Structural and Functional Identification of Two Human, Tumor-derived Monocyte Chemotactic Proteins (MCP-2 and MCP-3) Belonging to the Chemokine Family

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Summary

Cytokine-stimulated human osteosarcoma cells (MG-63) secrete several related chemotactic factors, including the neutrophil-activating protein interleukin 8 (IL-8) and the monocyte chemotactic protein (MCP)-1. We describe the isolation and characterization of two novel monocyte chemotactic factors from this tumor cell line. Although these proteins copurified with MCP-1 and IL-8 on heparin-Sepharose, they could be separated by cation-exchange fast protein liquid chromatography and reverse-phase high-performance liquid chromatography. The corresponding 7.5- and 11-kD proteins were NH2-terminally blocked but were identified by sequencing peptide fragments. They showed a primary structure mostly related to that of MCP-1 and were therefore designated MCP-2 and MCP-3, respectively. These molecules can be classified in a subfamily of proinflammatory proteins characterized by the conservation of cysteine residues. MCP-2 and MCP-3 are also functionally related to MCP-1 because they specifically attract monocytes, but not neutrophils, in vitro. The chemotactic potency (specific activity) was comparable for all three MCPs. Intradermal injection of these proteins in rabbits resulted in selective monocyte recruitment in vivo. Since tumor cells are good producers of leukocyte chemotactic factors, it could be questioned whether these molecules can indirectly control tumor growth by attracting leukocytes or whether they rather promote invasion by the secretion of proteases from the attracted cells.

Cell migration is an important phenomenon in inflammation and in the invasive behavior of tumors. In response to infection, phagocytes are attracted to the inflammatory focus through the generation of chemotactic gradients. Recently, chemotactic factors belonging to a novel family of proinflammatory proteins have been identified (1-7). In contrast to the classical chemoattractants (e.g., C5α, FMLP), these molecules specifically affect the migration of single types of leukocytes. Within this family of structurally related, low relative molecular mass factors, IL-8 is a well-characterized neutrophil-activating protein (1-5). IL-8 can be grouped into a class (designated CXC chemokines) based upon the position of the two NH2-terminal, conserved cysteines. Monocyte chemotactic protein 1 (MCP-1) belongs to a second class (CC chemokines), in which these cysteines are adjacent (6, 7). MCP-1 is an attractant for monocytes but not for neutrophils (6-8). Except for some platelet products, these inflammatory proteins can be induced in various cell types (fibroblasts, endothelial cells, monocytes, lymphocytes) in response to a variety of stimuli, including cytokines (1-8). Several transformed cell types have also been described to secrete these chemotactic factors. This implies that chemoattractants might play a role in tumor biology (9). We describe here the identification of two novel monocyte chemotactic factors isolated from stimulated osteosarcoma cells. Since the proteins were found to be biochemically and biologically related to MCP-1, they are designated MCP-2 and MCP-3.

Materials and Methods

Production and Purification of Monocyte Chemotactic Activity. Human MG-63 osteosarcoma cells (8, 10) were stimulated with pure human IL-1β or semi-purified cytokine derived from mitogen-stimulated mononuclear cells. After 5 h of induction, cell cultures were washed and incubated with serum-free or serum-containing (2% FCS) growth medium for 48 h at 37°C. MCP (3 liters/batch) was concentrated and partially purified by adsorption to controlled pore glass (CPG-10-350; Serva, Heidelberg, Germany). The CPG eluate was further purified by antibody affinity chromatography using a polyclonal antibody to fibroblast-derived cytokine (11). Alternatively, MCP was purified by heparin-Sepharose (CL-6B; Pharmacia, Uppsala, Sweden) chromatography. MCP was eluted in a

Abbreviations used in this paper: CPG, controlled pore glass; GCP, granulocyte chemotactic protein; MCP, monocyte chemotactic protein.

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linear NaCl gradient (0.05–2 M) in 50 mM Tris, pH 7.4 (8). MCP recovered by antibody or heparin-Sepharose chromatography was further purified by Mono S FPLC (Pharmacia) in 50 mM formate, pH 4.0. After an extensive wash with equilibration buffer, activity was eluted (1 ml/min) in a linear NaCl gradient (0–1 M) in 50 mM formate, pH 4.0.

Fractions were checked for purity by SDS/PAGE. Samples (3–20 µl) were loaded onto a linear gradient (10–25%) polyacrylamide gel, and silver stained (8, 11). The relative molecular mass markers (Bio-Rad Laboratories, Richmond, CA) used were phosphorylase b (M, 92,500), BSA (M, 66,200), OVA (M, 45,000), carbonic anhydrase (M, 31,000), soybean trypsin inhibitor (M, 21,500), lysozyme (M, 14,400), and the low relative molecular mass marker (Pierce Chemical Co., Rockford, IL) aprotinin (M, 6,500).

