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

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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 chemoattractants, 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-

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[1] *Abbreviations used in this paper:* DTT, dithiothreitol; EIgMC3b, 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 chemoattractant (a 1/200 dilution of endotoxin activated mouse serum [7]) and covered with a 10-μm thick polyvinylpyrrolidone-coated 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% CO_2, 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 wiped away from the nonmigrated side of the membrane. After air drying and staining with Diff-Quik (American Scientific Products, McGaw Park, IL), migrated cells 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 Na_2SO_4 per 100 ml of concentrated plasma at 20°C were added to precipitate unwanted high mol wt proteins. Gel filtration 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) to remove traces of IgG.

Immunization of Mice and Production of Monoclonal Anti-MSP. MSP, partially purified as described above and emulsified in CFA, was injected intraperitoneally into BALB/c mice. The amount of protein per injection was 280 μg. MSP in incomplete Freund's adjuvant was injected subcutaneously 2 wk later. The following week, serum from the mice was tested for anti-MSP activity by application to a minicolumn of Protein-A Sepharose and measurement of the capacity of the washed column to absorb applied MSP (9). 2 wk after this, 0.1 ml of MSP solution was injected intravenously; spleens of the two mice with the most anti-MSP serum activity were removed 4 d later. Fusion, cloning, and propagation of cell hybrids were done by published methods (10). Spleen cells were fused at a 5:1 ratio with NS-1 cells (11). Beginning at about 10 d, supernatants from the hybrid cells were tested by the immunoabsorbance assay (9) for anti-MSP activity. Cells from positive wells were cloned. Clones that produced IgG anti-MSP were passed intraperitoneally in mice, and IgG was purified from ascitic fluid on Protein-A Sepharose.

Purification of MSP by Immunoaffinity and HPLC Chromatography. Four 6 liter batches of outdated human plasma were used for large scale purification of MSP. A batch was thawed, filtered through sterile gauze, centrifuged at 20°C for 15 min at 10,000 rpm in a JA-10 rotor in a centrifuge (J2-21; Beckman Instruments, Fullerton, CA), filtered again through gauze to remove floating

lipid, and finally filtered through a glass fiber prefilter (Nalgene 280–5000; Nalge Co., Rochester, NY). The immunoaffinity column for the first purification step had a diameter of 2.5 cm and a bed height of 8 cm. It was packed with CNBr-Sepharose (Pharmacia LKB) that had been mixed with 240 mg of IgG monoclonal anti-MSP. Plasma was run through the column with a hydrostatic head of about 3 m, over a period of 4 h at 20°C. After the column was rinsed with 0.15 M NaCl, 0.5 M Tris buffer, pH 8.0, MSP was eluted with 0.1 M, pH 2.5 glycine buffer. Fractions of 5 ml were collected into tubes containing 1 ml of 0.5 M, pH 7.9, potassium phosphate buffer. The A_{280} peak, comprising about 110 ml, was dialyzed at 4°C overnight against 0.03 M NaCl, 0.02 M 3-[N-morpholino]propanesulfonic acid, pH 6.7, which was the starting buffer for CM-HPLC. It was centrifuged at 20,000 rpm in a Type 30 rotor of an ultracentrifuge (L8-70; Beckman Instruments) at 5°C, concentrated on an Amicon (Amicon Corp., Danvers, MA) YM-10 ultrafilter to 2 ml, dialyzed again against starting buffer, and filtered through a 5 ml, 0.45 μm Centrex unit (Schleicher and Schuell, Inc., Keene, NH). It was then applied through a 2-ml sample loop to a Beckman Spherogel-TSK CM-3SW dp 10 μm column (7.5 mm × 7.5 cm). Running conditions were flow of 1 ml/min, 2 min/fraction, A_{280} absorbance range of 1.28. After the flow-through, a linear gradient of NaCl in starting buffer was run to a limit concentration of 0.3 M. Fractions were assayed for MSP biological activity. We pooled and froze about 10 fractions across the MSP peak (HPLC–CM-1 MSP). After three more batches of plasma were processed, the four HPLC–CM-1 MSP peaks were pooled, dialyzed against HPLC-CM starting buffer, concentrated to 2 ml, dialyzed against starting buffer again, filtered, and chromatographed on the HPLC-CM column under the same conditions as the first run except for an A_{280} absorbance range on the chart recorder of 0.64. Fractions across the A_{280} peak were pooled (HPLC–CM-2 MSP).

Gel Electrophoresis. SDS-PAGE of MSP purified by immunoabsorption and HPLC-CM was in a Pharmacia Phastgel system (Pharmacia LKB) with a 10–15% gradient gel under both reducing and nonreducing conditions.

