Macrophage Stimulating Protein: Purification, Partial Amino Acid Sequence, and Cellular Activity

By Alison Skeel,* Teizo Yoshimura,* Stephen D. Showalter,† Shuji Tanaka,§ Ettore Appella,§ and Edward J. Leonard*

From the*Immunopathology Section, Laboratory of Immunobiology, National Cancer Institute, Frederick, Maryland 21702; the Program Resources Inc., NCI-Frederick Cancer Research Facility, Frederick, Maryland 21702; and the *Chemistry Section, Laboratory of Cell Biology, National Cancer Institute, Bethesda, Maryland 20892

Summary

Macrophage stimulating protein (MSP) was purified to homogeneity from human blood plasma by selection of biologically active fractions obtained by sequential immunoaffinity and high pressure liquid ion exchange chromatography. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis the molecular mass of MSP was 70 kilodaltons (kD); under reducing conditions two gel bands were seen, at 47 and 22 kD. The disulfide-linked two-chain structure of MSP was confirmed by separation of reduced and alkylated MSP chains. A computer search comparison of six partial sequences of MSP digests showed that MSP has not been recorded in data banks of protein sequences. Two MSP fragments had >80% identity in overlaps of 12–16 residues to sequences in the protein family that includes human prothrombin, plasminogen, and hepatocyte growth factor. The concentration of purified MSP required for half-maximal biological activity was the order of 10^{-10} M. In addition to making mouse resident peritoneal macrophages responsive to chemotactants, MSP caused the appearance of long cytoplasmic processes and pinocytic vesicles in freshly plated macrophages. MSP also caused phagocytosis via the C3b receptor, CR1. Whereas resident peritoneal macrophages bind but do not ingest sheep erythrocytes opsonized with IgM anti-Forssman antibody and mouse C3b, addition of MSP caused ingestion. Thus, MSP causes direct or indirect activation of two receptors of the mouse resident peritoneal macrophage, CR1 and the C5a receptor.

The inflammatory or cytotoxic activity of leukocytes is subject to complex regulation. A well-studied example is the mouse peritoneal macrophage, for which distinctions have been made between resident, inflammatory, and immunologically activated populations. Generation of a macrophage capable of tumor killing requires a priming signal that acts on an inflammatory macrophage, but not on a resident macrophage; this creates a transient state during which contact with the target cell initiates the cytotoxic response (1). Other examples abound, including neutrophil priming that increases the magnitude of an agonist-induced metabolic burst (2). We found that a stimulating signal was required for resident peritoneal macrophages from C3H mice to make a chemotactic response to C5a. Macrophages in RPMI-1640 medium did not migrate to C5a unless serum was added to the cell suspension (3). The stimulating effect of serum was concentration dependent, over a range of about 2 to 12% heat-inactivated serum. Sera from different mammals, including mice and humans, were effective. Fractionation of human serum for the stimulating effect led to partial purification of a protein (4), which we called macrophage stimulating protein (MSP)1. In addition to making resident macrophages responsive to C5a, MSP induced morphological changes within 1 h after macrophages were plated in tissue culture dishes: the cells assumed elongated shapes, with long processes, and increased numbers of vacuoles were evident.

The low concentration of MSP in serum precluded complete purification by conventional techniques. We have now achieved this by a combination of immunoaffinity and HPLC chromatography. Screening of partial sequences of MSP in the protein data bank (5) shows that MSP is a new entry on the list of described proteins.

Materials and Methods

MSP Bioassay. The assay for MSP is based on a concentration-dependent increase in the chemotactic response of resident perito-

