Published in final edited form as:

Circulation. 2012 October 30; 126(18): 2227–2235. doi:10.1161/CIRCULATIONAHA.112.123968.

Endogenous and Natural Complement Inhibitor Attenuates Myocardial Injury and Arterial Thrombogenesis

Vasile I. Pavlov, MD¹, Mikkel-Ole Skjoedt, PhD², Ying Siow Tan, BS¹, Anne Rosbjerg, B Sci², Peter Garred, MD, DMSc², and Gregory L. Stahl, PhD¹

¹Center for Experimental Therapeutics and Reperfusion Injury, Department of Anesthesiology, Perioperative and Pain Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston MA

²Laboratory of Molecular Medicine, Department of Clinical Immunology-7631, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark

Abstract

Background—Coagulation disorders and reperfusion of ischemic myocardium are major causes of morbidity and mortality. Lectin pathway initiation complexes are composed of multimolecular carbohydrate recognition subcomponents and three lectin pathway specific serine proteases. We have recently shown that the lectin pathway specific carbohydrate recognition subcomponent mannose-binding lectin (MBL) plays an essential role in the pathophysiology of thrombosis and ischemia/reperfusion injury. Thus, we hypothesized that the endogenous MBL associated protein, MAP-1, that inhibits complement activation *in vitro* also could be an *in vivo* regulator by attenuating myocardial ischemia/reperfusion injury and thrombogenesis when used at pharmacologic doses in wild type mice.

Methods and Results—MAP-1, in two mouse models, preserves cardiac function, decreases infarct size, decreases C3 deposition, inhibits MBL deposition and prevents thrombogenesis. Further, we also demonstrate that MAP-1 displaces MASP-1, MASP-2 and MASP-3 from the MBL complex.

Conclusions—Our results suggest that the natural, endogenous inhibitor, MAP-1 effectively inhibits lectin pathway activation *in vivo*. MAP-1 at pharmacologic doses represents a novel therapeutic approach for human diseases involving the lectin pathway and its associated MASPs.

Keywords

myocardial infarction; coagulation; infarction; immunology

INTRODUCTION

The innate immune response is a 'perfect' system, which through evolution makes the seminal decision to respond against foreign pathogens¹. Host defense to foreign invaders is mediated by a repertoire of innate immune molecules and receptors able to recognize pathogen-associated molecular patterns (PAMPs), including bacterial surface mannans and glycans, LPS and bacterial DNA CpG motifs^{1,2}. Components of innate immunity can

Correspondence: Gregory L. Stahl, PhD, Center for Experimental Therapeutics and Reperfusion Injury, Harvard Institutes of Medicine, HIM 845A, 77 Avenue Louis Pasteur, Boston, MA 02115, Phone: (617) 525-5021, Fax: (617) 525-5026, gstahl@zeus.bwh.harvard.edu.

DISCLOSURES: MOS and PG are listed as inventors on a patent application about the use of MAP-1 as an anti-inflammatory agent. The other authors declare no conflicts of interest.

recognize self-tissue following various insults observed in human disease (i.e., during autoimmunity, transplant rejection, and allergy), as well ²⁻⁴. Recent evidence from our laboratories shows that in addition to mannose and N-acetylglucosamine PAMPs, mannose-binding lectin (MBL) an initiation molecule in the lectin complement pathway (Fig. 1) recognizes endogenous ligands, resulting in induction of inflammatory mediators, tissue injury, vascular remodelling and thrombogenesis in several models of human disease *in vivo* ⁴⁻¹⁰.

