Brief Definitive Report

PROPERTIES OF MONOCYTE CHEMOTACTIC AND ACTIVATING FACTOR (MCAF) PURIFIED FROM A HUMAN FIBROSARCOMA CELL LINE

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We recently succeeded in purifying a monocyte chemotactic factor (MCF) from the conditioned media of a myelomonocytic cell line (THP-1) (1). We have molecularly cloned the cDNA of MCF (2), and shown that MCF, in addition to its monocyte chemotactic activity, also activates monocytes to be more cytostatic against several types of human tumor cells in vitro (1). Therefore, we termed this molecule monocyte chemotactic and activating factor (MCAF). Subsequently, MCAF has been shown to be produced by several additional cell types, namely fibroblasts and endothelial cells (3). We describe here that MCAF, which was purified from human fibrosarcoma cell line-conditioned media, also induces superoxide anion and lysosomal enzyme release from human monocytes and has potent in vivo monocyte recruitment activity.

Materials and Methods

Purification of MCAF. Confluent fibrosarcoma cells, 8387 (4), were stimulated with 100 ng/ml rTNF-α in serum-free media overnight. The culture supernatants were centrifuged, concentrated, and dialyzed against 0.05 M Tris-HCl, pH 7.5, before being applied to a 50-ml heparin Sepharose column. MCAF was eluted with 0.05 M Tris-HCl, pH 7.5, containing 0.5 M NaCl. The eluate was further concentrated and loaded on to a gel filtration column of Sephacryl S-200 (Pharmacia Fine Chemicals, Uppsala, Sweden), which was equilibrated with D-PBS. The fractions containing monocyte chemotaxis activity in the molecular mass range of 10–20 kD were pooled and then applied to a carboxymethyl-silica gel column (7.5 × 150-mm CM-3SW) (Toyo Soda, Tokyo, Japan) connected to an HPLC system (2150; LKB Instruments, Inc., Uppsala, Sweden). The starting buffer was 0.02 M 3-[*N*-morpholino] propanesulfonic acid (MOPS), pH 6.5, and MCAF was eluted with a linear increase of salt concentration up to 0.5 M NaCl. Finally, samples were applied to a reverse phase chromatography column (4.6 × 750-mm TMS-250 Ultropac column, 10 μm; Toyo Soda) with the starting

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solvent, water containing 0.01% trifluoroacetic anhydride (TFA) (Pierce Chemical Co., Rockford, IL), and the limiting solvent, 60% acetonitrile (J. T. Baker Chemical Co., Philipsburg, NJ) in water containing 0.01% TFA.

Western Blotting Analysis of MCAF. The samples were first separated on 15% SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane for 16 h at 100 mA using 0.025 M Tris-glycine buffer, pH 8.3. After the proteins were transferred to a membrane, MCAF was reacted with the rabbit anti-MCAF antibody, which was generated by immunizing a rabbit with synthetic MCAF (10th to 54th amino acid of mature MCAF) (1:20 dilution), followed by ^{125}I-labeled protein A (4 μCi/20 ml). After extensively washing, the membrane was dried and exposed to X-Omat AR film (Kodak, Rochester, NY) for 4 d at −70°C.

MCAF-Receptor Binding Assay. THP-1-derived pure MCAF (1) was radiolabeled by monoiodinated Bolton-Hunter reagent, as previously described (5). In the binding inhibition assay, 10^7 monocyte-enriched mononuclear cells diluted in RPMI 1640 with 10 mg/ml BSA and 20 mM Hepes were added to a total volume of 200 μl, then incubated in duplicate with ^{125}I-MCAF (~70,000 cpm/ng). Serial dilutions of unlabeled MCAF and rIL-8 (6) dissolved in PBS containing 10% glycerol were added to the PBMC and incubated for 15 min at 37°C. After centrifugation, the cell pellet was resuspended in binding buffer, spun through 10% sucrose in PBS, and the resulting pellet was assayed to determine the amount of bound MCAF. The percentage inhibition of binding of ^{125}I-MCAF by unlabeled ligands was determined.

Intradermal Injection of MCAF. 20 μl of various concentrations of MCAF in PBS was injected intradermally into the ears of 60 Lewis rats according to a randomized double blind protocol. Gentamicin cream was placed over the needle puncture to prevent infection. Groups of four rats for each of the five doses of MCAF (including PBS controls) were killed 3, 6, and 18 h after injection. The ears were excised, fixed in 10% buffered formalin, processed for routine histology, and stained with hematoxylin-eosin.

