Alpha 1-antichymotrypsin is the human plasma inhibitor of macrophage ectoenzymes that cleave pro-Macrophage Stimulating Protein

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Running title: α1-antichymotrypsin inhibits macrophage ectoenzymes
Summary

Macrophage Stimulating Protein (MSP)\(^1\) is secreted as 78 kDa single chain pro-MSP, which is converted to biologically active, disulfide-linked \(\alpha\beta\) chain MSP by cleavage at \(\text{Arg}_{483}\)-\(\text{Val}_{484}\). Murine resident peritoneal macrophages have two cell surface proteolytic activities that cleave pro-MSP. One is a pro-MSP convertase, which cleaves pro-MSP to active MSP; the other degrades pro-MSP. The degrading protease is inhibited by soybean trypsin inhibitor or by low concentrations of blood plasma, which allows the convertase to cleave pro-MSP to MSP. Using pro-MSP cleavage as the assay, we purified the inhibitor from human plasma. The bulk of the plasma protein was removed by salting out and by isoelectric precipitation of albumin. Highly purified inhibitor was then obtained in three steps: dye-ligand binding and elution, ion exchange chromatography, and HPLC gel filtration. After SDS-PAGE and transfer to a polyvinylidene membrane, N-terminal sequencing of the product identified it as \(\alpha_1\)-antichymotrypsin. The mean concentration of \(\alpha_1\)-antichymotrypsin in human plasma is 7 \(\mu\)M. At this concentration, \(\alpha_1\)-antichymotrypsin inhibits both macrophage enzymes. A concentration of 0.4 \(\mu\)M, which is in the expected concentration range in extracellular fluid, preferentially inhibits the degrading enzyme, which allows for cleavage to active MSP by the pro-MSP convertase.

\(^1\)Abbreviations. ACT: \(\alpha_1\)-antichymotrypsin; MSP: Macrophage Stimulating
Protein; STI: soybean trypsin inhibitor.
Introduction

Macrophage Stimulating Protein (MSP) is a pleiotropic 78 kDa growth and motility factor that is structurally related to several proteins of the coagulation system (1, 2). MSP acts on a number of cell types including tissue macrophages, epithelia, and hematopoietic cells (3). Actions on macrophages include stimulation of motility (4), induction of phagocytosis of serum complement-coated erythrocytes (1), and inhibition of the upregulation of NO synthase by inflammatory stimuli (5, 6). MSP induces adhesion, motility and replication of epithelial cells, and it can prevent the apoptosis that occurs when epithelial cells are prevented from attachment to a substrate (7). MSP mediates its effects by binding to and activating a cell receptor tyrosine kinase known as RON in humans (8, 9) and STK in mice (10, 11).

Human MSP is a disulfide-linked heterodimer comprising an α and β chain with molecular masses (not including N-linked carbohydrate) of 53 and 25 kDa, respectively. It is a member of a plasminogen-related family of proteins characterized by multiple copies in the α chain of a highly conserved triple disulfide loop structure (kringle). The kringle protein family includes plasminogen (12), prothrombin (13), urokinase (14), and hepatocyte growth factor/scatter factor (HGF/SF) (15, 16). These proteins are secreted as single-chain precursors, which have no biological activity until the protein is cleaved into α and β chains by specific serine proteases at an Arg-X bond (17), in which X is most frequently Val. The αβ chain junction of MSP is Arg483-Val484 (18). The β chain of MSP is homologous to the β chain catalytic domain of the serine protease members of the kringle family.
However MSP and HGF/SF are devoid of proteolytic activity, because of amino acid substitutions of the three β chain protease active site residues His, Asp and Ser. They are thought to have evolved from an ancient coagulation protein (19) to become growth and motility factors, while retaining the protease-dependent activation mechanism of the zymogens of the family.

Like kringle proteins of the coagulation system, MSP is constitutively synthesized by hepatic parenchymal cells and is secreted into the circulating blood as biologically inactive pro-MSP. The mean concentration of pro-MSP in the plasma of a series of normal human subjects is 5 nM (20). The level is not changed in the course of an acute phase reaction (21). To act on target cells in extravascular sites, pro-MSP must diffuse into tissues and be proteolytically cleaved to the biologically active disulfide-linked αβ-chain heterodimer (22). The EC50 for the action of the MSP heterodimer on macrophages is 0.25 nM (1).