Identification of Monocyte Chemotactic Proteins by Sequence Analysis. MCPs were purified to homogeneity by reverse-phase HPLC (8). Active fractions from FPLC were analyzed on a 220 x 2.1-mm C-8 Aquapore RP-300 column (Applied Biosystems, Inc., Foster City, CA) equilibrated with 0.1% TFA. Chemotactic activity was eluted with an acetonitrile gradient in 0.1% TFA at 0.4 ml/min.

Tryptic fragments from 4 µg MCP were prepared by digestion with 0.2 µg bovine trypsin (sequencing grade; Boehringer Mannheim, Mannheim, Germany) at 37°C for 18 h (8). Alternatively, 4 µg of MCP was digested by addition of 0.2 µg of endoproteinase Asp-N, asparaginylendopeptidase, or endoproteinase Lys-C (sequencing grade; Boehringer Mannheim) at 37°C for 18 h. The MCP fragments obtained were separated by reverse-phase HPLC, as described for the final purification of MCP. Amino acid sequences of proteins and peptides were determined in duplicate with an on-line sequencing system with asparaginyl- or Lys-C endoproteinase. Sequence analysis revealed that all these MCP fragments obtained were NH2-terminally blocked. Identity with authentic MCP-1 was confirmed by sequencing fragments (from residues 3–26 and 54–60 of the mature protein) of an Asp-N endoproteinase digest (Fig. 3).

Asays for Chemotaxis and Activation of Cells. PBMC were isolated by hydroxyethyl starch sedimentation and Ficoll-sodium metrizoate centrifugation (8). Neutrophils were further purified by hypotonic shock and Percoll gradient centrifugation (8).

Chemotaxis under agarose was measured according to the method of Nelson et al. (12) and as previously described (8, 13). The titration end-point, corresponding to 1 U/ml, was calculated from a dilution resulting in the half-maximal effective migration distance as compared with that obtained with human granulocyte chemotactic protein (GCP)/IL-8 (13) or MCP-1 (8).

Migration of monocytes and granulocytes was also assessed by the microchamber (Neuro Probe Inc., Cabin John, MD) technique (8, 14). An optimal concentration of purified human MCP-1 (8) or GCP/IL-8 (13) was used as a reference chemotacticant. The chemotactic index was calculated from the number of cells migrated to the test sample divided by the number of cells migrated to the control medium.

Release of gelatinase B was used as a parameter to measure monocyte or granulocyte activation. Purified granulocytes (5 x 10⁶ cells/ml) or adherent monocytes (2 h/37°C) were stimulated (in serum-free medium) with test reagents for 45 min or 18 h, respectively. Supernatants were centrifuged to remove cells, and gelatinase activity was determined by SDS/PAGE zymography (15).

In Vivo Infiltration of Leukocytes in Rabbit Skin. New Zealand white rabbits were shaved at the abdomen and injected intradermally with the indicated doses of homogeneous, pyrogen-free MCP preparations (100 µl/site, eight sites/rabbit). After 18 h, rabbits were killed and the injection sites were excised. Skin biopsies were processed for routine histology, stained with hematoxylin-eosin, and microscopically examined.

### Results

Isolation and Identification of Human MCP-2 and MCP-3. The monocyte chemotactic activity from stimulated MG-63 cells was concentrated by adsorption to CPG beads and further purified by antibody affinity chromatography or heparin-Sepharose chromatography. Distinct monocyte chemotactic activities could subsequently be separated by cation-exchange FPLC (Fig. 1). The predominant activity eluted at 0.5 M NaCl in the gradient and corresponded to two major protein bands (10 and 16 kD) previously identified as MCP-1α and MCP-1β (6, 8). In addition, a second and a third MCP peak were recovered at 0.7 and 0.6 M NaCl, and were designated MCP-2 and MCP-3, respectively (Fig. 1).

To investigate the nature of these additional MCP peaks from FPLC, their activities were purified to homogeneity (in parallel with MCP-1) by reverse-phase HPLC. Fig. 2A shows that MCP-1 eluted on HPLC at ~26% acetonitrile, whereas MCP-2 and MCP-3 eluted at 30% and 27.5% acetonitrile, respectively. This further indicated that the latter activities represent distinct factors. Homogeneous MCP-1 remained heterogeneous upon SDS-PAGE in that multiple molecular mass bands (10, 12, 14, and 16 kD) were eluting in single HPLC fractions (Fig. 2B). Sequence analysis revealed that all these MCP-1 proteins were NH2-terminally blocked. Identity with authentic MCP-1 was confirmed by sequencing fragments (from residues 3–26 and 54–60 of the mature protein) of an Asp-N endoproteinase digest (Fig. 3).