1Abbreviations used in this paper: DTT, dithiothreitol; ElgMC3b, sheep erythrocytes opsonized with IgM anti-Forssman antibody and mouse C3b; HGF, hepatocyte growth factor; MSP, macrophage stimulating protein; VBS, veronal buffered saline.
neal C3H mouse macrophages to endotoxin-activated mouse serum (3). Macrophages obtained by peritoneal lavage with 7–8 ml of RPMI 1640 medium containing 2% BSA were centrifuged at 4°C for 10 min at 250 g and resuspended in RPMI 1640 without added protein at a cell concentration of 10^6/ml. Bottom wells of a multiwell chemotaxis chamber (6) were filled with chemotactant (a 1/200 dilution of endotoxin activated mouse serum [7]) and covered with a 10-μm thick polycarbonate membrane with 5-μm holes. After gasket and top plate were added to complete assembly of the chamber, upper wells were filled with 50 μl volumes of macrophage suspensions in RPMI 1640 medium containing the stimulating protein to be assayed. During the incubation period of 3 h at 37°C in humidified air with 5% CO₂, macrophages migrated through holes in the membrane and remained attached to the attractant side of the membrane. The chamber was then disassembled, and cells were washed away from the nonmigrated side of the membrane. After air drying and staining with Diff-Quik (American Scientific Products, McGaw Park, IL), macrophages were counted with an image analyzer (8). MSP fractions were assayed in duplicate, and results were expressed as the percentage of input macrophages that migrated. A unit of MSP per ml of test solution was defined as the reciprocal of the dilution required to induce ~30% of the maximal chemotactic response obtained at the plateau of the dose-response curve.

Isolation of MSP Antigen for Monoclonal Antibody Production. Purification of MSP from 3,600 ml of outdated frozen plasma (Blood Bank, Clinical Center, National Institutes of Health, Bethesda, MD) was based in part on published methods (4). Plasma was lyophilized and reconstituted to 1/3 of the starting volume. 8 g of Na₂SO₄ per 100 ml of concentrated plasma at 20°C were added to precipitate unwanted high mol wt proteins. Gelfiltration of the supernatant on Sephadex G-200 (Pharmacia LKB, Piscataway, NJ) yielded fractions with MSP activity in a region corresponding to a molecular mass of ~100 kD. MSP was further purified by DEAE-cellulose chromatography, flat bed isoelectric focusing, another G-200 gel filtration, and equilibration with formalin-fixed Cowan strain Staphylococcus aureus (Zymed Laboratories, San Francisco, CA) morpholino]propanesulfonic acid, pH 7.8. The concentration of guanidine HCl in the heavy and light acid analysis and SDS-PAGE.

Reduction and Pyridylethylation of MSP. A 4.5 nmol sample of HPLC-CM-2 MSP was dialyzed against 10 mm NH₄HCO₃, lyophilized and dissolved in 200 μl water. From amino acid analysis of a 5 μl sample of this material, there were 2 nmol or approximately 215 μg of total MSP. We lyophilized 180 μl and reconstituted the MSP in 25 μl of 6 M guanidine HCl, 0.5 M Tris HCl, pH 8.2. Approximately 6.5 μmol of dithiothreitol (DTT) in a volume of 50 μl of the guanidine-tris diluent was added. After a 4-h equilibration at 20°C, 19 μmol of iodoacetamide in a volume of 36 μl was added, and the solution was held at 20°C in the dark for 20 min. The reduced and alkylated MSP heavy and light chains were separated on a Superose 12 FPLC column (Pharmacia LKB) that was equilibrated with 6 M guanidine HCl, 0.05 M Tris HCl, pH 7.8. The concentration of guanidine HCl in the heavy and light chain pool fractions was reduced by ultrafiltration on a Centricon 10 filter and refiltered after addition of Tris buffer. Each sample was then dialyzed against 0.1% acetic acid, lyophilized, and reconstituted in 200 μl of 1% acetic acid. Aliquots were used for amino acid analysis and SDS-PAGE.

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Reduction and Alkylation of MSP. 4 ml of purified MSP (HPLC-CM-2 MSP) were dialyzed against 10 mm NH₄HCO₃, lyophilized and dissolved in 200 μl water. From amino acid analysis of a 5 μl sample of this material, there were 2 nmol or approximately 215 μg of total MSP. We lyophilized 180 μl and reconstituted the MSP in 25 μl of 6 M guanidine HCl, 0.5 M Tris HCl, pH 8.2. Approximately 6.5 μmol of dithiothreitol (DTT) in a volume of 50 μl of the guanidine-tris diluent was added. After a 4-h equilibration at 20°C, 19 μmol of iodoacetamide in a volume of 36 μl was added, and the solution was held at 20°C in the dark for 20 min. The reduced and alkylated MSP heavy and light chains were separated on a Superose 12 FPLC column (Pharmacia LKB) that was equilibrated with 6 M guanidine HCl, 0.05 M Tris HCl, pH 7.8. The concentration of guanidine HCl in the heavy and light chain pool fractions was reduced by ultrafiltration on a Centricon 10 filter and refiltered after addition of Tris buffer. Each sample was then dialyzed against 0.1% acetic acid, lyophilized, and reconstituted in 200 μl of 1% acetic acid. Aliquots were used for amino acid analysis and SDS-PAGE.
perose 12 column in pH 8.0, 0.05 M Tris-HCl containing 6 M guanidine hydrochloride. Separated heavy and light chain peaks were dialyzed against 0.1% acetic acid and then lyophilized.