The R483-V484 scissile bond of pro-MSP is a typical cleavage site for trypsin-like serine proteases. Several such proteases of the coagulation system, including factors XIa and XIIa and serum kallikrein cleave pro-MSP to active MSP in vitro (23). However, cleavage is minimal when blood clots, indicating that pro-MSP is not a preferred substrate for these enzymes (22). We have described two pro-MSP convertase activities in extravascular sites, one in wound fluid exudates (20) and the other associated with murine resident peritoneal macrophages (22).

Cleavage of pro-MSP by resident peritoneal macrophages involves a
minimum of two surface proteolytic activities: one, a pro-MSP convertase, cleaves pro-MSP to active MSP; the other degrades pro-MSP (22). When pro-MSP is added to macrophages in culture wells in serum-free medium, the activity of the degrading enzyme predominates. However, if soybean trypsin inhibitor (STI) is present, the degrading enzyme is preferentially inhibited, and the pro-MSP convertase cleaves the protein to active MSP, as shown by SDS-PAGE of the products and by induction of characteristic shape changes in the macrophages. The effect of STI could be reproduced by adding human or mouse serum to the cultures. A number of human serum protease inhibitors, including α1-antitrypsin, C1-inhibitor, anti-thrombin III and α2-macroglobulin, had no effect on macrophage cleavage of pro-MSP (22). Identification of the serum inhibitor and approaches to determining its physiological role are the subjects of this communication.
Experimental Procedures

Reagents and chromatography columns. Recombinant human pro-MSP and MSP were supplied by Drs. W. Yoshikawa and T. Takehara of Toyobo, Osaka, Japan. Soybean trypsin inhibitor (STI) was from Boehringer Mannheim, Indianapolis, IN. AEBSF and Bovine albumin, Fraction V, Fatty Acid-poor, Nuclease- and Protease-free were from Calbiochem, Palo Alto, CA. Alpha-1 antichymotrypsin was from Athens Research and Technology, Athens, GA. ¹²⁵I Bolton Hunter Reagent (NEX120) and Enlightning were from NEN Life Science Products, Boston, MA. Serotonin was from Sigma-Aldrich. [³H] dextran, 70,000 kDa, was from Amersham. Affi Gel blue gel was from BioRad, Hercules, CA. Immobilon-P transfer membranes (polyvinylidene fluoride) were from Millipore/Waters, Bedford, MA. LeukoStat stain was from Fisher Scientific. HPLC-DEAE, Protein Pak DEAE5PW was from Millipore/Waters. HPLC TSK-3000, TSK gel, Type G3000SW was from Tosohaas, Montgomeryville, PA.

Labeling of human pro-MSP with ¹²⁵I. Ten micrograms of pure recombinant human pro-MSP in 30 µl of 0.1 M borate buffer, pH 8.5, were added to 250 µCi of ¹²⁵I-labeled Bolton-Hunter reagent and equilibrated on ice for 60 min. The reaction was terminated by addition of 300 µl of borate buffer containing 0.5 M glycine. After 10 min on ice the reaction mixture was applied to an Excellulose GF-5 desalting column (Pierce, Rockford, Illinois) that had been equilibrated with
PBS containing 0.25% gelatin. The iodinated protein was eluted with 1 ml of PBS-gelatin buffer, and counted in gamma-counter (Gamma 5500, Beckman). The specific activity of the labeled protein was approximately 400 Ci/mmol.

Partial degradation of iodinated pro-MSP occurred during storage at –20°C, as shown by the appearance on SDS-PAGE of a band that migrated to about the same position as the MSP α-chain and a band corresponding to small fragments (Figure 2, lane C). To detect the capacity of the macrophage pro-MSP convertase to cleave pro-MSP to the αβ-chain heterodimer, we used 125I-pro-MSP stored for a limited time (Figure 4) or freshly prepared (Figure 5).