MCP-2 and MCP-3 were found to reside in a 7.5- and 11-kD protein, respectively (Fig. 2B). MCP-2 and MCP-3 also had a blocked NH2-terminus, but could be identified through fragmentation of (twice) 4 µg pure MCP-2 and MCP-3 with trypsin or Asp-N endoproteinase and (once) 4 µg MCP-3 with asparaginyl- or Lys-C endoproteinase. Sequence analysis of the obtained fragments, separated by HPLC, demonstrated that both MCP-2 and MCP-3 have a primary struc-

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**Figure 1.** Separation of MCP-1, MCP-2, and MCP-3 by cation-exchange FPLC. Concentrated MG-63 cell supernatant was loaded on a Mono S column at pH 4.0 and eluted (1-ml fractions) with a linear NaCl gradient (- - -). Absorbance (---) was monitored at 280 nm. Fractions were tested for monocyte chemotactic activity (histograms) in the agarose assay.
Figure 2. Purification of MCP-1, MCP-2, and MCP-3 to homogeneity by reverse-phase HPLC. (A) MCP-1, MCP-2, and MCP-3 from FPLC were loaded separately on a C-8 Aquapore RP-300 column and eluted (0.4-ml fractions) with an acetonitrile gradient (--.--). Absorbance was monitored at 220 nm (---). (B) SDS/PAGE of HPLC-purified MCPs. Active fractions were run with 5 µl (MCP-1α and β) or 20 µl (MCP-2 and MCP-3) on a linear gel under reducing conditions and silver stained. Relative molecular mass markers were as indicated in Materials and Methods. Numbered arrows indicate MCP activity (histograms) measured in the agarose assay and the corresponding protein bands, respectively.

Figure 3. Amino acid sequence alignments of MCPs from different species. The MCP-2 and MCP-3 sequences were experimentally determined from overlapping peptide fragments obtained after trypsin, endoproteinase Asp-N, asparaginyl- and Lys-C endoproteinase digestions. The other protein sequences are cDNA derived (17, 18, 21-24). Values in parentheses indicate the percentage of sequence similarity with human MCP-1. The sequence data of MCP-2 and MCP-3 are available from EMBL/GenBank/DDBJ under accession numbers P80075 and P80098, respectively.

Biological Characterization of MCP-2 and MCP-3 In Vitro and In Vivo

In the assay measuring migration under agarose, MCP-2 and MCP-3 showed specific activities (1 U corresponding to a half-maximal migration distance) of ~10^4 U/mg, comparable with that of MCP-1. In contrast, at the highest dose tested, no neutrophil chemotactic activity could be detected with all three MCP species (<10^3 U/mg), whereas IL-8 had a specific activity of 10^6 U/mg for neutrophils.

Fig. 4 compares the chemotactic potency (minimum effective concentration) and efficacy (maximal effect at optimal concentration) of MCP-1, MCP-2, and MCP-3 in the microchamber migration assay. All three MCPs are most efficacious for monocyte chemotaxis at 1 nM, the effect of MCP-3 being somewhat lower. The minimum effective concentration was 0.1-0.3 nM. The specific activity (1 U corresponding to a stimulation index of 2.5) in this monocyte microchamber assay was calculated to be 7 × 10^5, 4 × 10^5, and 4 × 10^5 U/mg for MCP-1, MCP-2, and MCP-3, respectively. In contrast, their specific activity for neutrophils was found to be < 5 × 10^4 U/mg, whereas IL-8 scored > 2 × 10^6 U/mg.

It was verified whether MCP-1, -2, and -3 could activate monocytes to secrete gelatinase B. Although by their adherence monocytes can spontaneously secrete gelatinase activity,
The production of the 85-kD enzyme could be enhanced by treatment with either MCP-1, -2, or -3 (data not shown). In contrast, on neutrophils no gelatinase B release was observed with any of the MCP molecules, whereas these cells were found to release this enzyme after treatment with IL-8 (15).

Finally, the three factors were compared for their chemotactic potency after administration in vivo (Fig. 5). Purified, endotoxin-free material was therefore injected intradermally into rabbit skin (10–500 ng/site). A selective and prominent infiltration of monocytes was noticed with MCP-1, -2, and -3. At 10 ng/site, MCP-2 and MCP-3 were still able to elicit an intradermal monocyte recruitment. The appropriate control (pyrogen-free saline) did not influence leukocyte infiltration. The monocyte infiltration was characterized by clustering of cells, visible monocyte adherence to endothelia (margination), and accumulation of monocytes around the site of injection (Fig. 5).