The number of neutrophils, lymphocytes, and monocytes/macrophages present in each of eight 3-μm sections cut through injection sites from the ears of four rats were counted on coded slides by light microscopy. Cells present in the two high-powered fields on either side of the field containing the injection site were counted. The resultant data were analyzed by regression analysis using the SAS General linear models procedure (PROC GLM, SAS; 1988, Version 6.03).

Monocyte chemotaxis assay, SDS-PAGE, superoxide anion assay, *N*-acetyl β-D-glucosaminidase assay, and tumor growth inhibition assay were performed as described (1, 3, 7, 8).

Results

Purification of MCAF. MCAF was purified to homogeneity from eight liters of conditioned media from a TNF-stimulated fibrosarcoma cell line, 8387, by sequential chromatography, including heparin-Sepharose affinity chromatography, gel filtration, carboxymethyl (CM)-HPLC, and reverse-phase (RP)-HPLC. The biological activities of each fraction was monitored by both in vitro monocyte chemotactic activity and β-glucosaminidase-releasing activity. On CM-HPLC, MCAF was eluted at ~0.3 NaCl, corresponding to two distinct peaks (CM fractions 25 and 26, data not shown). MCAF from each fraction was applied to RP-HPLC. As shown in Fig. 1, *a* and *b*, monocyte chemotactic activity and β-glucosaminidase-releasing activity coeluted at a discrete absorbance peak located in both RP-HPLC eluates. We have also confirmed that these two activities coeluted with monocyte cytostatic augmenting activity (RP-HPLC; Fig. 1), suggesting that all these activities are due to a single molecular species. SDS-PAGE analysis of the purified MCAF showed microheterogeneity in molecular mass at ~15 kD (Fig. 1 *c*). As shown in Fig. 1 *d*, all bands detected on SDS-PAGE analysis showed the immunological reactivity with MCAF on Western-blotting analysis. No endotoxin activity was detected by Limulus lysate assay (<0.05 ng/ml). Fractions from CM 25, RP 21-22 contained 10.6 μg protein, and

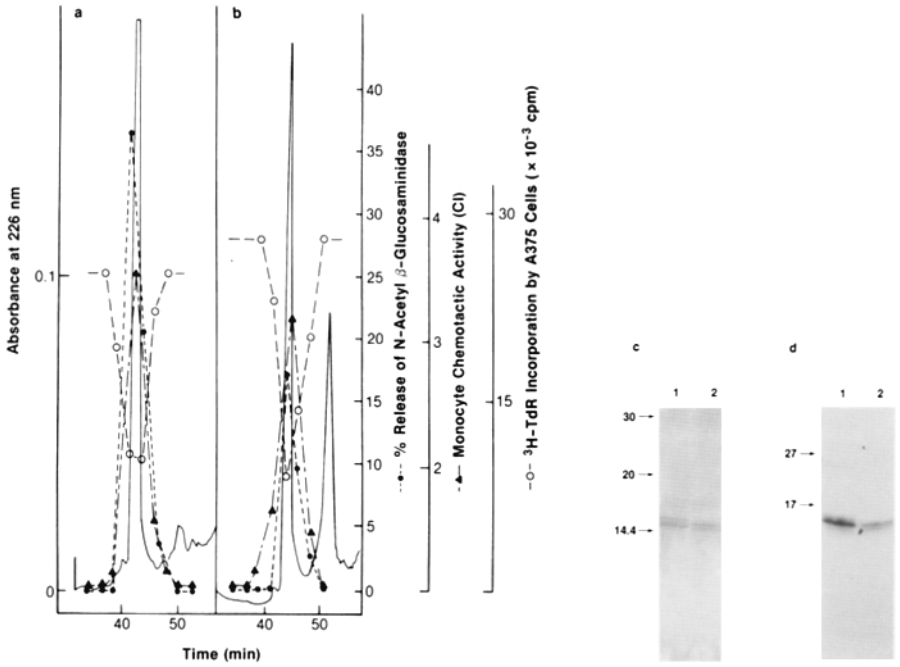

FIGURE 1. Monocyte chemotactic activity, *N*-Acetyl β-glucosaminidase release, and augmentation of monocyte cytostatic activity against tumor cells at a 1:1,000 dilution of fractions obtained from CM-HPLC (*a*) fraction 25 and (*b*) fraction 26; and run on a reverse-phase column. Absorbance is shown by the solid line. (*c*) SDS-PAGE of purified MCAF (*1*) CM 25, RP 21-22; and (*2*) CM 26, RP 22-23. Approximately 70–100 ng of protein was applied to a 15% polyacrylamide gel followed by silver staining. (*d*) Western blotting of the purified MCAF: (*1*) CM 25, RP 21-22; and (*2*) CM 26, RP 22-23.

fractions from CM 26, RP 22-23 contained 6.8 μg protein, based on amino acid composition analysis.