**Assay for pro-MSP cleavage by macrophages.** Resident peritoneal macrophages were obtained from C3H/HeN male mice by lavage of the peritoneal cavity with DMEM containing 2% fetal bovine serum. Cells were washed twice and resuspended in DMEM without serum at a concentration of 8x10⁵ total cells/ml. Wells of a 96 well microtiter plate (Costar 3596, Corning, Inc., Corning, NY) were loaded with 200 µl volumes of cell suspension. After 30 min for cell settling and adherence, well fluid was replaced with 50 µl DMEM containing 1 mg/ml of nuclease-protease-free BSA, about 150 fmoles of 125I-pro-MSP, and fractions being tested for inhibition of pro-MSP cleavage by macrophages. The plate was incubated at 37°C for 3-4 hrs. Proteins of the well fluids were separated by SDS-PAGE under reducing conditions on a
polyacrylamide gel. MSP, pro-MSP and BioRad pre-stained protein standards were also applied to the gel as reference markers. Radioautographs of the dried gel were made to determine the presence of $^{125}$I-pro-MSP cleavage products.

For determination of morphological effects of pro-MSP cleavage products, murine resident peritoneal cells in DMEM were distributed into wells of a 48-well tissue culture plate (400,000 cells/well). After incubation at 37°C for 1 hr, well fluids were replaced by 400 µl DMEM containing 3 nM MSP as a positive control or 5 nM pro-MSP with protease inhibitors being tested. The plate was incubated for 3-4 hrs, and then the adherent cells were stained with LeukoStat for microscopic evaluation and photography.

**Protease inhibitor activity in mouse peritoneal fluid.** Into the peritoneal cavity of a series of mice, we injected 4 ml of 0.15M NaCl with or without 4x10⁻⁵M serotonin (5HT). After an interval of 1 minute for the NaCl mice and 30 minutes for the 5HT mice, the animals were killed by cervical dislocation and fluid was aspirated from the peritoneal cavity. The recovered peritoneal cavity washout fluids were concentrated by ultrafiltration. $A_{280}$ was measured before and after ultrafiltration, and fluids were tested for capacity to affect the pattern of $^{125}$I-pro-MSP cleavage by mouse peritoneal macrophages. After incubation, reaction products were resolved by SDS-PAGE and visualized by radioautography.

To estimate the volume of fluid in the peritoneal cavity of normal mice or
30 min after intraperitoneal serotonin, we injected 0.5 ml of 70,000 kDa $[^3]$H dextran into a series of 3 mice each. After massage of the abdomen, the mice were killed and peritoneal fluid was aspirated and counted in a scintillation counter. From a comparison of the aspirate cpm and the injected cpm, we calculated that the mean peritoneal fluid volume was $140 \pm 30 \mu l$ (s.e.m.) for normal mice and $900 \pm 30 \mu l$ 30 min after intraperitoneal serotonin. The $900 \mu l$ figure for the serotonin-treated mice was obtained after subtraction of the injected serotonin volume. From the recovered $A_{280}$ in the peritoneal cavity washouts and the pre-washout peritoneal fluid volumes, we calculated values for pre-washout $A_{280}$.

**Initial steps to isolate inhibitor from human plasma.** To 200 ml of pooled outdated human blood plasma were added 53 gm (NH$_4$)$_2$SO$_4$ to make a molarity of 1.5. The slurry was stirred at 20°C for 30 min, and centrifuged at 25,000 x g for 30 min. The precipitate was discarded. To precipitate albumin from the supernatant, 0.3N HCl was added until the pH was 4.1. The precipitate was removed by centrifugation, and the supernatant was ultrafiltered on an Amicon YM-10 filter from a volume of about 250 ml to 150 ml. The solution was dialyzed at 4°C against water to remove the (NH$_4$)$_2$SO$_4$ and then against 0.02M pH 7.1 sodium phosphate buffer.
**Column chromatography of albumin-free inhibitor.** The 150 ml of albumin-free inhibitor solution was applied to a 3x23 cm column containing 75 ml of Affi Gel Blue gel. After unbound protein was washed through the column, bound protein was eluted with 1.4M NaCl. Total $A_{280}$ of the pooled eluate was 26% of the applied $A_{280}$. The 100 ml eluate was dialyzed against 0.02M, pH 8.0 tris buffer and concentrated on an Amicon YM 10 and Centricon 10 filter to a volume of about 3 ml, and an $A_{280}$ of 42. A 2.5 ml volume of this solution was applied to an HPLC-DEAE column, and bound proteins were eluted with a NaCl gradient from 0 to 0.3M in starting buffer over a period of 60 min. Fractions with inhibitor activity were pooled, ultrafiltered to a volume of 2.5 ml, dialyzed against 0.02M, pH 8.0 tris buffer and rechromatographed on the HPLC DEAE column under the same conditions. In the final chromatography step, the peak DEAE inhibitor fraction was dialyzed against DMEM and applied to an HPLC TSK 3000 gel filtration column.
Results