Initiation molecules involved in lectin complement pathway activation in humans include MBL and ficolins (i.e., ficolins-1, -2, and -3), whereas in mice MBL-A and -C are the preeminent initiation molecules ¹¹. Each of these initiation molecules are associated with serine proteases termed mannose-binding lectin/ficolin associated serine proteases (i.e., MASPs 1, 2, and 3), which are involved in the direct activation of C4 and C2 in the lectin complement pathway and conversion of pro-Factor D to Factor D and cleavage of factor B in the alternative complement pathway ¹²⁻¹⁵. The direct activation of the alternative pathway has so far only been clearly documented in rodents. Moreover, they have also been shown to be involved in cleavage of prothrombin to thrombin, cleavage of fibrinogen, activation of FXIII, and cleavage of kininogen (Fig. 1) ¹⁶⁻²¹. Inhibition of MBL or MASP-2 protects against ischemia/reperfusion injury, whereas the natural endogenous inhibitor C1-inhibitor, which also inhibits MASPs in addition to other enzymes (i.e., C1r/s, factor XIIa and kallikrein) also preserves myocardial function following ischemia/reperfusion ^{7;9;10;22;23}. Thus, initiation molecules of the lectin complement pathway and their associated MASPs are involved in the activation of multiple biologic pathways involved in human disease. Specific inhibition of the lectin pathway has the functional capacity to regulate and attenuate many different biologic pathways and cascade systems *in vivo*.

Additional truncated protein variants of the MASPs are also associated with MBL and ficolin complexes, including small MBL-associated protein (sMAP or MAP19) ²⁴, and MBL/ficolin-associated protein-1 (MAP-1) ²⁵ also named MAP44 ²⁶. MAP-1 and sMAP are alternative splice variants originating from the *MASPI* and *MASP2* genes, respectively, and lack the serine protease domains. MAP-1 displaces MASP-2 and inhibits MBL- and Ficolin-3-dependent complement activation *in vitro* ²⁵, while no conclusive function has been attributed to sMAP. Thus, we hypothesized that MAP-1 is an inhibitor of the lectin pathway *in vivo* and could be used as a pharmacological inhibitor of MASP mediated diseases. In the present study, we investigated the use of MAP-1 as an inhibitor of lectin pathway activation in two different *in vivo* models that activate MASP-1 and/or MASP-2 and are MBL-dependent.

METHODS

All procedures were reviewed and conducted according to the Institute's Animal Care and Use Committee. All experiments were performed under the standards and principles set forth in the Guide for Care and Use of Laboratory Animals, published by National Institute of Health (NIH Publication No. 85-23, Revised 1996).

Animals

C57BL/6 (WT) mice (8–12 weeks old, Taconic Farms) were used as background controls for genetically modified MBL null mice, as described previously ^{7;8}. The following groups were investigated in the *in vivo* studies: 1) WT, 2) MBL null, 3) WT + bovine serum albumin (BSA; control protein at 500 µg/mouse; ip), 4) WT + MAP-1 (300 or 500 µg/mouse; ip), 5) MBL null + rhMBL (30 µg/mouse; ip), 6) MBL null + rhMBL (30 µg/mouse;

ip) + MAP-1 (160 µg/mouse; ip). Mice were housed 4 per cage and had unlimited access to water and standard mouse chow.

Competition ELISA assay

Mannan (Sigma-Aldrich M7504) was immobilized on Maxisorp ELISA plates (Nunc, Denmark) at 10 µg/ml overnight at 4°C and served as a ligand for MBL. The plates were washed and blocked in TBS with 0.05% Tween-20 and 2 mM CaCl₂ before incubation with 0.5 µg/ml rMBL for 2 hours at 20°C. In three different experimental settings, serial dilutions of rMAP-1 and rMASP-1, rMASP-2 or rMASP-3, respectively, were mixed in non-adsorbent 96 well plates preceding 2 hours co-incubation at 20°C on the rMBL/mannan ELISA plates.

Hereafter, immuno-detection was used to assess the binding of rMASP-1, rMASP-2 or rMASP-3 to rMBL. Binding of rMASP-1 and rMASP-3 was detected with 0.5 µg/ml of a monoclonal antibody (mAb) F3-46 reacting with a shared epitope of MASP-1 and -3, but does not cross-react with MAP-1. Similar results were also obtained with a series of other specific MASP-1/-3 mAbs. The mAb producing hybridomas were generated as described previously²⁷. Incubation of the primary mAbs was done overnight at 4°C. A signal was obtained with 45 minutes incubation of HRP-conjugated rabbit anti-mouse IgG at 1:1500 (P0260, Dako, Denmark) and 15 minutes development using ortho-phenylene-diamine (Dako, Glostrup/Denmark). The enzyme reaction was stopped with 1M H₂SO₄. Optical density levels were measured using a V-max Kinetic-reader (Molecular Devices, U.S.A.).

