Conversion of Monocyte Chemoattractant Protein-1 into a Neutrophil Attractant by Substitution of Two Amino Acids*

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The small cytokine monocyte chemoattractant protein-1 has structural similarity to the neutrophil chemoattractant interleukin-8, but each protein is specific in attracting its own target cell. To investigate the structural basis of this cell type specificity, we have developed an Escherichia coli expression system for the monocyte chemoattractant and mutagenized selected amino acid residues to ones found at the corresponding positions of interleukin-8. We find that a double mutation of tyrosine 28 and arginine 30 to leucine and valine, respectively, causes a drastic decrease in chemotactic activity toward monocytes with the appearance of a novel (interleukin-8-like) neutrophil chemotactic activity. Computer graphic analysis predicts that, with the double substitution, a putative receptor binding groove of the monocyte chemoattractant protein would become topographically similar to that of interleukin-8. We therefore postulate that one or both of these amino acid residues are part of the binding contact of these small cytokines and their receptors.

The recruitment of monocytes to areas of inflammation is a key factor in normal wound healing and in various diseases including atherosclerosis. Macrophages derived from monocytes are the precursors of foam cells that play a major role in the formation of atherosclerotic plaques (1). The cytokine monocyte chemoattractant protein-1 (MCP-1, also known as MCAF) may be an important mediator in the recruitment of monocytes. This 77-amino acid protein shows chemoattractant activity for monocytes but does not attract neutrophils (2). It has been reported to be expressed in atherosclerotic lesions in vivo (3) and induced in vitro by oxidized low density lipoproteins (4) that are considered to be a risk factor in atherosclerosis. The DNA and gene for MCP-1 were recently cloned, and the encoded protein was shown to have structural similarity to a family of cytokines and related proteins (5–7). Probably the best characterized member of this family is interleukin-8 (IL-8), which is a chemoattractant for neutrophils but has no activity for monocytes. Furthermore, the receptors for the two cytokines are different, as neither blocks the binding of the other to their respective target cells (8, 9). X-ray crystallography (10) and nuclear magnetic resonance studies (11) showed that an IL-8 dimer consists of an antiparallel β sheet topped by two α helices, forming a groove. Computer modeling studies predict that MCP-1 is very similar in structure to IL-8 (12). A diagram of the predicted structure of MCP-1 is shown in Fig. 1. Since the groove in these two proteins resembles a structure in the human leukocyte antigen-A2 molecule that mediates peptide binding (13), it has been postulated that this region might be involved in binding of the cytokines to their receptors. However, the exact structural features critical to the cell type specificity of these molecules remain unknown.

Since MCP-1 and IL-8 are predicted to have a very similar backbone structure but bind to different receptors, we postulated that specific side chains in the structure would control receptor binding and hence cell specificity. To investigate this idea, we have substituted amino acid residues 28 and 30 of this protein with the corresponding residues found in IL-8. We report that this double mutant showed chemotactic activity for neutrophils.

MATERIALS AND METHODS

Construction of Expression Clones—We used a human MCP-1 cDNA clone which was isolated from an endothelial cell cDNA library† to express the protein in Escherichia coli. We engineered the human MCP-1 cDNA to insert an Ndel restriction site (CATATG, which includes an initiation codon) just upstream of the point where the signal sequence is processed off during secretion of the natural MCP-1 product. This was done by performing a polymerase chain reaction (PCR) with the MCP-1 cDNA as a template and primers complementary to the 3' and 5' MCP-1 sequences and including appropriate restriction sites (primer sequences GGGAATCA-TATGCGAGCGATGAACT and GGGAATCTCAAGTCTC-GGAGTTT). We purified the product, cut it with BamHI and EcoRI, subcloned it into pUC 18, and sequenced it to ensure that no mutations were introduced during the PCR. We subsequently cut out the fragment with Ndel and BamHI and cloned it into the pET3a expression vector (14) in strain DH5α. We then transformed the bacterial expression strain BL21(DE3) pLYS-S with the constructs.

To produce MCP-1 mutants, we altered the coding sequence by a two-step PCR procedure (15) to introduce a single mutation of codon 30 from AGA encoding arginine to GTA encoding valine, and a double change including the Arg to Val change plus a mutation of codon 28 from TAT (tyrosine) to TTG (leucine). We subcloned the mutated sequences into pUC 18 and completely sequenced them. We then inserted the fragments into pET3a and transformed the strain BL21(DE3) pLYS-S as above.

