The vector was transfected into Sf9 insect cells (ATCC CRL 1711) together with linearized baculovirus DNA (BaculoGOLD™; Pharmingen, San Diego, CA) using the lipofectin method. rMCP-4 was purified from the supernatant of serum-free Sf9 cell cultures in the presence of protease inhibitors (20 μg/ml Pefabloc SC; Boehringer, Mannheim, Germany, 1 μg/ml leupeptin, 1 μg/ml E64, and 1 mM EDTA) by three chromatography steps: cation exchange, heparin affinity, and size exclusion (poros 50 HS, poros 20 HE1, Perseptive Biosystem, and Sephacryl S200 HR; Pharmacia Fine Chemicals, Piscataway, NJ). The purified material was analyzed by laser desorption mass spectrometry (matrix-associated laser desorption ionization-time of flight) and sequenced by Edman degradation. In addition, trypsin fragments were prepared (17), separated on reverse-phase HPLC, and peak fractions were analyzed by laser desorption mass spectrometry. The material was further purified by chromatography on a MiniS column (Pharmacia) equilibrated in 30% CH₃CN, 20 mM potassium phosphate, pH 6.0, and eluted in a KCl gradient. Peak fractions were subjected to reverse-phase chromatography on a μRPC C2/C18 column (Pharmacia) and analyzed by laser desorption mass spectrometry.

Chemokine Standards. The chemokines used for comparison, MCP-1, MCP-2, MCP-3, RANTES, MIP-1α, and eotaxin, were chemically synthesized by Dr. I. Clark-Lewis (Hanson Centre for Cancer Research, Institute of Medical and Veterinary Science, Adelaide, Australia) according to established protocols (18).

Cells. Monocytes (4) and neutrophils (19) were isolated at >90% purity from donor blood buffy coats supplied by the Central Laboratory of the Swiss Red Cross (Bern, Switzerland). The same source was used for the isolation of blood lymphocytes (5). Human CD4⁺ and CD8⁺ T cell clones were maintained in culture and used according to Loetscher et al. (5). Fresh blood of healthy individuals was used to purify eosinophils by dextran sedimentation followed by Percoll density-gradient centrifugation and negative selection with anti-CD16 mAb-coated magnetic beads (11).

In Vitro Chemotaxis. Chemotaxis was assessed in 48-well chambers (Neuro Probe, Cabin John, MD) using polyvinylpyrrolidone-free polycarbonate membranes (Nucleopore Neuro Probe, Cabin John, MD) with 5-μm pores for monocytes and eosinophils, and 3-μm pores for lymphocytes. RPMI 1640 supplemented with 20 mM Hepes, pH 7.4, and 1% pasteurized plasma protein solution (the Central Laboratory of the Swiss Red Cross Laboratory) was used to dissolve the chemokines, which were placed in the lower well, and to suspend the cells (50,000 monocytes or eosinophils and 100,000 lymphocytes per upper well). After 60 min at 37°C, the membrane was removed, washed on the upper side with PBS, fixed, and stained. All assays were done in triplicate, and the migrated cells were counted in five randomly selected fields at 1,000-fold magnification. Spontaneous migration was determined in the absence of chemoattractant.

Enzyme Release. The release of elastase was tested in neutrophils exactly as described previously (19). The release of

N-acetyl-β-D-glucosaminidase was tested in monocytes (4). In brief, samples of 1.2 × 10⁶ monocytes in 0.3 ml prewarmed medium (136 mM NaCl, 4.8 mM KCl, 1.2 mM KH₂PO₄, 1 mM CaCl₂, 20 mM Hepes, pH 7.4, 5 mM D-glucose, and 1 mg/ml fatty acid-free BSA) were pretreated for 2 min with cytochalasin B (2.7 μg/ml) and stimulated with a chemokine. The reaction was stopped after 3 min by cooling on ice and centrifugation (6,000 g, 3 min), and enzyme activity was determined in the supernatant.