Detection of rMASP-2 binding to rMBL was performed with an anti-MASP-2 rat mAb 8B5 (Hycult Biotechnology, Netherlands) as described above, with the exception that HRP-conjugated rabbit anti-rat (P0450, Dako, Denmark) was used as a secondary antibody.

Recombinant proteins

For these assays we used in-house generated human recombinant proteins that were all expressed in CHO-DG44 cells (rMBL, rMASP-1, rMASP-2, rMASP-3 and rMAP-1) as previously described^{25,27}. MAP-1 used in the animal experiments was also produced by the same technique, while recombinant human MBL used in the animal experiments was a gift from Enzon, Inc. USA.

Murine MI/R model

The murine MI/R model was performed using modifications as described^{7;9;28}. Briefly, mice were anesthetized with sodium pentobarbital (60 mg/kg) for intubation, then ventilated with positive pressure on a SAR Small Animal Ventilator (Model 683, Harvard Apparatus, Holliston, MA, USA) and maintained under anesthesia with isoflurane (1.7 MAC). The chest was opened through a sternotomy and the chest wall retracted using a 5-0 black-braided silk suture. An 8-0 black-braided silk suture (USSDG, Norwalk, CT) was passed beneath the left anterior descending (LAD) coronary artery approximately 2 mm from the tip of the left atrium. A 1–2 mm piece of 0-0 suture (Deknata, Fall River, MA) was placed on the LAD, and the ligation tightened to occlude the artery. After 45 minutes of ischemia, the ligation was loosened and the 0-0 suture removed. Drainage (20GA I.V. Catheter, BD Insyte) was placed through the skin beneath the sternum. The chest was closed using a 5-0 black-braided silk suture (Ethicon). The skin was sutured using a 5-0 black-braided silk suture (Ethicon). The drainage catheter was removed from the thoracic cavity after air was removed. The animal was removed from the respirator and allowed to reperfuse for 4 hours. EKG changes (i.e., ST segment) were monitored and used to establish ischemia and reperfusion.

Infarct size measurements

Following reperfusion, mice were anesthetized with sodium pentobarbital. The chest cavity was opened and the LAD coronary artery ligation retightened. The heart was flushed retrograde through the thoracic aorta with 500 μ l of PBS and then perfused with 200 μ l of 5% Brilliant Blue G (Acros). Hearts were excised and cross-sectioned from base to apex into 1-mm slices using a coronal acrylic matrix (Roboz). Sections were incubated in 1% triphenyltetrazolium chloride (Acros) at 37°C for 15 minutes as described^{7;10;29}. Following triphenyltetrazolium chloride staining, sections were fixed in 10% formalin (Sigma-Aldrich) at 4°C overnight. Each section of the heart was imaged using a Nikon SMZ800 stereoscopic zoom microscope, digital SPOT Insight camera (Diagnostic Instruments, Inc. USA), and areas calculated using Image J software (National Institutes of Health, Bethesda, MD). Infarct size was determined by calculating total areas of left ventricular free wall, infarcted tissue, non-ischemic tissue, and ischemic area at risk (AAR). AAR was not significantly different between groups (data not shown).

Echocardiography

Additional mice underwent experimental MI/R as described above. Transthoracic echocardiographic measurements were used to evaluate cardiac function as described^{7;9;28;30}. Echocardiography was performed 4 hours after reperfusion using a Philips Sonos 5500 (Andover, MA, USA) with a 7–15 MHz probe. Ejection fraction (EF) was calculated via long axis length and short axis area measurements of the left ventricle (LV) during systole and diastole as described^{7;9;28;30}.

Collection of Blood and Tissue

Following reperfusion and echocardiography, the chest cavity was opened, the inferior cava vein cut and blood collected from the thoracic cavity. Hearts were excised and embedded in OCT and frozen in liquid nitrogen cooled 2-methylbutane.