Sequencing of Endopeptidase-Digested Pyridylethylated Heavy and Light Chains. The lyophilized pyridylethylated heavy and light chains were dissolved in 50 µl of 0.1 M ammonium bicarbonate, pH 9.5, containing 4 M urea. The chains were digested at 35°C by addition of 0.2 µg lysylendopeptidase, followed by another 0.2 µg after 8 h, for a total incubation time of 24 h. The digests were separated on a 0.21 x 3 cm BU-300 reverse phase column (Thomson Instrument Co., Springfield, VA). The larger, well-separated peaks were sequenced by N-terminal Edman degradation on an Applied Biosystems (Foster City, CA) 470A peptide sequencer equipped with an on-line 120A phenylthiohydantoin analyzer.

Measurement of IgMC3b Binding and Phagocytosis by Resident Mouse Peritoneal Macrophages. Reagents included sheep blood, anticoagulated with acid-citrate-dextrose; veronal buffered saline with 0.1% gelatin (VBS-gel): 0.14 M NaCl, 0.1 mM veronal, pH 7.4, 1 mM MgCl2, 0.15 mM CaCl2; rabbit IgM anti-Forssman antibody, kindly supplied by Dr. Tibor Borsos (Dept. of Pathology, Uniformed Service U. Health Science, Bethesda, MD); C5-deficient AKR mouse serum, stored at -80°C; DMEM; and ammonium chloride lysis buffer (0.16 M NH4Cl, 0.01 M KHCO3, 0.001 M EDTA, pH 7.4). Sheep erythrocytes opsonized with IgM anti-Forssman antibody and mouse IgC3b (IgMC3b) were prepared as follows: sheep E were washed twice with VBS-gel and suspended to a concentration of 2 x 10⁸ E/ml. To 1 ml E were added 0.1 ml IgM anti-Forssman antibody. After 45' at 37°C, the cells were washed and resuspended in 10 ml VBS-gel + 0.6 ml AKR mouse serum. After 20' at 37°C, the cells were washed 3 x in cold VBS-gel and then stored at 4°C in 4 ml VBS-gel.

For phagocytosis experiments, 0.5 ml aliquots of mouse resident peritoneal cells at a concentration of 8 x 10⁵ cells/ml DMEM without FCS (obtained as described for the MSP bioassay) were added to 24-well polystyrene plates (Costar, Cambridge, MA). Macrophages comprised about 70% of total peritoneal cells. After 1 h at 37°C, the plates were washed twice with DMEM, and then 0.5 ml ElgMC3b were added, followed by 0.1 ml of DMEM with different concentrations of MSP. The ratio of erythrocytes to peritoneal cells was 50:1. After incubation for specified intervals, nonadherent ElgMC3b were washed out of the wells, and 0.5 ml aliquots of ammonium chloride lysis buffer were added. This buffer was removed after 2.5 min, and the monolayers were stained with Diff-Quik. The bottoms of the wells were then cut out and mounted on glass slides so that cells could be examined with a 100x oil immersion objective.

