

PURIFICATION AND CHARACTERIZATION OF A NOVEL
MONOCYTE CHEMOTACTIC AND ACTIVATING FACTOR
PRODUCED BY A HUMAN MYELOMONOCYTIC CELL LINE

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The capacity of various cell types to infiltrate inflammatory sites suggests the existence of specific chemotactic cytokines for each cell type. Snyderman et al. (1) first described that leukocytes from human peripheral blood when stimulated in vitro with a specific antigen (purified protein derivative [PPD]) or a nonspecific mitogen (PHA) produced a soluble factor that was chemotactic for homologous monocytes (2). The production of this lymphokine proved to be a sensitive in vitro correlate of delayed hypersensitivity (1). We have previously reported the identification, purification, and cDNA cloning of a novel neutrophil chemotactic factor (NCF) produced by LPS-stimulated human monocytes (3-5). In the course of identifying NCF, we detected in the same LPS-stimulated human monocyte-conditioned media a chemotactic activity for monocytes that was distinct in molecular weight from NCF. Since the level of monocyte chemotactic factor (MCF) in these conditioned media was at least 10 times less than that of NCF, we used conditioned media from a human myelomonocytic cell line (THP-1) as a source of MCF. The identical conditioned media were previously used for the purification of human IL-1 β (6). We now report the purification of human MCF produced by THP-1 cells to homogeneity and describe the potent monocyte activating factor (MAF) activity of this MCF.

Materials and Methods

Chromatography Procedures for the Purification of MCF. (a) For Heparin Sepharose chromatography, the 200-ml DEAE Sephacel breakthrough fraction was applied to 50 ml Heparin Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden), that had been equilibrated with 0.05 M Tris-HCl, pH 8.0, with 0.2 M NaCl. After washing the column extensively, MCF was eluted with 50 mM Tris HCl, pH 8.0, with 0.75 M NaCl. (b) For gel filtration, the 4-ml concentrate from Heparin Sepharose was loaded onto a 2.6 \times 90-cm column of Sephacryl S-200 (Pharmacia Fine Chemicals) equilibrated with D-PBS at 4°C. The flow rate was adjusted to 15 ml/h and 8-ml fractions were collected. (c) For cation HPLC, fractions corresponding 10-25 kD in gel filtration were concentrated and dialyzed against 0.02 MOPS, pH 6.5, and applied to a 7.5 \times 150-mm CM-3SW column (Toyo Soda, Tokyo, Japan) equipped to an HPLC system (model 2150; LKB Instruments, Inc., Sweden) that had been equilibrated with the same buffer. The starting buffer was 0.02 M MOPS, pH 6.5, and the limiting buffer

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was 0.02 M MOPS, pH 6.5, with 0.5 M NaCl. The flow rate was 1.0 ml/min and 2.0-ml fractions were collected. MCF was eluted at \sim 0.25 M NaCl. (d) For reverse phase chromatography, the active fractions from CM-HPLC were directly applied to a 4.6×750 -mm TMS-250 Ultropac column ($10 \mu\text{m}$; Toyo Soda). The starting solvent was water with 0.01% trifluoroacetic acid (TFA) (Pierce Chemical Co., Rockford, IL), and the limiting solvent was 60% acetonitrile (J. T. Baker Chemical Co., Phillipsburg, NJ) with 0.01% TFA.

Monocyte Chemotactic Assay. Monocyte chemotactic activity was measured in a multiwell Boyden chemotaxis chamber (Neuro Probe Inc., Cabin John, MD), as reported (1, 2). MCF was serially diluted in RPMI 1640 medium with 0.5% BSA and added to the lower chamber. Monocyte-enriched leukocytes were obtained from the National Institutes of Health Clinical Center Transfusion Medical Department (Bethesda, MD) and further separated on Ficoll-Hypaque. The purity of monocytes in this preparation was 30–50% and no neutrophil contamination was detected after staining with Giemsa solution. These mononuclear cells at 10^6 /ml RPMI 1640 medium with 0.5% BSA were added into upper chambers. Two chambers were separated by an $8\text{-}\mu\text{m}$ pore size polyvinyl pyrrolidone-free polycarbonate filter (Nucleopore, Pleasanton, CA). The chamber was incubated at 37°C for 90 min. The migrated cells that adhered to the lower surface of the membrane were fixed with methanol and stained with Giemsa solution. The migrated cells consisted of $>95\%$ monocytes by microscopic analysis.

Tumor Growth Inhibition Assay. 10^5 monocyte-enriched cells from leukapheresis (supplied by the NIH Clinical Center Transfusion Medical Department) in $100 \mu\text{l}$ RPMI 1640 medium with 5% FCS (Hyclone Laboratories, Logan, UT) were plated in 96-microwell culture plates. After incubating cells for 1 h at 37°C , nonadherent cells were removed by aspiration and washed twice with the same medium warmed to 37°C . The adhered monocytes were $>90\%$ pure by nonspecific esterase staining. Human tumor cell lines, all of which were obtained from the American Type Culture Collection (Rockville, MD), were detached by EDTA/trypsin treatment, washed twice with the same RPMI medium, and 10^4 cells in $100 \mu\text{l}$ RPMI medium were added into monocyte-containing wells. Serially diluted purified MCF was then added and cells were cultured for 72 h at 37°C in a CO_2 incubator. The cells were incubated for the final 6 h with $0.5 \mu\text{Ci}$ [^3H]TdR, where incorporation of thymidine into cells was measured.