The identity of the purified MCAF from a human fibrosarcoma cell line with the previously purified MCAF from a human myelomonocytic cell line, THP-1, was further supported by specific inhibition of binding at 37°C of ^{125}I-labeled MCAF obtained from THP-1 to human PBMC by the unlabeled fibrosarcoma-derived MCAF (Fig. 2). Another basic polypeptide chemoattractant, IL-8, at concentrations from 0.1 ng/ml to 10 μg/ml, failed to inhibit the binding of ^{125}I-labeled MCAF to human PBMC at 37°C.

In Vitro and In Vivo Biological Activities of Purified MCAF. The purified MCAF from fraction CM 25, RP 21-22 and fraction CM 26, RP 22-23 was assayed for monocyte chemotactic activity, *N*-acetyl β-glucosaminidase release, and superoxide anion release. The half-maximal monocyte chemotactic response occurred at doses of ~1 ng/ml for both fractions, while the *N*-acetyl β-glucosaminidase release showed an EC_{50} of ~3–5 ng/ml (data not shown). MCAF was able to release superoxide anions at concentrations >20 ng/ml. MCAF exhibited no chemotactic activity for neutrophils at any concentration tested (data not shown).

To evaluate whether the purified MCAF also had in vivo cell recruitment effects,

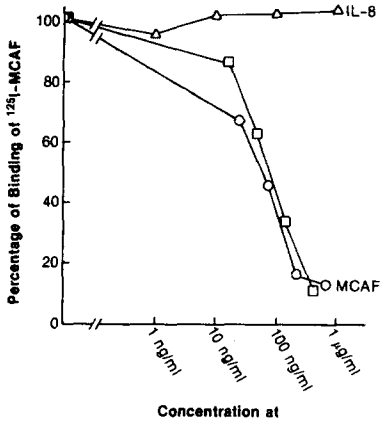

FIGURE 2. Competition for the binding of ^{125}I-MCAF (THP-1 derived) to human PBMC by unlabeled purified MCAF. (□) CM 25, RP 21-22 (10.6 μg/ml); (○) CM 26, RP 22-23 (6.8 μg/ml); and (Δ) rIL-8 at different dilutions was added together with 70,000 cpm (1 ng) ^{125}I-MCAF and incubated at 37°C for 15 min. The binding is expressed as a percentage of binding obtained with ^{125}-MCAF (THP-1 derived) alone (2,330 ± 187 cpm). The data shown are representative of two independent experiments.

serial dilutions of MCAF in endotoxin-free PBS (fraction CM 25, RP 21-22) were injected into the ears of Lewis rats at various times. MCAF showed selective monocyte/macrophage recruitment activity in vivo when assayed at 3, 6, and 18 h. There was a significant dose- and time-dependent accumulation of total leukocytes in the perivenular connective tissue underlying the intradermal injection sites of purified MCAF in the ears of Lewis rats. Dose-dependent accumulation was found for monocyte/macrophages (p = 0.002) but not for neutrophils (p = 0.18) or lymphocytes (p = 0.14) at each of the time points tested (data not shown). No monocyte infiltration was detected at 3 min, 30 min, or 90 min after injection, regardless of the dose of purified MCAF used (data not shown). Fig. 3 *A* shows the site of PBS injection at 18 h to contain a significant macrophage infiltrate, but a sparse infiltration of lymphocytes and neutrophils. Fig. 3 *B* shows that 5.3 ng/ml of MCAF induced a much more marked monocyte/macrophage infiltration at 18 h.

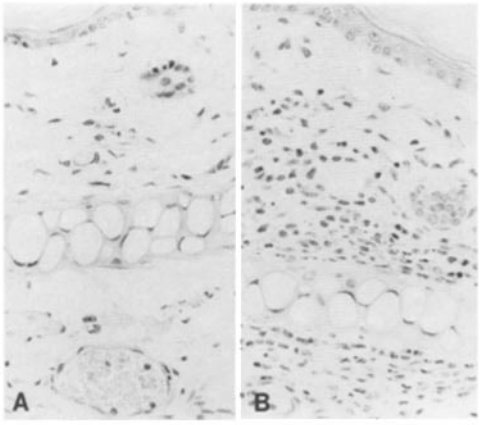

FIGURE 3. The histological appearance (×175) of rat ear dermis 18 h after injection of (*A*) 20 μl PBS and (*B*) 20 μl MCAF (CM 25, RP 21-22), at 5.3 ng/ml. PBS inoculation (*A*) results in variable degrees of margination and infiltration of neutrophils, lymphocytes, and monocytes, whereas MCAF inoculation (*B*) produces marked infiltration with monocyte/macrophages and few, if any, neutrophils or lymphocytes.