Results

Purification of MSP by Immunoaffinity and CM-HPLC Chromatography. MSP was partially purified by passage of human plasma down a Sepharose monoclonal anti-MSP column. No MSP biological activity was found in the pass-through volume. After the column was washed with PBS to remove unbound protein, MSP was eluted with, pH 2.5, glycine buffer. This single step achieved an approximately 5,000-fold purification (Table 1). However, since the preparation showed a large number of bands in SDS-PAGE gels stained with Coomassie blue, MSP was further purified by CM-HPLC. As shown in the upper panel of Fig. 1, most of the macrophage stimulating activity coeluted with a well-defined A280 peak. This was the last major protein peak to be eluted by the NaCl gradient, which reflects the fact that during fractionation the isoelectric point of MSP shifts upward to a value that is high relative to most serum proteins (12). The small peak of biological activity that eluted earlier may represent MSP with a pI intermediate between the native value of 5.8 and the pI in 6 M urea of 7.6 (12). To minimize contaminating protein, the MSP peak was rechromatographed, as shown in the lower panel of Fig. 1. A summary of MSP purification and yield is shown in Table 1. Most of the starting plasma protein was removed in the immunoaffinity column step. How-

Table 1. Purification of MSP

| Protein* | MSP/protein† | Total MSP |
|----------|--------------|-----------|
| mg       | U/mg         | U         |
| 5 liters human plasma | 35 x 10⁴ | 6 | 2 x 10⁶ |
| Anti-MSP column eluate | 67 | 3 x 10³ | 2 x 10⁵ |
| HPLC-CM-1 eluate | 1.5 | 7 x 10⁴ | 10⁵ |
| HPLC-CM-2 eluate | 0.6 | 2 x 10⁵ | 10⁵ |

* A280
† Frozen aliquots from each fractionation step were thawed and assayed with a single preparation of mouse peritoneal macrophages. A unit of MSP/ml test solution is defined as the reciprocal of the dilution required to induce ~ 30% of the maximal bioassay response. Based on a Mr of 70 kD, 30% of the maximal bioassay response for HPLC-CM-2 MSP occurs at 6 x 10⁻¹¹ M.

Figure 1. HPLC-CM purification of MSP. (Upper panel) chromatography of MSP eluted from immunoaffinity column. See Materials and Methods for running conditions. (O) MSP activity of fractions. (Lower panel) rerun of MSP peak.
ever, as indicated by Fig. 1 and by the data in the table, MSP was only about 1% of the total anti-MSP column eluate protein. A 100-fold increase in specific activity was achieved by the two sequential HPLC-CM runs.

**Polyacrylamide Gel Electrophoresis of MSP.** SDS-PAGE of purified MSP on a 10-15% polyacrylamide gradient gel, followed by staining with Coomassie blue, showed a single band under nonreducing conditions and two bands under reducing conditions (Fig. 2). This indicated that MSP comprised two disulfide-linked chains (α and β). By comparison with Mr markers, estimates for native MSP, α chain and β chain were 70, 47, and 22 kD, respectively. From the specific activity of HPLC-CM-2 MSP (Table 1) and an approximate molecular mass of 70 kD, we estimated that the concentration of pure MSP required for a half-maximal response in the bioassay is the order of 10^-10 M.

**Table 2. Amino Acid Composition of MSP**

| Amino Acid | Native MSP | α Chain | β Chain |
|------------|------------|---------|---------|
| D          | 45         | 35      | 12      |
| E          | 65         | 39      | 24      |
| S          | 33         | 21      | 16      |
| G          | 117        | 78      | 54      |
| H          | 17         | 12      | 4       |
| R          | 33         | 26      | 9       |
| T          | 41         | 26      | 10      |
| A          | 59         | 40      | 17      |
| P          | 64         | 40      | 14      |
| Y          | 11         | 11      | 4       |
| V          | 52         | 25      | 16      |
| M          | 8          | 4       | 3       |
| I          | 13         | 7       | 6       |
| L          | 47         | 26      | 15      |
| F          | 18         | 14      | 5       |
| K          | 22         | 16      | 7       |
| Total      | 645        | 420     | 216     |

* Based on estimated molecular masses from SDS-PAGE of 70, 47, and 22 kD for native MSP, α chain and β chain.
Figure 4. Partial amino acid sequences of MSP. Sequences with asterisks (*) were compared in the protein data bank. Identity of residues in brackets ([ ]) was uncertain because of low yield. Dashes (—) represent undetermined residues. Amino acid letter codes in the second row of a sequence indicate a contaminating fragment in the purified material. The underlined sequence in α chain fragments BU-11 and BU-12 is a highly conserved kringle motif (see Discussion).