Ferric chloride (FeCl₃) coagulation model

The mouse model of localized thrombus formation was used as described^{6;21;31;32}. Mice were induced and maintained with isoflurane anesthesia and placed in a supine position. An incision was made and the right common carotid artery exposed by blunt dissection. Carotid blood flow was measured with a Doppler flow probe (Transonic) as described^{6;21}. Whatman filter paper saturated with 3.5% FeCl₃ was applied to either side of the carotid artery, proximal to the Doppler flow probe. The filter paper was removed 3 minutes after 3.5% FeCl₃ application and the carotid blood flow measured continuously for 30 minutes. Carotid arteries were removed, embedded in OCT and frozen in liquid nitrogen cooled 2-methylbutane.

Histology and Immunohistochemistry

Frozen sections of carotid arteries (5 μ m) were fixed with 4% paraformaldehyde for 10 minutes, rinsed with PBS, and followed by incubation for one hour with monoclonal rat anti-mouse MBL-A and MBL-C (Hycult Biotech, The Netherlands; 1:100 in PBS/0.05% Triton X-100 supplemented with 1 mM CaCl₂). To control for non-specific staining, isotype control antibody (i.e., rat IgG, Vector Laboratories, Burlingame, CA) was used in place of the rat anti-mouse MBL-A and MBL-C mAbs. After a brief rinse, slides were incubated with biotinylated polyclonal rabbit anti-rat IgG (Dako, Carpinteria, CA; 1:600 in PBS for 45 minutes). Tissue sections were incubated with a Vectastain ABC-AP kit (Vector Laboratories) and MBL was detected with a Vector Red alkaline phosphatase substrate kit (Vector Laboratories). All images were captured using a Nikon Eclipse E400 microscope and analyzed using SPOT imaging software (Diagnostic Instruments).

Myocardial sections (7 μ m) were fixed with acetone for 10 minutes, rinsed with PBS and followed by one hour incubation with polyclonal goat anti-mouse C3 (MP Biomedicals, Solon, OH; 1:500 in PBS/0.05% Triton X-100). After a brief rinse, C3 deposition was detected using donkey anti-goat IRDye800 (Rockland Immunochemicals, Gilbertsville, PA; 1:2000 in PBS for one hour). C3 deposition was visualized using an Odyssey infrared imaging system (LI-COR, Lincoln, NE) and analyzed using Image J software (National Institutes of Health, Bethesda, MD).

MBL and C3 deposition fluorochrome immunosorbent assay (FLISA)

MBL and MBL-dependent C3 deposition on mannan-coated 384 microtiter plates was performed as previously described³³. Human sera (2%) were incubated with vehicle (VBS), anti-MBL mAb (3F8, 10 μ g/ml) or MAP-1 (5 or 10 μ g/ml) for 1 hr at 37°C and was then placed in mannan-coated wells and processed for MBL and C3 deposition as described³³. An additional FLISA assay was also performed by replacing the mannan with N-acetylglucosamine-BSA (GlcNAc-BSA) as the MBL ligand coated to the wells as described¹⁰. Experimental groups for the GlcNAc-BSA FLISA were vehicle (VBS), D-mannose (30 mM) or MAP-1 (1, 5 and 10 μ g/ml). Results from the GlcNAc-BSA FLISA assay were processed the same way as the mannan FLISA assay. Background integrated intensity (II) from the Odyssey readings in both assays consisted of wells coated with VBS only and subtracted from all groups. All groups were performed in triplicate and mannan FLISA was repeated three times (N=3) and the GlcNAc-BSA FLISA was repeated 5 times (N=5).

Statistical Analysis

All statistical analysis was performed using SigmaStat software (SPSS, Chicago, IL, USA). Data are presented as means $\pm$ SEM. Normality and equal variances were checked in each statistical analysis and the one way ANOVA followed by the Student-Newman-Keuls test was used to establish significance between groups in all Figures with the following exceptions. One way repeated measures ANOVA followed by the Holm-Sidak test was used to compare groups in the FeCl₃ study. The t-test was used to compare the two WT groups in the analysis of infarct area. C3 deposition on N-acetylglucosamine-BSA plates was analyzed by a Kruskal-Wallis one way ANOVA on Ranks, followed by the Dunn's method to find differences between groups. $P < 0.05$ was considered statistically significant.