Results and Discussion

21 liters of conditioned media from THP-1 cells was cultured at 2×10^6 /ml for 2 d in spinner culture flasks in the presence of $1 \mu\text{g}/\text{ml}$ LPS, $50 \mu\text{g}/\text{ml}$ silica, and 2 mM hydroxyurea, as previously described (6). The conditioned media were concentrated, dialyzed against 10 mM sodium phosphate buffer (NaPB), pH 7.5, by a hollow fiber system, and applied to a DEAE Sephacel column that was equilibrated with the same buffer. Monocyte chemotactic activity in the breakthrough fraction was further purified by sequential chromatography using heparin Sepharose, Sephacryl S-200 gel filtration, CM-cation exchange HPLC, and C-1 reverse phase HPLC. Fig. 1 *a* shows the results of the final chromatography on reverse phase HPLC. There was complete identity between one of the major protein absorbance peaks and monocyte chemotactic activity. The material yielded only one band by silver staining on SDS-PAGE analysis with an estimated molecular weight of 15,000, as shown in Fig. 1 *b*. The overall recovery of MCF activity was 7.3% and $2 \mu\text{g}$ of pure MCF was obtained from 21 liters of conditioned media. Since no significant amount of cleaved amino acid could be detected at the NH_2 terminal by Edman degradation sequence analysis on a gas phase sequencer, the purified material was cleaved by cyanogen bromide (CNBr) and sequenced again. The partial amino acid sequence of one of the CNBr-cleaved peptides was NH_2 -(Met)-Asp-His-Leu-Asp-Lys-Gln-Thr-Gln-X-Pro-Lys-Thr. The computer-assisted comparison of this peptide sequence with other

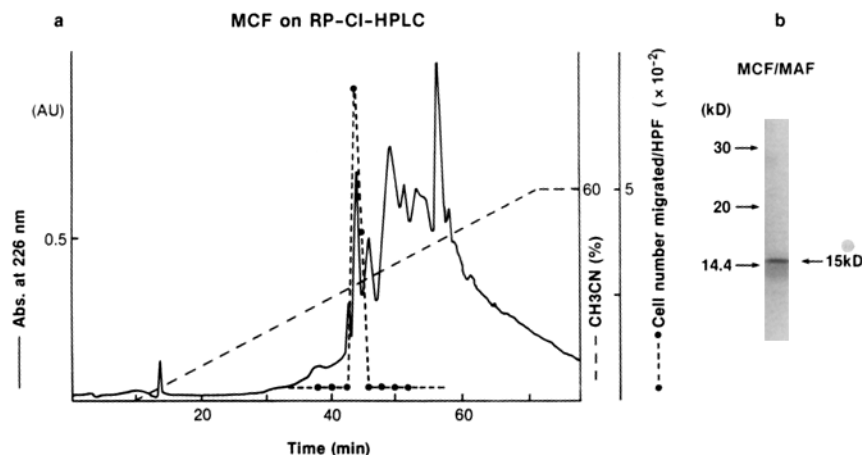


FIGURE 1 (a) Reverse phase HPLC of human MCF. After evaporation of samples from each fraction, MCF activity was measured. (b) SDS-PAGE analysis of purified human MCF. 15% acrylamide gel with stacking gel was used. After electrophoresis, the gel was stained with silver staining. Low molecular weight marker protein mixture (Pharmacia Fine Chemicals) was used as standard.

known polypeptides did not show any significant homology/similarity. The purified MCF was free from endotoxin (<0.1 ng/ml in fraction 45 and 46 from reverse phase HPLC as measured by Limulus amoebocyte lysate assay) and gave a half maximal chemotactic activity of 0.46 ng/ml for human unstimulated monocytes using a multi-well Boyden chamber system. MCF had a specific of 2.1×10^6 U/mg, defining 1 U as the reciprocal of the dilution at which half maximal chemotactic activity was obtained. MCF failed to show any significant chemotactic activity at 0.01 ng/ml to 100 ng/ml for human peripheral blood resting T lymphocytes or for normal neutrophils (data not shown).