Initial purification steps. A flow chart that outlines the initial steps in purification of the inhibitor is shown in Figure 1. The rationale was to separate the bulk of the plasma proteins from the inhibitor prior to final purification. In the first step, about 70% of the total A$_{280}$ was precipitated at a concentration of 1.5M (NH$_4$)$_2$SO$_4$, leaving the inhibitor and plasma albumin in the supernatant. Exploratory experiments showed that in two different high resolution fractionation trials, Affi-gel blue dye-ligand chromatography and ion exchange chromatography, albumin and the inhibitor behaved similarly. Therefore, albumin was removed by precipitation near its isoelectric point at pH 4.1, leaving the inhibitor in the supernatant. The inhibitor was then purified by sequential chromatography on Affi-gel blue and HPLC DEAE columns as outlined in Methods.

Final purification and analysis. A relatively large amount of protein was eluted from the HPLC-DEAE column just prior to the inhibitor. To minimize admixture of this protein, pooled inhibitor fractions were equilibrated with starting buffer and re-run on the HPLC-DEAE column (DEAE-2). Proteins were eluted with a linear NaCl gradient. A range of fractions from 38 to 54, corresponding to a portion of the NaCl gradient from 0.15M to 0.23M was assayed for capacity to inhibit proteolysis of $^{125}$I-pro-MSP by macrophages. SDS-PAGE and radioautography after incubation with macrophages showed that proteolysis of pro-MSP was inhibited by fractions in the 44-48 range (Fig. 2).
SDS-PAGE under reducing conditions of fractions 44-48 showed a major band at about 66 kDa and a number of less intense bands over the 14 to 97 kDa marker range (data not shown). To determine if the major band was the inhibitor, we applied DEAE-2 fraction 44 to an HPLC TSK-3000 gel filtration column. Figure 3 shows maximal inhibitor activity in fractions 35 and 36, which corresponded to a protein peak that eluted at the position of serum albumin (68 kDa). After SDS PAGE of the peak fractions, the gel was lightly stained with Coomassie blue to locate the 68 kDa band (data not shown). This was repeated with a pool of fractions 35-37, the protein band was transferred to immobilon P, and a piece of membrane corresponding to the 68 kDa band was cut out for N-terminal sequencing. The sequence of the first 15 N-terminal residues [NSPLDEENLTXENXD] had 87% identity to a 15 residue sequence of human α1-antichymotrypsin. Travis et al reported that the apparent molecular mass of purified human α1-antichymotrypsin on SDS-PAGE gels was 68 kDa (24).

**Alpha1-antichymotrypsin preferentially inhibits the macrophage pro-MSP degrading enzyme.** The inhibition of macrophage pro-MSP cleavage by α1-antichymotrypsin is shown in Figure 4. After a control incubation of $^{125}$I-pro-MSP without cells for 180 min, SDS-PAGE under reducing conditions shows by radioautography a predominant 78 kDa band that represents uncleaved pro-MSP (Fig. 4, lane 6). The small band below it reflects a pro-MSP degradation product that occurs on storage of the iodinated protein. In all other lanes, which
are radioautographs of pro-MSP after incubation with macrophages, the two bands of interest are at 53 kDa and 46 kDa. The 53 kDa band corresponds to the $\alpha$ chain of MSP, generated by the macrophage pro-MSP convertase; the 46 kDa band is a fragment generated by the macrophage pro-MSP degrading enzyme (22). Without inhibitors of the macrophage enzymes, the 46 kDa degradation product band is more intense than the 53 kDa MSP $\alpha$-chain band (Fig. 4 lane 1). STI, a selective inhibitor of the degrading enzyme, allows exclusive cleavage by the macrophage pro-MSP convertase to MSP (Fig. 4 lane 2, 53 kDa band). The action of 3 different concentrations of $\alpha_1$-antichymotrypsin is shown in Figure 4, lanes 3-5. Lane 3 shows detectable inhibitor action at a concentration of 0.07 $\mu$M as shown by an increase, compared to lane 1, in the 78 kDa and 53 kDa bands and a decrease in the 46 kDa band and small fragments (F). These changes are more pronounced for 0.4 $\mu$M $\alpha_1$-antichymotrypsin (lane 4). At a concentration of 5 $\mu$M, $\alpha_1$-antichymotrypsin almost completely inhibited both enzymes, as shown by the large amount of uncleaved pro-MSP (lane 5) and a band pattern comparable to the incubation control (lane 6).