RESULTS

MAP-1/MASP-1, -2 and -3 compete for MBL binding

We investigated the direct competition for MBL binding using checkerboard dilutions of rMAP-1 together with rMASP-1, -2 or -3. The MAP-1/MASP preparations were co-incubated on the MBL/mannan surface in order to mimic the *in vivo* situation of the formation of an MBL complex with associated proteins on a natural ligand surface. Similar to all three MASPs, we observed a clear tendency that MAP-1, in concentrations above 80 ng/ml, inhibited MASP binding to MBL (Fig. 2). We observed a very strong dose-dependent inhibitory effect of MAP-1 with an almost complete inhibition of the MASP binding in concentrations above 7 μ g/ml. We also assessed the inhibition range in MAP-1 concentration < 30 ng/ml, where no inhibition was observed. At concentrations above 20 μ g/ml, no additional inhibitory effect was evident (Fig. 2).

MAP-1 preserves myocardial function

Hearts from MBL null mice are protected from MI/R induced loss of cardiac function compared to WT mice⁷. In contrast, MBL null mice developed a WT phenotype and

significantly decreased myocardial ejection fraction following MI/R (Fig. 3A) when given rhMBL similar to that previously observed⁷. MAP-1 (160 µg/mouse) significantly prevented MI/R induced loss of cardiac function in rhMBL (30 µg/mouse) supplemented MBL null mice (Fig. 3A, left panel). Ejection fractions following MI/R in WT mice treated with MAP-1 were significantly higher compared to WT mice treated with saline or a control protein, BSA (Fig. 3A, right panel). We observed a non-significant difference in the ejection fraction in WT mice following MI/R when using 500 µg MAP-1/mouse compared to 300 µg MAP-1/mouse (Fig. 3B). The data demonstrate a MAP-1 induced protection from MBL induced loss of cardiac function following MI/R.

MAP-1 reduces infarct size

We also investigated whether MAP-1 protects the myocardium from infarction following MI/R. As we have previously demonstrated, MBL null mice have very small myocardial infarctions following MI/R (Fig. 4A and 4C)⁷. Addition of rhMBL (30 µg/mouse) to MBL null mice increased myocardial infarction size compared to MBL null mice (Fig. 4C). MAP-1 treatment (160 µg/mouse) of MBL null mice supplemented with rhMBL significantly protected mice from myocardial infarction compared to MBL null + rhMBL mice. Similarly, WT mice treated with control protein (i.e., BSA) undergoing MI/R displayed larger myocardial infarctions compared to MAP-1 treated WT mice (Fig. 4B and 4C). Thus, the natural, endogenous inhibitor, MAP-1, preserves myocardial function, as well as myocardial tissue from the tissue damage associated with MI/R.

MAP-1 prevents complement activation following MI/R

Complement activation following MI/R results in C3 deposition via an MBL complex and MASP-2 dependent mechanism^{7,23}. WT mice treated with MAP-1 displayed significantly less C3 deposition following MI/R compared to WT mice treated with control protein (i.e., BSA; Supplementary Fig. 1 online). Thus, as previously demonstrated *in vitro*^{25,26}, MAP-1 also prevents MBL and MASP-2 dependent complement activation *in vivo* following MI/R.

MAP-1 prevents occlusive arterial thrombogenesis

Previous studies have demonstrated that FeCl₃-induced occlusive thrombogenesis is mediated by the MBL complex and MASP-1 *in vivo*^{6,21}. WT + BSA mice developed occlusive thrombogenesis and succession of carotid artery blood flow 15 minutes following application of 3.5% FeCl₃. WT mice treated with MAP-1 (500 µg/mouse) demonstrated no decrease in carotid artery blood flow following FeCl₃ application. These results demonstrate that MAP-1 significantly prevents coagulation *in vivo* (Fig. 5).

Carotid arteries were harvested to evaluate MBL deposition on the arterial wall as demonstrated previously⁶. MBL-A and MBL-C deposition were observed on the arterial wall of WT mice treated with BSA (Fig. 6, left panel). In contrast, WT mice treated with MAP-1 (500 µg/mouse) displayed significantly reduced deposition of murine MBL and the absence of a thrombus within the artery compared to BSA treated WT mice (Fig. 6, right panel). A lack of staining was observed in isotype control stained arteries, demonstrating the specificity of the mAb rat anti-mouse MBL-A/-C staining (Fig. 6, middle panel). These data support the hypothesis that in addition to preventing assembly of MASP-1, -2 and -3 within the MBL complex, that the MBL complex is also prevented from depositing onto oxidatively stressed arteries.