To establish other possible effects of MCF on the function of human monocytes, we examined whether this factor stimulated human monocytes to be growth inhibitory for tumor cells. As shown in Fig. 2, purified MCF in a dose-dependent manner augmented the growth inhibitory effects of human monocytes on several human tumor cell lines, including a melanoma cell line A375 C-5 (IL-1 resistant), A375 C-6 (IL-1 sensitive) (7-9), a mammary tumor cell line MCF7, a colon carcinoma cell line HT 29, a rhabdomyosarcoma cell line HTB 82, and a leiomyosarcoma cell line HTB 88. No significant inhibition of tumor cell growth was observed by MCF-activated monocytes on human glioma cell lines HTB 16, U373, and HTB 14; and human bladder carcinoma cell lines HTB 3 and HTB 4. The effective dose yielding half-maximal MAF activity was similar to that for chemotactic activity. Polymyxin B at 10 μ g/ml did not block the activation of monocytes by MCF. We did not observe any direct growth inhibitory activity of this factor on these tumor cells. Time course experiments showed the growth inhibitory activity of MCF-treated monocytes to begin at 2 d and become maximal at 3 d of incubation (data not shown). Prolonged culture of A375 cells with monocytes in the presence of MCF led to lysis of only tumor cells at 4-5 d as determined by light microscopy. Conditioned media from MCF-stim-

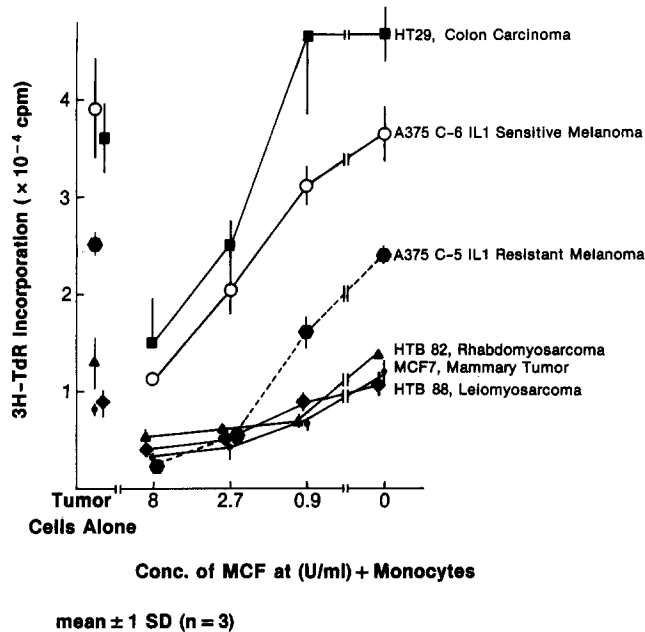


FIGURE 2. Augmentation of tumor growth inhibitory activity of human monocytes by purified MCF. [^3H]TdR incorporation by monocytes was <300 cpm.

ulated human monocytes failed to inhibit the growth of A375 cells and antibodies to IL-1 α , IL-1 β , TNF- α , and IL-6 did not block the capacity of MCF-treated monocytes to inhibit the growth of A375 cells, suggesting that these cytokines do not mediate either monocyte activation by MCF or the cytostatic/cytocidal effect of MCF-stimulated monocytes on tumor cells. MCF also induced morphological changes in monocytes such as irregularity of shape and increased agglutination but not spreading by several hours to 3 d (data not shown).

We have described the purification of a novel MCF with considerable MAF activity from the conditioned media of a human myelomonocytic cell line. Although the myelomonocytic cell line source of this factor was surprising and suggested that MCF may be an autostimulatory factor, a similar phenomenon has been observed with other monocyte/macrophage products, such as IL-1 (9), TNF- α (10), and IFN- β (11).

There appear to be distinct chemotactic factors for each inflammatory cell type. We have recently reported the purification and cDNA cloning of a NCF derived from LPS-stimulated human monocytes (4, 5). In addition, our recent purification of T lymphocyte chemotactic factor from PHA-stimulated human PBMC-conditioned media showed it to be identical to NCF (12). It has also been reported that IL-5 (T cell-replacing factor) is chemotactic for mouse eosinophils (13) and that NCF is not chemotactic for eosinophils (14). Therefore, it is clear that there are several leukocyte-derived chemotactic polypeptides that attract distinct cell populations *in vitro*. The target cell specificity of these factors could be based on the exclusive expression of the receptor for these factors on target cells. The *in vivo* effect on cell migration of these chemotactic polypeptides remains to be established. The bifunctional capability of MCF (chemotactic activity and monocyte activating activity) suggests that MCF may have local and perhaps systemic *in vivo* inhibitory effects

on tumor growth. The effects of MCF on other functions of monocytes and the growth and differentiation of immature myeloid cells and leukemic cells also merit study. The cDNA cloning of this novel MCF/MAF is in progress to permit further structure-function studies and to produce recombinant MCF molecules. The identification of MCF-producing cells in inflammatory site, delayed-type hypersensitivity site, and certain diseases will be examined when specific antibodies to MCF and cDNA probe become available.

Summary

A novel basic heparin-binding monocyte chemotactic factor (MCF) was purified to homogeneity from the conditioned media of human myelomonocytic cell line THP-1 based on its in vitro monocyte chemotactic activity. The purified MCF was homogeneous and estimated to be 15 kD on SDS-PAGE. Purified MCF stimulated normal human monocytes to be growth inhibitory in vitro at 2–3 d for several human tumor cell lines. This represents the first report of the identification and purification of a chemoattractant cytokine that also activates monocytes but is distinct from interferons and other known cytokines.

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