Ca²⁺ Changes. Changes in the cytosolic free Ca²⁺ concentration ([Ca²⁺]_i) were measured in monocytes, eosinophils, and lymphocytes loaded with fura-2 by incubation for 30 min at 37°C with 0.2 nmol fura-2 acetoxyethyl ester per 10⁶ cells in a buffer containing 136 mM NaCl, 4.8 mM KCl, 1 mM CaCl₂, 5 mM glucose, and 20 mM Hepes, pH 7.4. After centrifugation, loaded cells were resuspended in the same buffer (10⁶ cells/ml), stimulated with the indicated chemokine at 37°C, and the [Ca²⁺]_i-related fluorescence changes were recorded (20).

Receptor Usage. Receptor desensitization was tested in monocytes and eosinophils by monitoring [Ca²⁺]_i changes upon repeated chemokine stimulation at 90-s intervals exactly as described for monocytes by Ugucioni et al. (4).

Results

MCP-4 Expression and Analysis. rMCP-4 that was released into the culture supernatant of Sf9 cells was readily purified by cation exchange, heparin affinity, and size exclusion chromatography (see Materials and Methods). On SDS-PAGE (21), the purified preparation yielded a single band with a molecular mass of ~8,000 D and no evidence for contaminant proteins. Laser desorption mass spectrometry resolved a predominant peak and two very small peaks with masses of 8,575 ± 30, 8,760 ± 30, and 9,314 ± 30, respectively. Edman degradation of the mixture yielded two NH₂ termini, FNPQGLAQPDA . . . and LAQPDA . . . , corresponding to the two minor components. No sequence was obtained for the major component. The purified material was therefore digested with trypsin and the resulting peptides were separated by reverse-phase chromatography and analyzed. The largest fragments, which were derived from the NH₂ termini (Fig. 1), separated well from the smaller digestion peptides. Mass spectrometry of these fragments revealed a major sharp peak corresponding to *pyro*-QPDALNVPSTCCFTFSSK and two minor peaks representing LAQPDALNVPSTCCFTFSSK and FNPQGLAQPDALNVPSTCCFTFSSK. These results are consistent with the sequencing information, since pyroglutamic acid, which is derived from deamidation and cyclization of the NH₂-terminal glutamin, is resistant to Edman degradation. The term MCP-4 was attributed to the major form (75 residues, mol wt = 8,577) and the minor forms (77 and 82 residues, mol wt = 8,779 and 9,324) were designated (LA)MCP-4 and (FNPQGLA) MCP-4. In Fig. 1, the sequences of the three forms are aligned with those of MCP-3 and eotaxin, which share 60% amino acid identity with MCP-4. The identity of MCP-4 with

MCP-4

QP▽DALNVPSTCCFTFSSKKISLQRLKSYVITT.SRCPQKAVIFRTKLGKEICADPFKEKWVQNYMKHLGRKAHTLKT

FNPQGLA-----CC-----C-----C-----

LA-----CC-----C-----C-----

MCP-3

--VGI-TST-CCYR-IN--PK--E--RR--S-HC-RE---K--D--C--TQ---DF---DK-TQ-P-L

Eotaxin

GPASV-T-CC-NLANR--P---E--RRI-SGKC-----K--A-D-C---K---DS--Y-DQ-SP-P-P

Figure 1. Amino acid sequences of the three NH₂-terminal variants of MCP-4 aligned with MCP-3 and eotaxin. Identical amino acids are represented by hyphens. The cleavage site of trypsin yielding larger NH₂-terminal fragments is indicated (arrowhead).

RANTES, MIP-1 α , and MIP-1 β was only 29, 39, and 41%, respectively.

Enzyme Release in Monocytes. MCP-4 and (LA)MCP-4 were isolated by reverse-phase chromatography for biological testing, but (FNPQGLA)MCP-4 could not be obtained in sufficient amounts. The activity was compared on monocytes, which respond readily to MCP-1, MCP-2, and MCP-3 (4), by measuring the release of the lysosomal enzyme *N*-acetyl- β -D-glucosaminidase, a test that is particularly reliable for a quantitative assessment. As shown in Fig. 2 a, MCP-4 induced abundant release while (LA)MCP-4 was at least 30 times less potent. In view of these results, the 75-amino acid protein with NH₂ terminal pyroglutamic acid was used for further testing. In Fig. 2 b, the effects of MCP-4 are compared with those of other CC chemokines. The response induced by MCP-4 was very similar to that observed with MCP-2, which was always somewhat less potent than MCP-1 and MCP-3. RANTES was markedly less active, and eotaxin was inactive.