MAP-1 attenuates MBL deposition on GlcNAc-BSA

We have previously demonstrated that MAP-1 does not prevent MBL deposition on mannan-coated plates²⁵, so the inhibition of MBL deposition on carotid arteries was an

unexpected observation (Fig. 6). MBL and C3 deposition on mannan-coated plates are summarized in Fig. 7A. MBL and C3 deposition on mannan were significantly inhibited by mAb 3F8. In contrast, MBL deposition on mannan was not inhibited by MAP-1. Consistent with our findings in Fig. 2, MAP-1 significantly attenuated C3 deposition on mannan-coated plates in a dose-related manner. We also investigated whether MAP-1 could inhibit MBL deposition on a structurally different ligand, GlcNAc-BSA. As shown in Fig. 7B, MAP-1 (10 μ g/ml) significantly attenuated MBL deposition and the resulting C3 deposition on GlcNAc-BSA. These data demonstrate that in addition to inhibiting MASPs incorporation into the MBL complex, MAP-1 also inhibits MBL deposition on some MBL ligands and inhibits the resulting complement activation and C3 deposition.

Discussion

MAP-1, also known as MAP44²⁶, is a recently discovered protein that inhibits lectin complement pathway activation and is highly expressed in striated muscle, including the myocardium²⁵. While the functional aspects of this protein is largely unknown, the current study demonstrates that at pharmacologic doses, MAP-1 functions as a novel, endogenous and natural inhibitor of the lectin pathway via several mechanisms. First, the *in vitro* studies demonstrate that MAP-1 competitively inhibits all three MASPs from interacting with the MBL complex. Second, *in vivo* MAP-1 also inhibits MBL deposition in the FeCl₃ model of occlusive thrombogenesis. Third, MAP-1 effectively inhibits MBL complex mediated pathophysiologic outcomes *in vivo* following MI/R and inhibits thrombogenesis. Thus, MAP-1 is a novel, endogenous and natural inhibitor of the lectin complement pathway *in vivo*.

Our data extend previous studies which demonstrate an important role of the MBL complex in myocardial infarction^{7;9;10;30;34}. MAP-1 in the present study significantly preserved myocardial function, inhibited C3 deposition and decreased myocardial infarct size in WT mice following MI/R. Along these lines, MASP-2 inhibition or deletion also decreases myocardial infarct size and is responsible for formation of a C3 convertase necessary for C3 deposition following MBL complex interactions with its ligand^{23;35}. MAP-1 dose-dependently inhibits MASP-2 association with the MBL complex in the present study and also significantly decreased C3 deposition *in vivo* following MI/R. Thus, MAP-1, when given at pharmacologic doses, functionally inhibits formation of a functional MBL complex and the resulting complement activation following MI/R *in vivo*.

While many studies have investigated the role and function of MASP-2 in the activation of the lectin complement pathway, the functions of MASP-1 and MASP-3 have only recently been demonstrated. MASP-1 and -3 activate the alternative complement pathway^{12–14;36}. Since MAP-1 dose-dependently inhibits assembly of MASP-1 and -3 into the MBL complex, MAP-1 also likely inhibits this amplification loop, which plays a major role in tissue damage and inflammation following ischemia/reperfusion injury³⁷. MAP-1's multiple inhibitory mechanisms of action within the complement system make it potentially a very effective and efficient inhibitor before, during and following initiation of complement activation.

MASP-1 and MASP-2 play significant roles in coagulation. MASP-2 and MASP-1 contribute to generation of thrombin from prothrombin^{19;38}. MASP-1 dose-dependently cleaves/activates fibrinogen and FXIII albeit at a slower rate than thrombin *in vitro*³⁸. MASP-1 may also stabilize clot formation by activation of TAFI and thus inhibiting fibrinolysis³⁸. We have previously demonstrated that MASP-1 is responsible for FeCl₃-induced occlusive thrombogenesis *in vivo*⁶. In the present study, MAP-1 dose-dependently inhibited incorporation of both MASP-1 and MASP-2 into the MBL complex. Further,

MAP-1 inhibited occlusive thrombogenesis of the carotid artery following FeCl₃ application in WT mice. Thus, MAP-1 is a functional, native and endogenous inhibitor of coagulation *in vivo*.