To check that no additional mutations had arisen in the double mutant plasmid, we reisolated the plasmid and repurified the expression cells. We isolated DNA from the original expression cells, transformed DH5α, and completely sequenced the insert from a single

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‡ The abbreviations used are: MCP-1, monocyte chemoattractant protein-1; IL-8, interleukin-8; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; MIP-2, macrophage inflammatory protein-2; gro/MGSA, melanoma growth stimulatory activity; NAP-2, neutrophil attractant protein-2; HPLC, high performance liquid chromatography.

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clone with the two PCR primers to ensure that the sequence was as expected. We then retransformed BL21(DE3) pLYS-S with this DNA and isolated the recombinant protein as indicated below.

**Purification of Recombinant Human MCP-1 and Mutant Derivatives**—Bacterial cells containing expression plasmids were grown in a liter of LB broth containing 50 μg/ml ampicillin and 25 μg/ml chloramphenicol. They were induced with 0.4 mM isopropyl thiogalactoside at 37 °C for 4 h. The cell pellet was collected by centrifugation at 8000 rpm for 10 min at 4 °C, and the cells were suspended in 0.05 M phosphate buffer, lysed by sonication, and left on ice overnight at 4 °C. Guanidine hydrochloride was added to the cell lysate to a final concentration of 4 M to solubilize the protein, and the lysate was centrifuged at 100,000 × g. The supernatant was filtered through a 0.2-μm filter and subjected to gel filtration on a Sephadex G-75 column. In initial experiments, we assayed for the protein by Western blots with an antiserum prepared against baboon MCP-1. In later experiments we assayed for co-migration with previously purified material on SDS gels or by Western blots with rabbit antibodies prepared using the recombinant protein. The fractions containing MCP-1 were pooled, dialyzed, and lyophilized. The residue was dissolved in 0.1% (v/v) trifluoroacetic acid containing 7 M guanidine hydrochloride and injected onto a C4 reverse phase column (Brownlee Aquapore Butyl) equilibrated in 0.1% trifluoroacetic acid in water. The proteins were eluted by a 45-min linear gradient of 0-70% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.8 ml/min.

The purified MCP-1 peak was identified by Western blots and lyophilized.

**Monocyte and Neutrophil Chemotaxis Assays**—Monocytes were isolated from buffy coat (leukocyte-rich plasma) from the American Red Cross by centrifugation using Accuspin tubes (Sigma). In some cases this monocyte preparation was further purified by counterflow centrifugal elutriation. Both procedures gave similar results. The monocytes were collected, centrifuged, washed in phosphate-buffered saline, and resuspended in Gey’s balanced salt solution with 0.2% (w/v) bovine serum albumin. Human neutrophils were isolated from blood collected from healthy donors by centrifugation on Polymorphprep (Accurate Scientific & Chemical Corp.). The blood (5 ml) was carefully layered on 3.5 ml of Polymorphprep solution in a 12-ml centrifugation tube. The tubes were centrifuged at 450-500 × g for 35 min in a swing-out rotor at 20 °C. The neutrophil fraction was transferred into a tube containing 0.45% NaCl solution, centrifuged, washed in phosphate-buffered saline, and resuspended in the desired medium. The chemotaxis assays were performed in a 48-well microchemotaxis device (Neuroprobe) as described (16) with 5-μm pore size filters. The number of cells that migrated to a buffer control was subtracted as background. The endotoxin level of all purified proteins was less than 1 ng per mg of protein, as measured by the Limulus amoebocyte assay (Cape Cod Associates), which we found did not stimulate monocyte chemotaxis.

**Protein Chemistry**—To quantitate the number of thiol groups present in the recombinant MCP-1 and the mutants, these proteins were treated with 5,5'-dithiobis(2-nitrobenzoic acid) (Sigma) in Tris-HCl buffer (pH 8.3) (17). Absorbance was measured at 412 nm. For tryptic digestion of the double mutant Tyr-28 — Leu, Arg-30 — Val and 1 and 2, 100 μg each of the two protein fractions were incubated with 5 μg of sequencing grade trypsin (Boehringer Mannheim) for 2 h at 37 °C. The tryptic digests were treated with 10 mM dithiothreitol at 37 °C for 1 h. Performic oxidation of the proteins was performed by the method described by Hir (18). The oxidized proteins were then trypsin-digested and injected onto a C4 reverse phase column as described earlier.