Reduction and Alkylation of MSP. 4 ml of purified MSP (HPLC–CM-2 MSP) were dialyzed against 10 mM NH_4HCO_3, 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.

Reduction and Pyridylethylation of MSP. A 4.5 nmol sample of HPLC–CM-2 MSP in 300 μl of 0.5 M Tris-HCl, pH 8.2, containing 6 M guanidine hydrochloride and 20% 2-propanol was reduced by addition of 100 μg DTT. After 4 h at room temperature, it was alkylated by addition of 30 μl of 10% 4-vinylpyridine in 2-propanol. After 3 h, pyridylethylated MSP was applied to a Su-

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 an 0.21 × 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 EIgMC3b 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 $MgCl_2$, 0.15 mM $CaCl_2$; 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 NH_4Cl, 0.01 M $KHCO_3$, 0.001 M EDTA, pH 7.4). Sheep erythrocytes opsonized with IgM anti-Forssman antibody and mouse C3b (EIgMC3b) were prepared as follows: sheep E were washed twice with VBS-gel and suspended to a concentration of 2 × 10^8 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× 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 × 10^5 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 EIgMC3b 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 EIgMC3b 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 100× 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 A_{280} peak. This was the last major protein peak to be eluted by the NaCl
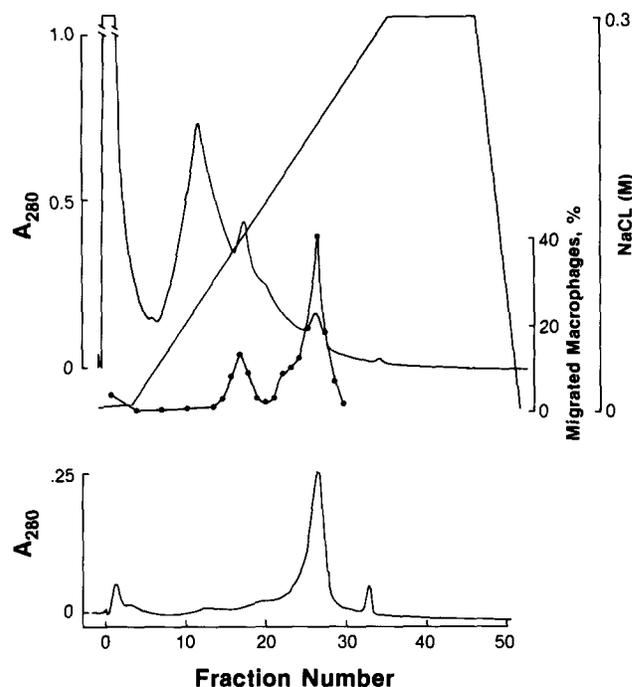
Table 1. *Purification of MSP*

|	Protein*	MSP/protein‡	Total MSP
	mg	U/mg	U
5 liters human plasma	35 × 10^4	6	2 × 10^6
Anti-MSP column eluate	67	3 × 10^3	2 × 10^5
HPLC–CM-1 eluate	1.5	7 × 10^4	10^5
HPLC–CM-2 eluate	0.6	2 × 10^5	10^5

* A_{280}

‡ 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 × 10^{-11} M.

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-

Figure 1. HPLC-CM purification of MSP. (*Upper panel*) chromatography of MSP eluted from immunoaffinity column. See Materials and Methods for running conditions. (●) MSP activity of fractions. (*Lower panel*) rerun of MSP peak.
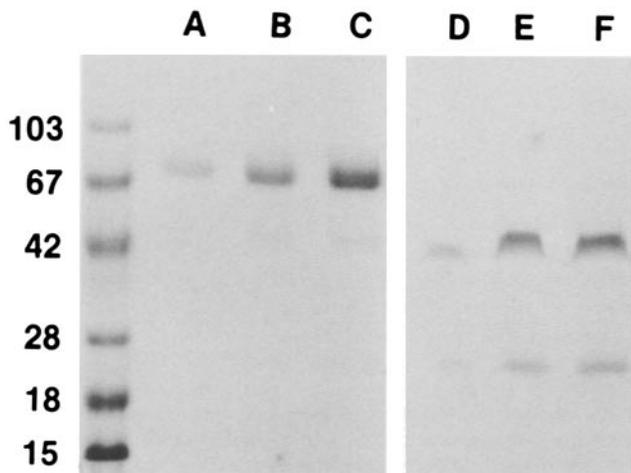
Figure 2. SDS-PAGE of purified HPLC-CM-2 MSP under nonreducing (lanes *A*, *B*, and *C*) and reducing (lanes *D*, *E*, and *F*) conditions. The amounts applied per lane were 125, 250, and 500 ng, respectively, for lanes *A*, *B*, and *C* and *D*, *E*, and *F*.