Interestingly, we also observed that MAP-1 inhibited MBL deposition on the vascular endothelium following FeCl₃ application. This was an unexpected observation, as MAP-1 does not prevent MBL binding to mannan (Fig. 7A) and is consistent with our previous findings²⁵. In contrast to mAb 3F8, which inhibits both MBL and C3 deposition on mannan, MAP-1 did not inhibit MBL binding to mannan-coated plates, but did dose-dependently attenuate C3 deposition (Figure 7A). The attenuation of C3 deposition on mannan-coated plates is consistent with the inhibition of MASPs incorporation into the MBL complex as shown in Fig. 2, but does not explain the inhibition of MBL binding in the FeCl₃ carotid artery study (Fig. 6). The binding avidity of MBL is significantly higher to mannan compared to GlcNAc-BSA³⁹. We hypothesize that MAP-1 may inhibit MBL binding on MBL ligands that display lower avidity binding. Indeed, we observed that MAP-1 significantly attenuated MBL and C3 deposition on GlcNAc-BSA coated plates. The GlcNAc-BSA complex is a MBL ligand that displays lower avidity compared to mannan, probably due to the irregular spacing of the GlcNAc attached to BSA. These data suggest that MAP-1, in addition to inhibition of MASPs incorporation into MBL complexes, also attenuates MBL binding onto some ligands, which could particularly be relevant towards endogenous ligands.

In the present study, we demonstrate multiple functional aspects of MAP-1 *in vivo*, which are graphically represented in Fig. 1. In addition to attenuating myocardial injury and complement activation following myocardial ischemia and reperfusion, MAP-1 functionally inhibits occlusive thrombogenesis *in vivo*. Thus, MAP-1 has a variety of inhibitory properties associated with the MBL complex, particularly MASP-1, MASP-2 and MASP-3. These three serine proteases are also associated with the ficolins, which leads to complement activation and likely other biological activities⁴⁰. We cannot exclude additional inhibitory functions of MAP-1 that may be associated with MASPs activity. The present data clearly demonstrate the native, natural, endogenous inhibitor MAP-1 has multiple functional outcomes associated with coagulation and complement activation *in vivo*. These pharmacologic actions may have a significant impact in the treatment of cardiovascular diseases associated with complement and coagulation abnormalities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We acknowledge the expert technical assistance of Margaret Morrissey during the course of these studies. We thank Dr. Lea Munthe-Fog for help with graphical design.

FUNDING SOURCES: This work was supported in part by NIH grants AI089781, HL056086, HL099130, The Novo Nordisk Research Foundation, The Svend Andersen Research Foundation, The Danish Medical Research Council and The Research Foundation of the Capital Region of Denmark.

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Figure 1.

A model of the recognition molecules MBL and the ficolins in association with the MBL/ficolin associated serine proteases (MASPs) is shown. The possible roles of the MASPs in activation of the complement system and other cascade systems and subsequent MBL/ficolin associated protein-1 (MAP-1) inhibition are indicated. The direct MASP mediated activation of the alternative pathway has so far only been shown in rodents. In addition, MAP-1 also appears to attenuate binding of the recognition molecules to certain ligands as indicated. Hmw = high molecular weight.

Fig A: MAP-1 compact on MBL:MASP-1 complex formation **Fig. B: MAP-1 impact on MBL:MASP-2 complex formation**

Fig. C: MAP-1 impact on MBL:MASP-3 complex formation **Figure 2.**

Influence of MAP-1 on complex formation between MBL and MASP-1 (**Panel 2A**), MASP-2 (**Panel 2B**) and MASP-3 (**Panel 2C**). rMBL was pre-incubated on immobilized mannan before application of pre-mixed serial dilutions of MAP-1 with MASP-1, -2, or -3. The level of MASP binding to MBL was detected by anti-MASP mAbs, and measured as OD_{490–650nm}. Error bars indicate two times the standard deviation of duplicate determinations.

Panel A

Panel B

Figure 3.

Myocardial ejection fraction (EF) measurements. EF was assessed after 45 minutes ischemia and 4 hours of reperfusion.