Chemotaxis. MCP-4 induced the migration of monocytes, eosinophils, and lymphocytes with a typical bimodal concentration dependence (Fig. 3). The activity on monocytes was comparable to that of MCP-3, both in terms of efficacy and potency, as indicated by the numbers of migrating cells and the concentration (100 nM) at which maximum effects were observed. In agreement with a former study (4), MCP-1 was slightly more efficacious and

considerably more potent as it reached its maximum activity already at 1 nM. In eosinophils, MCP-4 was tested in comparison with eotaxin and MCP-3, which are both potent attractants for these cells, while MCP-1 served as negative control. MCP-4 and eotaxin elicited similar migration responses, with a maximum at 10–30 nM. MCP-3 had comparable efficacy, but its maximum effective concentration was 100 nM. MCP-4 also induced strong migration of CD4⁺ and CD8⁺ T lymphocytes. Its efficacy was similar to that of MCP-1, but 10–100 nM MCP-4 was required for maximum effects, as compared to 1 nM MCP-1. Some migration of both types of T cells was also observed with eotaxin at concentrations between 10 nM and 1 μ M. Freshly prepared blood lymphocytes did not migrate in response to any of the chemokines that were effective on cloned cells.

[Ca²⁺]_i Changes. A rapid and transient rise in [Ca²⁺]_i was observed after MCP-4 stimulation of monocytes, lympho-

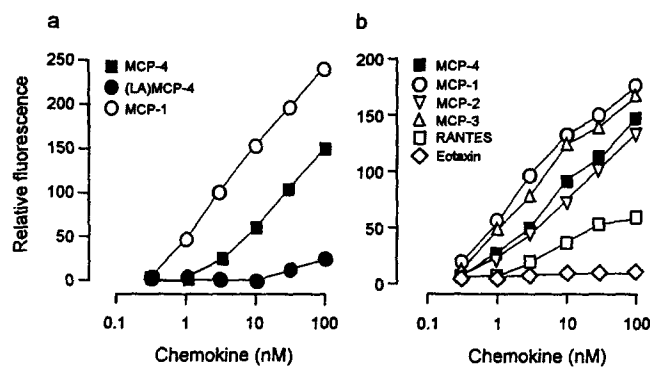


Figure 2. Release of *N*-acetyl- β -D-glucosaminidase from cytochalasin B-treated human blood monocytes in response to MCP-4 and other CC chemokines. Enzyme activity is presented in arbitrary fluorescence units. One out of three similar experiments performed with cells from different donors is shown.

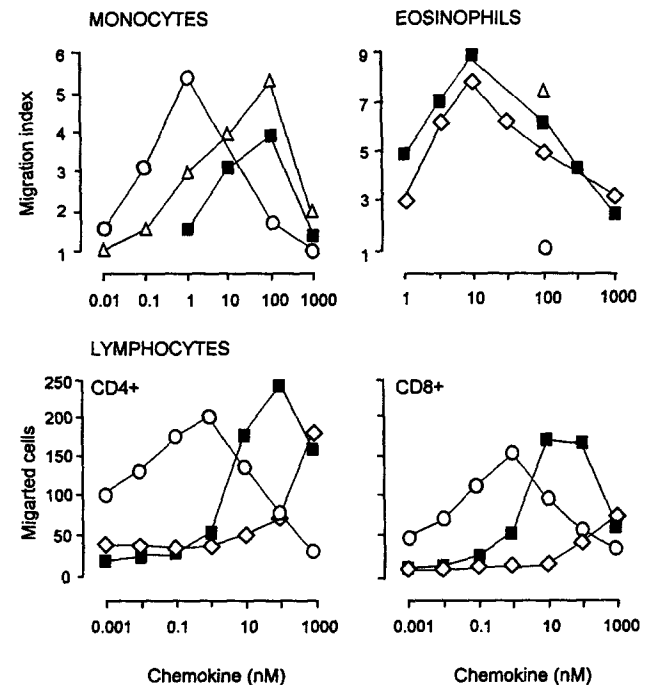


Figure 3. Chemotactic responses of human leukocytes to MCP-4 and other CC chemokines. Numbers of migrating cells per five high power fields, rather than migration index, are given for lymphocytes. (■) MCP-4, (○) MCP-1, (△) MCP-3, (◇) eotaxin. One out of three similar experiments performed with cells from different donors is shown.