**RESULTS AND DISCUSSION**

We developed an expression system and a two-step method that enabled us to purify milligram quantities of recombinant MCP-1. Aliquots from gel filtration were tested for cross-reactivity with anti-MCP-1 antiserum, and the fractions showing reactivity were pooled (Fig. 2A), dialyzed against distilled water, and lyophilized. The HPLC column profile of

![Fig. 1. Diagrammatic representation of the proposed structure of an MCP-1 dimer (12). The β sheets of the protein are represented by the ribbons with arrowheads and α helices by the cylinders. The positions of amino acid residues 28 and 30 are shown represented by the double-headed arrows in each of the monomers. The amino and carboxyl termini are indicated by NH₂ and COOH, respectively.](image-url)
this lyophilized fraction showed a major peak corresponding to the MCP-1 protein (Fig. 2B). This protein peak was collected and lyophilized. SDS gel electrophoresis of the purified protein showed a single band at around 12 kDa (Fig. 2C). The protein was confirmed to be MCP-1 by a Western blot using antiserum to MCP-1. The purity of the recombinant protein was further confirmed by amino acid analysis and N-terminal sequencing (not shown).

After inspecting sequence conservation patterns and performing structural modeling, we concentrated on two residues, tyrosine 28 and arginine 30 in the MCP-1 sequence. In the MCP-1 structure these residues might make only small changes in the three-dimensional structure of the protein.

The chemotactic activities of the recombinant MCP-1 and mutant proteins were analyzed by in vitro assays. The recombinant MCP-1 showed monocyte chemotactic activity identical to that of the natural product (Fig. 5A; the natural protein kindly provided by Dr. T. Yoshimura). This result indicates that the glycosylation reported to be present in the natural product (19) does not play a direct role in the biological activity, as previously found for other cytokines (20). The additional methionine present in the recombinant protein also did not affect the chemotactic activity. The Arg-30 → Val mutant showed almost equal monocyte chemotactic activity to the wild type protein. However, the activity of fraction Tyr-28 → Leu, Arg-30 → Val-1 was substantially less, and the Tyr-28 → Leu, Arg-30 → Val-2 fraction showed no detectable monocyte chemotactic activity (Fig. 5B). These results indicate that the amino acids at positions 28 and 30 of the protein sequence are important in the role of MCP-1 as a monocyte chemoattractant and could be involved in binding of the protein to its receptor.

The recombinant MCP-1 showed no detectable chemotactic activity toward neutrophils, just as the natural product (2). The Arg-30 → Val mutant also showed no activity toward neutrophils. However, both the Tyr-28 → Leu, Arg-30 → Val-1 and -2 forms showed a new chemotactic activity toward neutrophils (Fig. 6A). The neutrophil chemotactic activity could be removed from both Tyr-28 → Leu, Arg-30 → Val-
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Fig. 5. Monocyte chemotaxis in response to recombinant MCP-1 and its mutants. Chemotactic assays were performed in multiwell chambers as described (20). The number of monocytes that migrated were counted in three high power fields. The number of cells migrating to the background control of Gey's-bovine serum albumin mixture alone was subtracted. To compare samples from different donors, we expressed the numbers as the percentage of the migration to recombinant MCP-1 at $10^{-10}$ M in each separate experiment. This concentration showed peak activity under our conditions. A, a representative experiment showing the chemotactic response of monocytes to natural MCP-1 from glioma cells and recombinant MCP-1 at different protein concentrations. B, the chemotactic response of monocytes to recombinant MCP-1, the single mutant Arg-30 → Val, and the double mutant fractions Tyr-28 → Leu, Arg-30 → Val-1 and Tyr-28 → Leu, Arg-30 → Val-2. Values represent the arithmetic means of the data from five separate experiments, and error bars represent the standard errors of the mean.