**Macrophage protease inhibitor activity in mouse peritoneal fluid.** The above data show that over the concentration range from 0.07 $\mu$M to 5 $\mu$M $\alpha_1$-antichymotrypsin preferentially inhibited the macrophage pro-MSP degrading enzyme at the low end of the range and completely inhibited both the degrading enzyme and the convertase at 5 $\mu$M, a value that approaches the mean human
serum concentration of 7 µM (25). Thus, the effect of this inhibitor on macrophage proteolytic activity in extravascular sites will be critically dependent on its concentration. Using the peritoneal cavity as an accessible extravascular site, we assayed inhibitor activity of peritoneal fluid from normal mice and from mice treated with intraperitoneal serotonin to induce a moderate increase in vascular permeability. The results are shown in Figure 5. Lane 1 shows almost complete degradation of 125I-pro-MSP by macrophages in medium alone. Three percent mouse serum (A280 = 1.2) is in the partial inhibition range, which reveals cleavage by the convertase to MSP (53 kDa line) and by the degrading enzyme to the 46 kDa product (lane 2). Fluid washed out of the peritoneal cavity and ultrafiltered to an A280 of 2.2 (lane 3) also allowed partial cleavage to MSP (53 kDa line). Lane 4 shows the effect of concentrated (A280 6.1) peritoneal fluid from serotonin-treated mice. It minimized activity of the pro-MSP degrading enzyme (note low amount of the fragment band) and favored the action of the convertase as shown by the relative sizes of the 53 and 46 kDa bands.

**Inhibition of the macrophage pro-MSP degrading enzyme allows sufficient cleavage of pro-MSP to MSP to stimulate macrophage shape change.**

Pro-MSP, with or without protease inhibitors, or MSP as a positive control, was added to murine peritoneal macrophages in tissue culture wells, and the cells were periodically observed by phase microscopy during a 4 hr incubation at
37°C. In the presence of pro-MSP without protease inhibitors (Fig. 6A), macrophages did not change their rounded morphology, characteristic for cells in DMEM alone or DMEM and protease inhibitors without pro-MSP (not shown). MSP induced macrophages to become elongated in shape (Fig. 6B). Similar macrophage morphology was observed in wells with pro-MSP in the presence of STI (Fig. 6C) or α1-antichymotrypsin (Fig. 6D). These findings correlate with the data in Figure 4, showing that inhibition of the degrading enzyme by STI or α1-antichymotrypsin resulted in conversion of pro-MSP to MSP.
**Discussion**

We reported that murine resident peritoneal macrophages have two cell surface proteolytic activities that can cleave pro-MSP. One cleaves pro-MSP to active MSP, the other degrades pro-MSP (22). The degrading protease was inhibited by STI and by normal human or mouse serum. It was not inhibited by a number of plasma protease inhibitors, including $\alpha_1$-antitrypsin, C1-inhibitor, anti-thrombin III and $\alpha_2$-macroglobulin (22). We have now shown that the inhibitor in human plasma is $\alpha_1$-antichymotrypsin. It is therefore probable that the macrophage pro-MSP degrading enzyme is a serine protease with chymotrypsin or cathepsin-G like substrate preferences, which cleaves at sites with aromatic or large aliphatic residues. Inasmuch as the initial major macrophage inactivating cleavage product of pro-MSP is a 46 kDa fragment ((22) and Fig. 4, lane 1), our results suggest that this fragment is formed by cleavage at Phe$_{418}$, which would yield a polypeptide with a calculated mass of 45.7 kDa. Phe$_{418}$ is located within the triple disulfide loop structure of kringle 4, and therefore release of the fragment would require reduction. Consistent with this prediction, the amount of 46 kDa fragment was minimal after SDS-PAGE under non-reducing conditions (data not shown).