Figure 5. Effect of MSP on the chemotactic response of mouse resident peritoneal macrophages to C5a. Results are expressed as the percentage of input macrophages that migrated to the attractant side of the polycarbonate membrane. Error bars are SEM’s for triplicate cell counts of 1 mm² of membrane. Migration in the absence of MSP was 2%. The EC50 in this experiment was \(3 \times 10^{-10} \text{ M}\).

Table 3. MSP Stimulation of Ingestion of ElgMC3b

| Min. of incubation | 0 MSP | 10⁻⁹ M MSP |
|-------------------|------|-----------|
| 30                | 2 ± 0.3 | 52 ± 3 |
| 60                | 1 ± 0.3 | 60 ± 2 |
| 90                | 1 ± 0.3 | 60 ± 4 |
| 30                | 1 ± 0.3 | 48 ± 4 |
| 60                | 2 ± 0.3 | 48 ± 4 |
| 60                | 2 ± 0.3 | 63 ± 5 |
| 90                | 2 ± 0.6 | 54 ± 2 |

* To monolayers of mouse resident peritoneal macrophages were added ElgMC3b, with or without 10⁻⁹ M MSP. After the indicated times at 37°C, nonadherent erythrocytes were washed away; phase microscopy showed massive rosetting of erythrocytes to all macrophage monolayers. Adherent erythrocytes were then lysed, and monolayers were stained with Diff-Quick. Erythrocytes within macrophages survived the lysis buffer. Monolayers were examined with a 100 x oil immersion objective for the percentage of macrophages with at least one ingested erythrocyte; the mean ± the SEM of three 100-cell counts was calculated for each monolayer. Results are shown for two experiments.
Phagocytic activity ranged from 64% to 70% of macrophages caused 50-60% of the macrophages to ingest EIgMC3b. Phagocytosis was rapid; no significant increase in the percentage of macrophages with at least one ingested erythrocyte; the mean of three 100-cell counts was calculated for each monolayer. Results are shown for three experiments.

Discussion

Screening of MSP α and β chain partial sequence data in the protein data bank (5) shows that MSP is a newly described protein. The sequence similarities to portions of human prothrombin, plasminogen, and HGF are of great interest. Prothrombin, plasminogen, and the precursor form of HGF (18) are all single chain proteins which can be activated by cleavage at one site to form α and β chains. For example, conversion of plasminogen to active plasmin occurs when a single Arg-Val bond is cleaved, resulting in a two chain structure held together by a disulfide bond (19). In addition to their sequence similarities, common features of MSP, HGF, and plasmin include comparable Mr of the α and β chains, linkage of these chains by a single disulfide bond, and a valine residue at the N-terminus of each β chain. Another structural motif shared by plasmin, HGF, and prothrombin is a series of α chain triple disulfide loops, called kringles. The kringle repeats twice in prothrombin, four times in HGF and five times in plasminogen. The sequence NYCRNPD, which forms the base of one of the disulfide loops, is identically conserved in plasmin, HGF and prothrombin, except for F instead of Y in prothrombin (20). Our finding of this motif in the BU-11 and BU-12 fragments of the MSP α chain strongly suggests that there are kringles in the α chain, and provides added evidence for the relationship of MSP to this protein group. Since kringles are thought to mediate binding of plasmin to lysine on its target molecule, it will be of interest to determine if epsilon amino acid caproic acid (which inhibits binding of kringles to lysine residues [21]) inhibits MSP binding or biological activity.

The possible structural relationship of MSP to coagulation-fibrinolysis proteins suggests a mechanism for regulation of the potent activity of MSP, which is present in all normal mammalian plasma or serum. In analogy to prothrombin and plasminogen, MSP in circulating blood could be in the form of a single chain inactive precursor. When blood is shed, MSP could be cleaved by a proteolytic enzyme of the clotting cascade to form the disulfide-linked α-β chain active complex. In the intact organism, the source of an activating proteolytic enzyme could be the extrinsic coagulation pathway. For example, LPS causes macrophages to elaborate a procoagulant (22), which initiates activation of clotting cascade proteolytic enzymes.