Figure 3. Separation on Superose 12 of reduced and alkylated MSP α and β chains in the presence of 6 M guanidine hydrochloride. See Materials and Methods for details.
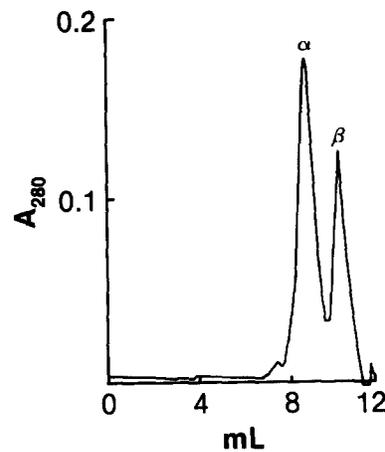
ever, as indicated by Fig. 1 and by the data in the table, MSP was only about 1% of the total anti-MSP column eluate protein. A 100-fold increase in specific activity was achieved by the two sequential HPLC-CM runs.

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

Table 2. *Amino Acid Composition of MSP*

Amino Acid	Residues/mole*		
	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.

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.

Amino Acid Composition and Partial Sequence Data for MSP. The first column of Table 2 shows the amino acid composition of native MSP, based on a molecular mass of 70 kD. After reduction and alkylation of MSP, α and β chains were separated on Superose 12, as shown in Fig. 3. Amino acid composition data for α and β chains are shown in the second and third columns of Table 2. The sums for α and β chains show reasonable agreement, for the most part, with values for native MSP.

A partial NH$_2$-terminal sequence of reduced and alkylated β chain was obtained, but NH$_2$-terminal blockage of the α chain precluded sequencing. Therefore the chains were digested with lysylendopeptidase and separated into a series of well-resolved peaks by reverse phase chromatography. Partial sequences of five fragments from each of the chains were determined (Fig. 4). The longest sequences comprised 23 residues. The sequence of one of the β chain fragments was identical to that of the undigested β chain N-terminus. Additional sequence information was obtained from MSP that was cleaved with cyanogen bromide. The six sequences with the fewest ambiguities (indicated by asterisks in Fig. 4) were compared with sequences in the protein data bank (5). Two of the six fragments had highly significant sequence similarities to members of a protein family that include prothrombin, plasminogen, and hepatocyte growth factor (HGF). Like MSP, these three proteins have α and β chains. The α chain fragment BU-12 had 81% identity in a 16 residue overlap with residues 348–363 of human HGF and 92% identity with residues 152–164 of bovine prothrombin. The BU-11 frag-