Panel 3A. Summary of murine myocardial ejection fraction data for MBL null (n=3), MBL null + rhMBL (30 µg/mouse, ip; n=3), MBL null + rhMBL (30 µg/mouse, ip) + MAP-1 (160 µg/mouse, ip; n=3), and WT + bovine serum albumin (BSA, 500 µg/mouse, ip; n=5), WT + saline (n=3), WT + MAP-1 (500 µg/mouse, ip; n=3). All data are mean ±

SEM. * $P < 0.05$ compared to WT+saline or WT+BSA; ** $P < 0.001$ compared to MBL null or MBL null+rhMBL+MAP-1.

Panel 3B. Summary of murine myocardial ejection fraction data for WT + BSA (500 μg /mouse, ip), MBL null + MAP-1 (300 or 500 μg /mouse, ip) following MI/R. All data are mean $\pm$ SEM of N experiments/group. * $P < 0.05$ compared to WT + MAP-1 at 300 or 500 μg /mouse.

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Figure 4.

Assessment of myocardial infarction following MI/R in MBL null and WT mice. Brilliant Blue G dye (blue) denotes the non-ischemic area, whereas red and white demonstrate the area at risk (ischemic tissue). White (unstained) tissue denotes infarcted tissue and red denotes viable tissue.

Panel 4A. Representative myocardial sections (apex to base) from individual hearts following staining for infarcted tissue in MBL null mice treated with rhMBL, rhMBL + MAP-1 or saline.

Panel 4B. Representative myocardial sections (apex to base) from individual hearts following staining for infarcted tissue in WT mice treated with MAP-1 (500 μ g/mouse, ip) or control protein (BSA, 500 μ g/mouse, ip).

Panel 4C. Percentage of infarcted LV. The percentage of the infarcted area was calculated from weight of the LV, AAR, area of infarct, and non-infarct area. The AAR was not significantly different within the groups. All data are mean $\pm$ SEM of N=3/group.

**P<0.001 compared to MBL null + rhMBL + MAP-1 or MBL null; *P<0.01 compared to WT + rhMBL

Figure 5.

MAP-1 inhibits FeCl₃-induced thrombogenesis *in vivo*. WT mice were treated with MAP-1 or BSA (500 µg/mouse, ip) two hours before application of FeCl₃ to the carotid artery. Blood flow was continuously monitored in both groups for 30 minutes following FeCl₃ application. Data are mean ± SEM from N=4–6 mice/group. *P<0.001 compared to BSA.

Figure 6.

MBL-A and -C deposition on carotid artery endothelial cells following FeCl_3 application. Arteries were removed 30 minutes following FeCl_3 application and stained for murine MBL-A/C. Representative micrographs demonstrate MBL-A/C deposition (red) on the endothelium of the carotid arteries in BSA treated (left panel) mice compared to MAP-1 treated (right panel) mice. The middle panel is a control stained artery from a BSA treated mouse and stained with isotype control primary antibody (Control) instead of the anti-MBL-A and -C antibodies (magnification X40 for insert and X100 for the marked section).

Panel A

Panel B

Figure 7.

MBL and MBL-dependent C3 deposition on mannan (Panel A) or GlcNAc-BSA (Panel B) coated plates.

Panel A.

On mannan-coated plates (Panel A), mAb 3F8 (10 µg/ml), significantly inhibited MBL (upper panel) and C3 deposition (lower panel). MAP-1 did not inhibit MBL deposition on mannan-coated plates but significantly attenuated C3 deposition in a dose-related manner. *P<0.001 compared to all groups within the same panel. Other statistical comparisons are present within the figure panel. II=integrated intensity. N=3 in triplicate

Panel B. On GlcNAc-BSA coated plates D-mannose (d-Man; 30 mM) significantly attenuated MBL and C3 deposition. MAP-1 (10 µg/ml) significantly attenuated MBL deposition. MAP-1 (5 or 10 µg/ml) significantly attenuated C3 deposition on GlcNAc-BSA plates. D-mannose was used as a control, as it will not inhibit potential ficolin induced C3 deposition on GlcNAc-BSA. *P<0.001 compared to Control; II=integrated intensity; N=5 in triplicate