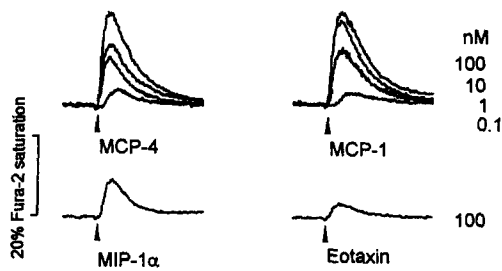


Figure 4. $[Ca^{2+}]_i$ changes in human $CD4^+$ lymphocytes after stimulation with increasing concentrations of MCP-4 and MCP-1 (upper graphs), or 100 nM MIP-1 α and eotaxin (lower graphs). Arrows indicate the addition of an agonist. Similar results were obtained with $CD8^+$ lymphocytes.

cytes, and eosinophils. The rate and magnitude of the rise increased with the concentration, and maximum values were obtained at chemokine concentrations between 10 and 100 nM. MCP-4 and MCP-1 induced similar concentration-dependent $[Ca^{2+}]_i$ transients in $CD4^+$ and $CD8^+$ T cells (see Fig. 4 for data on $CD4^+$ cells). Significant but much smaller changes were observed in both types of lymphocytes after stimulation with MIP-1 α and eotaxin (Fig. 4). The lower potency was expected since MIP-1 α (5) and eotaxin (see above) are weak lymphocyte attractants.

Desensitization Experiments. $[Ca^{2+}]_i$ changes after repeated stimulation were monitored in monocytes and eosinophils to assess receptor usage by MCP-4 and related chemokines. As shown in Fig. 5, stimulation of monocytes with MCP-1 or MCP-3 abolished responsiveness to MCP-4, indicating that the novel chemokine shares receptors with the monocyte chemotactic proteins. The response to MCP-4, by contrast, was not affected by stimulation with RANTES or MIP-1 α . When the monocytes were first stimulated with

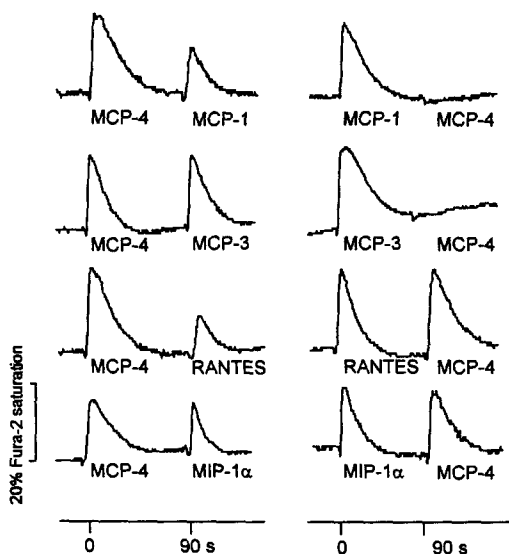


Figure 5. Cross-desensitization of human blood monocytes. Fura-2-loaded monocytes were stimulated sequentially at 90-s intervals with 50 nM MCP-4 and other CC chemokines, and $[Ca^{2+}]_i$ -dependent fluorescence changes were recorded. The tracings are representative for three to four separate experiments that were performed under identical conditions with cells from different donors.

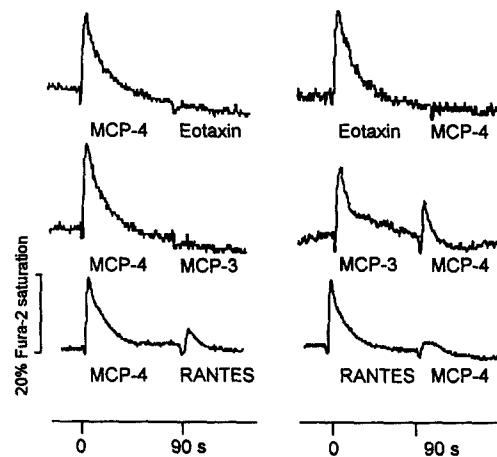


Figure 6. Cross-desensitization of human blood eosinophils. The conditions are identical to those in Fig. 5. Tracings are representative for three separate experiments that were performed with cells from different donors.