Inhibition of the macrophage pro-MSP degrading enzyme by STI (Fig. 4, lane 2) led to the expectation that the degrading enzyme would be a trypsin-like serine protease. It was thus a surprise to find that the serum inhibitor of the enzyme is $\alpha_1$-antichymotrypsin. A review of the literature showed that STI can
bind and inhibit chymotrypsin as well as trypsin. In fact, the inhibitor has two
binding sites, one for trypsin or chymotrypsin and another for chymotrypsin (26).

Since the macrophage pro-MSP convertase generates active MSP, the
cleavage site is expected to be Arg483-Val484, the αβ chain junction (18). This is a
preferred cleavage site for trypsin-like serine proteases. However, the inhibitor
profile for the macrophage pro-MSP convertase is unusual, in that it is not
inhibited by various inhibitors of trypsin-like serine proteases, including
leupeptin, aprotinin, FPR-chloromethylketone, FFR-chloromethylketone (data
not shown), and STI. And it is completely inhibited, along with the pro-MSP
degrading enzyme, by 5 µM α1-antichymotrypsin, a preferential inhibitor of
chymotrypsin-like proteases. Purification and characterization of the
macrophage pro-MSP convertase would therefore be of interest. It is possible
that the convertase and the degrading activities reflect dual specificities of a
single protein. An example is human cathepsin G, which has both trypsin- and
chymotrypsin-like specificities (27), and binds both trypsin and chymotrypsin
inhibitors (28). In addition to its well-known location in leukocyte azurophilic
granules, cathepsin G with dual specificity has been detected on cell membranes
isolated from the U-937 human promonocytic cell line (29). These cells, with or
without the maturational stimulus of phorbol esters, had no protease activity for
pro-MSP (data not shown).

Like the coagulation factors from which MSP is thought to have evolved (19),
regulation of its activity depends on proteolytic cleavage of the single chain
precursor pro-MSP, which is present in human circulating blood at a concentration of 5 nM (20), about 20x the EC50 of 0.25 nM (1). If pro-MSP and α1-antichymotrypsin diffuse from the circulation into the extravascular space, inhibition of pro-MSP degradation by tissue macrophages might allow the macrophage pro-MSP convertase to generate active MSP, which could act on cells expressing the RON MSP receptor, including macrophages themselves as well as epithelial cells (30).

Using the murine peritoneal cavity as an example of extravascular space, we showed that peritoneal fluid from normal or serotonin-treated mice could preferentially inhibit the macrophage pro-MSP degrading enzyme and allow cleavage by the convertase to MSP (Fig. 5, lanes 3 and 4). From the A280 of peritoneal washouts and determination of pre-washout peritoneal fluid volume by radioactive dextran dilution (as described in Methods), we estimated that the mean A280 in peritoneal fluid of normal and serotonin-treated mice was 6.8 ± 0.6 and 4.4 ± 0.3 respectively for a series of three mice each. The A280 of the fluids tested in the experiment illustrated in Figure 5 was 2.2 for lane 3 and 6.1 for lane 4. These data suggest that in both normal mice and mice with a moderate increase in vascular permeability, the concentration of inhibitor in this extravascular space is in the range that can inhibit proteolytic degradation of pro-MSP by macrophages, and allow generation of MSP by the pro-MSP convertase.
In contrast, since the concentration of plasma proteins in acute, severe inflammatory exudates approaches that of proteins in the circulation, the mean α1-antichymotrypsin concentration of 7 µM (25) is in the range that completely inhibits macrophage pro-MSP convertase activity (Fig. 4, lane 5). Mature MSP is present in these exudates (20), generated by a unique fluid phase pro-MSP convertase, purification of which is in progress. In contrast to the exudate fluid phase convertase, the potential significance of the macrophage pro-MSP convertase may be to generate MSP locally to act on cells expressing the RON receptor under physiological conditions, in the absence of inflammation.