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MSP, cleaved by CNBr

              5         10        15        20
   CB-5: P L T G Y E V - L G T L F Q N P Q - G E[S]L


α-CHAIN, lysylendopeptidase digest

   *BU-7: G E G Y[E]G T A - T T T A G - P[E]Q E - D A Q -


   BU-8: F N - E A A G V E V N N A E Y -
         S     Y V D             G E

   *BU-11: F L D Q G L D D N Y C R N P D G S E -
                          W

   *BU-12: D L R E N F - R N P D G[S]E A P -


   BU-14: G V Q - Q R[W]S A E T[P]- - - Q F -


β-CHAIN, lysylendopeptidase digest

   *BU-5: C E I A G W G E T K

   *BU-9: V V G G H P G N[S]P W[T]V - L - N N Q -


   BU-12: F L P A G P D D N -[C]R N P D G Q -
               D Q A L

   BU-13: D L - E N F - - N P D G[S]E - -

   *BU-16: L E R S V T L N Q R V A L I C L P P E[E]Y V[V]-


β-CHAIN, alkylated

        V V G G - P G N S P -[T]V[S]L R N R -
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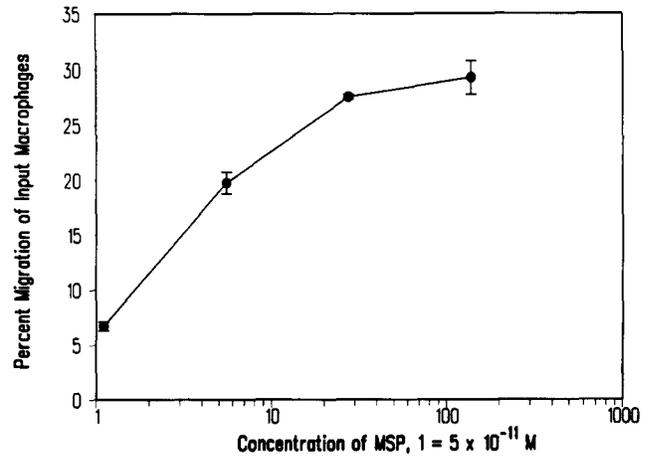
Figure 4. Partial amino acid sequences of MSP. Sequences with asterisks (*) were compared in the protein data bank. Identity of residues in brackets ([]) was uncertain because of low yield. Dashes (−) represent undetermined residues. Amino acid letter codes in the second row of a sequence indicate a 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 EC_{50} in this experiment was 3×10^{-10} M.

ment of the α chain had 83% identity with human plasminogen residues 505–516. The residues of the three related proteins with similarity to the MSP α chain fragments are all located in the α chains of these proteins. The 10-residue β chain fragment BU-5 corresponded, with three mismatches, to β chain residues 689–698 of human plasminogen.

Effects of MSP on Mouse Resident Peritoneal Macrophages. As noted in the Introduction, a stimulating signal was required for resident peritoneal macrophages from C3H mice to make a chemotactic response to C5a. The signal was present in mammalian serum, and a bioassay based on the stimulating effect was used for MSP purification. Fig. 5 shows this effect as a function of the concentration of pure MSP in the macrophage cell suspension. Whereas macrophages in RPMI 1640 did not migrate to C5a, the addition of MSP made them responsive. The EC_{50} was ∼5×10^{-11} M MSP.

The effect of MSP on resident peritoneal macrophage responsiveness to C5a led us to explore another receptor-mediated motility event that also requires an activating signal. Although EIgMC3b bind avidly to the C3b receptor (CR1) of mono-

cytes or resident peritoneal macrophages, binding is not followed by phagocytosis unless an additional signal (a T cell lymphokine (13), PMA (14), fibronectin (15), serum amyloid P (15), or laminin (16)) is provided, or unless cells are activated in vivo by induction of a peritoneal exudate (17). Table 3 confirms that mouse resident peritoneal macrophages do not ingest EIgMC3b (17), and shows that MSP stimulated ingestion. Whereas not more than 2% of resident macro-

Table 3. *MSP Stimulation of Ingestion of EIgMC3b*

Min. of incubation	Percentage of cells with ingested EIgMC3b	
	0 MSP	10^{-8} 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 EIgMC3b, 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 × 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.
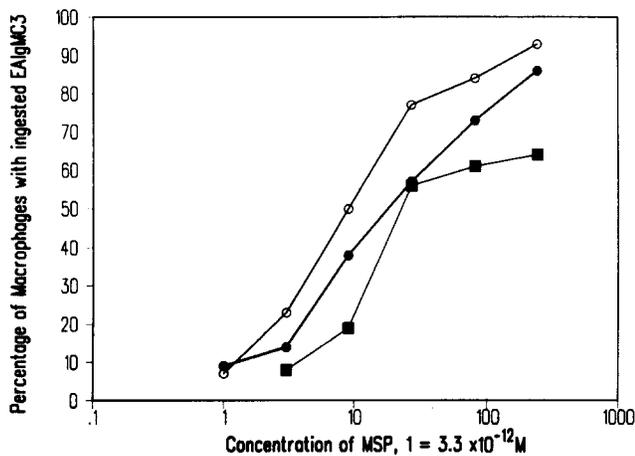
Figure 6. Effect of different concentrations of MSP on phagocytosis of EIgMC3 by mouse resident peritoneal macrophages. To macrophage monolayers were added EIgMC3, with or without MSP. After 1 h at 30°C, nonadherent erythrocytes were washed away; phase microscopy showed massive rosetting of erythrocytes to all macrophage monolayers. Adherent erythrocytes were then lysed, and monolayers were stained with Diff-Quik. Monolayers were examined with a 100× oil immersion objective for 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.

phages adherent to polystyrene tissue culture plastic ingested bound EIgMC3b, addition of MSP with the erythrocytes caused 50–60% of the macrophages to ingest EIgMC3b. Phagocytosis was rapid; no significant increase in the percentage of phagocytic macrophages was found after 30 min. Fig. 6 shows the percentage of macrophages with ingested erythrocytes as a function of MSP concentration. The peak percentage of phagocytic macrophages ranged from 64% to 93% in the three experiments, and the mean EC_{50} was 3×10^{-11} M.

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-

sive 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. 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 ap-

pearance 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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