MCP-4, a clear decline of the responses to MCP-1, RANTES, and MIP-1 α , as well as a slight attenuation of the response to MCP-3, were observed. This desensitizing effect increased with the concentration of MCP-4 (data not shown). These results confirm that MCP-4 shares receptors with the MCPs, and indicate that it also recognizes a receptor that binds RANTES and MIP-1 α .

As illustrated in Fig. 6, marked cross-desensitization between MCP-4 and MCP-3, RANTES, and eotaxin was observed in eosinophils. MCP-4 appears to be a major agonist for these cells since it abrogated the response to a subsequent stimulation with eotaxin and MCP-3, and markedly decreased responsiveness to RANTES. These results indicate that MCP-4 shares the receptors with MCP-3, RANTES, and eotaxin. Stimulation with MCP-4 did not affect eosinophil responses to MIP-1 α (data not shown), indicating that this chemokine acts through a receptor that does not recognize MCP-4. The same receptor is likely to bind RANTES because some activity of this chemokine was still observed in cells that had been pretreated with MCP-4.

Actions on Neutrophils. As a CC chemokine with similarity to the MCPs, MCP-4 was not expected to activate neutrophils. It was nevertheless tested in comparison with IL-8 as inducer of $[Ca^{2+}]_i$ changes and elastase release and found to be completely inactive in three independent experiments, up to a concentration of 300 nM.

Discussion

Our results show that MCP-4 has marked functional similarities to MCP-3 and eotaxin. Like MCP-3, MCP-4 is a chemoattractant of high efficacy but relatively low potency for monocytes and T lymphocytes. On eosinophils, MCP-4 is a very powerful attractant matching the effectiveness of eotaxin. MCP-4 shares receptors mainly with MCP-3, eotaxin, and RANTES, as shown particularly by cross-desensitization on eosinophils.

The preparation of rMCP-4 contained two variants with two and seven additional NH₂-terminal residues. (LA)MCP-4 had only weak activity on monocytes, suggesting that (FNPQGLA)MCP-4, which could not be tested, has little if any activity. It was formerly shown that addition of alanine, as well as minor changes at the NH₂ terminus of MCP-1, lead to major losses in biological activity (22). Of particular interest is the finding that MCP-4 has a pyroglutamic acid residue at the NH₂ terminus. This also applies to MCP-1, MCP-2, and MCP-3, which were all reported to be NH₂ terminally blocked (2, 3), suggesting that pyroglutamic acid is the biologically relevant NH₂ terminus of all MCPs.

On the basis of sequence similarity, CC chemokines can be subdivided into two groups, one including MCP-1, MCP-2, MCP-3, and eotaxin, and the other RANTES, MIP-1 α , and MIP-1 β . MCP-4, the novel CC chemokine described in this paper, clearly belongs to the first group since it shares sequence identities of 56–61% with the

MCPs and eotaxin, but only 29–41% with RANTES and the MIPs. In spite of the structural analogies, there is considerable functional heterogeneity within the two groups. Clear-cut differences in biological activity have been documented for MCP-1 on one hand, and MCP-2 and MCP-3 on the other (4, 10, 23). Eotaxin is even more distant, since it acts preferentially on eosinophils (12, 13), quite in contrast to the monocyte chemotactic proteins. Some important dissimilarities in potency and target selectivity also exist among RANTES, MIP-1 α , and MIP-1 β (1, 4, 5), and it is interesting to note that RANTES shares with MCP-3 some of the properties and receptor selectivities on eosinophils and monocytes (4, 11).

The present results suggest that MCP-4 may be of particular importance for its activity on eosinophils. In fact, while MCP-4 was somewhat less effective than MCP-1 and MCP-3 in monocytes and lymphocytes, it was equivalent to eotaxin as an eosinophil attractant and was superior to MCP-3 in desensitizing these cells toward eotaxin.

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