Discussion

It is well established that a large number of tumors are infiltrated with macrophages (9). The macrophages may either stimulate tumor growth through the production of growth factors or exert a suppressive effect through the production of "macrophage-activating factors," such as IFN-β, TNF, IL-1, and MCAF. A number of tumor-derived cell lines have been shown to produce monocyte chemotactic factors such as MCAF and related mediators (4). Such tumors are often infiltrated with macrophages in vivo, which is associated with a better prognosis (4). MCAF may contribute to inhibiting tumor growth by macrophages by attracting them to the tumor site and subsequently activating increased cytostatic activity. Since IL-1 and TNF induce MCAF production by certain tumor types as well as by normal fibroblasts and endothelial cells (3), the regulation of MCAF production in tumor cells, as well as in the connective tissues surrounding the tumor, may be regulated by locally produced IL-1 and TNF. Even though MCAF, unlike other cytokines such as IFN-γ and TNF, does not have a direct cytotoxic or cytostatic effect on tumor cells, MCAF may play an important role in control of local tumor growth either by its effect on monocytes or possibly by inducing other cytokines.

In this report, we have shown that MCAF induced *N*-acetyl β-D-glucosaminidase and superoxide release, supporting the concept that MCAF is not merely a chemoattractant for monocytes. Although the mechanism of augmentation of monocyte cytostatic activity against tumor cells by MCAF is not yet known, it may be mediated by lysosomal enzyme and/or superoxide anion release. IL-6, IL-1, and TNF are not responsible for this activity of MCAF, since MCAF does not induce mRNA expression for IL-1-α/β or TNF-α in monocytes in vitro, and antibodies to IL-1-α/β, TNF, and IL-6 did not block the cytostatic effect of MCAF (1).

We are also the first to show that MCAF has potent monocyte recruitment activity in vivo. This observation is consistent with and provides an explanation for previous reports of a correlation between monocyte chemotactic activity and macrophage content of tumors (4). Monocyte accumulation at the injection site of MCAF starts gradually at 3 h and increases up to 18 h. The potent in vivo monocyte recruitment activity of MCAF supports the hypothesis that MCAF is involved in the recruitment of monocytes to delayed-type hypersensitivity reaction sites.

We have also shown the specific binding of ^{125}I-MCAF to human PBMC at 37°C. Interestingly, another recently cloned chemoattractant, IL-8 (6), which has similar biochemical characteristics, did not compete with ^{125}I-MCAF in binding to monocyte-enriched mononuclear cells, suggesting that there are distinct receptors for MCAF on human monocytes. The binding of ^{125}I-MCAF does not occur at 4°C, but at 37°C, and starts rapidly with maximal binding after 15 min. The bound molecules are immediately internalized, as judged by treating cells with 0.1 M glycine-HCl, pH 3.0, 0.15 M NaCl buffered solution. This internalization can not be blocked by either ATP synthesis inhibitors (sodium azide; 2-4, dinitrophenol) or by transglutaminase inhibitors (bacitracin, monodansyl cadaverine). These phenomena are unusual for a polypeptide receptor, and, therefore, we have been unable to estimate the number or the binding affinity of the receptor for MCAF on any cell type.

Summary

A monocyte chemotactic and activating factor (MCAF) has been purified from TNF-stimulated 8387 human fibrosarcoma cell line-conditioned media. The purified

MCAF showed microheterogeneity yielding two bands on SDS-PAGE analysis. Fibrosarcoma-derived MCAF specifically competed with THP-1 (a human monocytic cell line)-derived ^{125}I-labeled MCAF in binding to human PBMC, whereas a similar basic heparin-binding leukocyte chemoattractant, IL-8, did not. The purified MCAF stimulated superoxide anion and *N*-acetyl β-D glucosaminidase-releasing activity in human monocytes, as well as monocyte cytostatic augmenting activity against tumor cells and chemotactic activity for monocytes. When injected subcutaneously into Lewis rat ears, the purified human MCAF also induced considerable in vivo local monocyte infiltration beginning at 3 h and becoming maximal at 18 h. In conclusion, the data presented in this paper indicate that MCAF is a potent activator of monocytes as well as a monocyte recruitment factor that acts through receptors that are specific for this novel molecule. This novel cytokine might have an important role in tumor growth control due to its ability to attract and activate monocytes.

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