**Discussion**

This study describes the identification of two novel monocyte chemotactic factors isolated from human tumor cells. Since the corresponding 7.5- and 11-kD proteins show high structural and functional similarity with MCP-1 (8, 17, 18), they are designated MCP-2 and MCP-3, respectively. Based on the conservation of four cysteine residues, these molecules can be classified in a family of small proinflammatory proteins (1–8). In accordance with most members of this chemokine family, MCP-2 and MCP-3 show affinity for heparin.

Almost the complete primary structure of MCP-2 (95%) could be disclosed by sequencing fragments of the NH2-terminally blocked mature protein. Out of 72 residues determined, 45 (62.5%) were found to be identical to those in MCP-1. Although the MCP-2 sequence was not contained in the EMBL/SSW/Prot Bank, the primary structure of this chemotactic factor fully corresponded to that of the cDNA-derived sequence of leukocyte-derived HC14. The HC14 gene encodes a protein of 99 amino acids, including a signal peptide of 23 residues (16). The primary structure of the mature MCP-3 protein, a third chemotactic factor for monocytes, was almost completely determined. From the fragments (67 residues) sequenced, it can be deduced that MCP-3 is more closely related to human MCP-1 (73% similarity) than to MCP-2 (60% similarity).

All three MCPs possess similar specific activities in monocyte chemotaxis assays. Concordantly with MCP-1, MCP-2 and -3 are selective attractants because no significant effect could be observed on neutrophils. The specific activity of MCPs for neutrophil chemotaxis is at least 50-fold lower than that of IL-8. The effect on other cell types (e.g., lymphocytes) needs to be investigated in more detail.

MCP-1 and IL-8 exert chemotactic activity in vivo in that they cause both locally and systematically a recruitment of monocytes and neutrophils, respectively (13, 19). Similarly, MCP-2 and MCP-3 were found to induce monocyte infiltration after intradermal injection in rabbit skin. Occasionally, few neutrophils (<5% of total leukocyte counts) were also noticed in the skin section. It was also consistently observed that the monocytes occurred in ‘clusters’ or ‘nests’ at the injection site, and marginating cells were sometimes seen in contact with the endothelial lining of the venules. This effect seemed most pronounced with MCP-3.

Murine macrophage inflammatory protein 1 (20) and other human molecules of this subfamily, such as RANTES (7), have been reported to possess monocyte chemotactic activity. However, the structural similarity of these proteins with MCP-1 is weaker than that of MCP-2 and -3. Since MCPs isolated from other species, such as the mouse (21) and the rat (22), show comparable sequence homology with the three human MCPs, it cannot be concluded whether these proteins actually represent the homologue of human MCP-1. In contrast, rabbit (23) and bovine (24) MCPs are structurally more related to human MCP-1 and MCP-3 than to MCP-2.

Unless the tumor cells were stimulated, none of these low molecular mass proteins, including MCP-1, -2, and -3, could be recovered by purification from cell supernatants. If cells were treated for 48 h with semi-purified leukocyte-derived cell supernatants (containing IL-1, IL-6, IFN-γ, and possibly other cytokines), secretion of MCP-3 and MCP-1 was rather consistent, whereas that of MCP-2 remained variable. IFN-γ has been reported to be a major inducer of HC14/MCP-2 mRNA in monocytes (16). Induction of MCP-3 was observed in MG-63 cells stimulated with pure IL-1β under serum-free conditions.
conditions (not shown). Addition of 2% FCS further increased the production level, indicating that the regulation of MCP secretion is complex. The possibility that MCP-3 could represent the bovine equivalent of MCP-1 (copurified from the serum additive) was excluded since both MCPs were produced under serum-free conditions or in the presence of human serum. As a control, MCP was isolated from bovine kidney cells and was found to possess a protein sequence distinct from that of the human MCPs (data not shown). Furthermore, isolation of a bovine MCP from seminal plasma (24) revealed a primary structure still different from that of bovine kidney cell–derived MCP. The molecular cloning of the MCP-2 and MCP-3 genes will enable a detailed study of the expression in various cell systems.

Tumor cells seem to be good producers of chemotactic factors. It could therefore be questioned whether the MCPs identified here play a role in the control of tumor cell growth and invasion. Indeed, tumor-derived monocyte chemotactic activity is reportedly correlated with the presence of tumor-associated macrophages (9). MCP-activated monocytes might...
thus be important in tumor immune surveillance by inhibiting cell growth (19). Alternatively, we speculate that chemotactic substances might promote invasion and metastasis by protease secretion from the attracted and activated leukocytes.

In conclusion, the structural and functional identification of two novel MCPs extends the growing list of molecules belonging to this superfamily of proinflammatory cytokines. Since both new chemokines are equally active in vitro and in vivo when compared with MCP-1, the question remains why nature provides several of these closely related chemotactic factors. It therefore remains essential to investigate whether some of these have still other unrelated biological functions or whether some could serve as antagonist of MCP or IL-8, for instance, through competition for receptor binding.

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