We have shown that MSP activates mouse resident peritoneal macrophages to ingest bound EIgMC3b. This observation links MSP to an important body of literature on the activation of the C3b receptor (CR1) of peritoneal macrophages and cultured human blood monocytes (13-17, 23-25). The possible significance of CR1 activation has been outlined in an interesting discussion by Griffin and Griffin (13); it includes host defense against microbial pathogens, cellular injury in immune complex disease, and destruction of neoplastic cells.

At this early stage in our knowledge, the biological role of MSP relates to the direct or indirect activation of two distinct receptors, CR1 and the C5a receptor. In both cases, ligation of the receptor leads to a cellular response (phagocytosis or chemotaxis) if an additional stimulus is provided by addition of MSP. It is not necessary to add MSP before ligation of CR1. EIgMC3b that are bound to macrophage monolayers in the absence of MSP are rapidly ingested when MSP is added (our unpublished observations). It is likely that MSP affects a specific step in the stimulus-response pathway of ligated CR1 or C5a receptors. Since activation of CR1 by PMA is associated with phosphorylation of CR1, it will be of interest to determine if CR1 or C5a receptors are phosphorylated after activation by MSP.

We reported that stimulation of resident macrophages to become responsive to C5a could be achieved not only by low concentrations of MSP, but also by relatively high concentrations (1-10 mg/ml) of serum albumin (26). The effectiveness of albumin depended on the method of purification, suggesting that albumin may be a carrier for an activating stimulus. When chemotactic responses of resident macrophages were compared with those of inflammatory exudate peritoneal macrophages (induced by thioglycollate or serum), we found that resident macrophages could be stimulated by either MSP or albumin, but exudate macrophages were respon-
wise, Wright et al. (15) reported that fibronectin-mediated when monocytes become mature tissue macrophages. Likewise, Wright et al. (15) reported that fibronectin-mediated activation of human monocyte C3b receptors was observed only after at least 3 d in tissue culture. If MSP activates human monocyte C3b receptors and the effect depends on the maturational stage of the cells, binding studies with labeled MSP could determine whether MSP-responsiveness is due to appearance of a receptor or to a post-receptor event. We expect that MSP-responsive cells have a high affinity MSP receptor, in view of the low concentration of MSP [10^{-10} M] that elicits biological activity (Figs. 5 and 6).

We can now also begin investigations to determine the in vivo significance of MSP. Does it affect resident populations of the monocyte lineage — such as tissue macrophages, lung macrophages, Kupffer cells or osteoclasts? Does it play a role in granuloma formation? In view of its presence in circulating blood, is it made by hepatic cells, and could it be an acute phase protein? We are developing MSP-specific probes suitable for ELISA of MSP in plasma and for immunohistochemical localization of MSP to determine its cellular source and sites of action.

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12. Leonard, E.J., A. Skeel, and S. Allenmark. 1982. Electrophoresis only to albumin. Thus, MSP responsiveness is a marker for the resident macrophage. It does not occur in either mouse exudate macrophages (26) or in freshly isolated and washed human blood monocytes (our unpublished observations), both of which represent an earlier stage of mononuclear phagocyte development. These observations suggest that MSP responsiveness may be associated with a receptor that appears when monocytes become mature tissue macrophages. The function of which represent an earlier stage of mononuclear phagocyte development. These observations suggest that MSP responsiveness may be associated with a receptor that appears when monocytes become mature tissue macrophages. Likewise, Wright et al. (15) reported that fibronectin-mediated activation of human monocyte C3b receptors was observed only after at least 3 d in tissue culture. If MSP activates human monocyte C3b receptors and the effect depends on the maturational stage of the cells, binding studies with labeled MSP could determine whether MSP-responsiveness is due to appearance of a receptor or to a post-receptor event. We expect that MSP-responsive cells have a high affinity MSP receptor, in view of the low concentration of MSP [10^{-10} M] that elicits biological activity (Figs. 5 and 6).

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Address correspondence to Dr. Edward J. Leonard, National Cancer Institute, Frederick Cancer Research Facility, Bldg. 560, Rm. 12-71, Frederick, MD 21702.

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