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Figure Legends

Figure 1. Flow chart for initial purification of inhibitor. Fractions with inhibitor activity are in the right leg of the flow chart.

Figure 2. Purification of inhibitor on anion exchange column. Re-run of DEAE-1 inhibitor pool on HPLC DEAE column (DEAE-2). Assay of fractions for capacity to inhibit cleavage of pro-MSP by macrophages. After $^{125}$I-pro-MSP was incubated with murine macrophages, reaction products were resolved by SDS-PAGE in an 8% gel under reducing conditions. Arrows on right point to Pro-MSP, a 53 kDa marker, and small mass proteins not resolved on the 8% gel (F). Lane C: control incubation without macrophages. Macrophages caused disappearance of the pro-MSP band unless inhibitor was present in fractions. Fraction 44 was selected for final purification, illustrated in Figure 3.

Figure 3. Final purification of inhibitor. Gel filtration of DEAE-2 inhibitor fraction 44 on an HPLC TSK-3000 column. Assay of fractions for capacity to inhibit cleavage of $^{125}$I-pro-MSP by macrophages. SDS-PAGE in an 8% gel under reducing conditions. Maximal inhibitor activity was detected in the A$_{280}$ elution peak, which was at the serum albumin marker location (fraction 35). Densitometry values in relative units for the pro-MSP band in fractions 33-39 were 3, 16, 21, 21, 18, 16 and 13 respectively.
Figure 4. Effect of STI and ACT on cleavage of pro-MSP by macrophages.

After \(^{125}\text{I}\)-pro-MSP was incubated with murine macrophages, reaction products were resolved by SDS-PAGE in a 10% gel under reducing conditions. Lane 6: 180 min incubation control without cells; all other lanes were from 180 min incubations with macrophages. Lane 1: no inhibitor. Lanes 3-5: 0.07 \(\mu\text{M}, 0.4 \mu\text{M}\) and 5 \(\mu\text{M}\) \(\alpha\)1-antichymotrypsin respectively. Lane 2 (from a different gel of the same experiment): 5 \(\mu\text{M}\) STI. Molecular mass markers are on the left. F: small fragments.

Figure 5. Effect of peritoneal fluid from normal or serotonin-treated mice on cleavage of pro-MSP by macrophages. Into the peritoneal cavity of a series of mice, we injected 4 ml of 0.15M NaCl with or without serotonin (5HT) as described in Methods. The recovered fluids were concentrated by ultrafiltration and tested for capacity to affect the pattern of \(^{125}\text{I}\)-pro-MSP cleavage by mouse peritoneal macrophages. After incubation, reaction products were resolved by SDS-PAGE and visualized by radioautography. Macrophage incubation medium composition was as follows. Lane 1: diluent. Lane 2: 3% mouse serum. Lane 3: (from a different gel): normal peritoneal fluid, \(A_{280}\) 2.2. Lane 4: 5HT peritoneal fluid, \(A_{280}\) 6.1. Lane 5: \(^{125}\text{I}\)-pro-MSP incubated without cells. Molecular mass markers are on the left. F: small fragments.
Figure 6. Morphology of macrophages in the presence of MSP, pro-MSP, or pro-MSP plus protease inhibitors. After 4 hrs in culture, cells were stained and observed by phase microscopy with a 40x objective. 

A: 5 nM pro-MSP; B: 3 nM MSP; C: 5 nM pro-MSP and 5 μM STI; D: pro-MSP and 0.4 μM ACT;
200 ml human plasma

1.5M (NH₄)₂SO₄

Ppt  Supernatant

Ppt (albumin)  pH 4.1  Supernatant

Pass-through  1.5 M NaCl eluate

Affi-gel blue  HPLC-DEAE

Inhibitor pool

Figure 1
Figure 2
Alpha1-antichymotrypsin is the human plasma inhibitor of macrophage ectoenzymes that cleave pro-macrophage stimulating protein—
Alison Skeel and Edward J. Leonard

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