Fig. 6. Neutrophil chemotaxis in response to recombinant MCP-1 and its mutants. Chemotactic assays were performed in multiwell chambers. The number of neutrophils that migrated through the pores was counted in three high power fields. For each experiment we determined the percentage of neutrophils that migrated as a fraction of migration to recombinant IL-8 at $10^{-9}$ M (the concentration giving peak activity). A, chemotactic response of neutrophils to IL-8, recombinant MCP-1, the single mutant Arg-30 → Val, and the double mutant fractions Tyr-28 → Leu, Arg-30 → Val-1 and Tyr-28 → Leu, Arg-30 → Val-2. The bars represent the arithmetic mean of the percent of neutrophils that migrated in five separate experiments with error bars defining standard errors of the mean. B, removal of neutrophil chemotactic activity of MCP-1 mutants by immunoprecipitation with anti-MCP-1 serum. IL-8, Tyr-28 → Leu, Arg-30 → Val-1, and Tyr-28 → Leu, Arg-30 → Val-2 were incubated with either non-immune rabbit serum (control) or anti-recombinant MCP-1 serum for 2 h followed by incubation with protein A-Sepharose for 2 h and centrifugation. The supernatants were assayed for chemotactic activity.

fractions by immunoprecipitation with an anti-MCP-1 serum but not with control preimmune serum (Fig. 6B). This showed that the observed activity was due to the mutant proteins and not due to any contaminant in our protein preparation.

The fact that the substitution of two amino acids in MCP-1 makes it attract neutrophils shows that the region of the molecule containing these 2 residues is an important determinant of cell type specificity of this cytokine. It is not surprising that the neutrophil chemotactic activity of this mutant form is lower than IL-8, as we expect that other residues of IL-8 also play a role in binding to the receptor. The result that both fractions of the Tyr-28 → Leu, Arg-30 → Val preparation show neutrophil chemotactic activity indicates that the structural differences between them are small. However, a difference is evident in the activity of the two fractions toward monocytes. We suggest that the Tyr-28 → Leu, Arg-30 → Val-1 fraction is likely to have the normal arrangement of disulfide bonds because of its higher monocyte chemotactic activity, while the Tyr-28 → Leu, Arg-30 → Val-2 fraction may be a variant form in which the disulfide bridges are formed by different cysteine pairs. The fact that the Tyr-28 → Leu, Arg-30 → Val-2 fraction is relatively inactive on
monocytes while retaining activity for neutrophils suggests that the MCP-1 receptor is sensitive to a perturbation in structure of the ligand that does not affect binding to the IL-8 receptor.

We conclude that residues 28 and/or 30 in MCP-1 and their counterparts in IL-8 are likely to be involved in interaction of these proteins with their receptors. This conclusion is consistent with a recent report that a synthetic peptide of residues 13-35 of MCP-1 showed monocyte chemotactic activity, albeit much less than the intact molecule (21). It suggests that a portion of the receptor may bind within the groove that is predicted by computer modeling. We have carried out a computer analysis of the structural changes caused by the mutation of residues 28 and 30. This analysis shows that the side chains of residues 28 and 30 of MCP-1 nearly fill the groove, whereas in the double mutant the side chains extend a much shorter distance and leave a significant space in the groove. The double substitution also renders the putative receptor binding groove more hydrophobic, making it more similar to IL-8 (Fig. 3).

We also found that aspartate 68 in MCP-1 extends from the $\alpha$ helix over the binding groove. In IL-8 the corresponding residue, number 66, is leucine making the putative binding region in IL-8 even more hydrophobic (Fig. 7). Since leucine 66 directly overlies residues 25 and 27 of IL-8, it is possible that this residue is also necessary for the optimal receptor binding. We suggest that the double mutant is not quite as effective as IL-8 as a neutrophil attractant because of the presence of aspartate 68 in place of leucine as in IL-8.

Our finding that residues 28 and 30 are important in determining cell specificity is consistent with patterns of sequence conservation between members of this cytokine family. Tyrosine 28 and arginine 30 are invariant in MCP-1 sequences from human, mouse, rat, and rabbit (22). The corresponding residues in IL-8, numbers 25 and 27, are leucine and valine in both the human and rabbit genes (22). Three other molecules, macrophage inflammatory protein-2 (MIP-2), melanoma growth stimulating activity (gro/MGSA), and neutrophil attractant protein-2 (NAP-2), are known to bind to IL-8 receptors (9, 23, 24). NAP-2 and MIP-2 have identical amino acids to IL-8 at the two positions, while gro/MGSA has valine at both positions (a conservative substitution) (24). The identification of structural features of these cytokine molecules that mediate binding to their receptors could lead toward the design of drugs that modulate the actions of these cytokines. Such drugs could be important research tools and might be valuable therapeutic agents.

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