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The Levels of the Lectin Pathway Serine Protease MASP-1 and Its Complex Formation with C1 Inhibitor Are Linked to the Severity of Hereditary Angioedema

Cecilie Bo Hansen,* Dorottya Csuka,† Lea Munthe-Fog,* Lilian Varga,† Henriette Farkas,† Karin Møller Hansen,* Claus Koch,‡ Karsten Skjødt,‡ Peter Garred,* and Mikkel-Ole Skjoedt*

C1 inhibitor (C1-INH) is known to form complexes with the lectin complement pathway serine proteases MASP-1 and MASP-2. Deficiency of C1-INH is associated with hereditary angioedema (HAE), an autosomal inherited disease characterized by swelling attacks caused by elevated levels of bradykinin. MASP-1 was shown to cleave high m.w. kininogen into bradykinin; therefore, we hypothesized that MASP-1 levels and the quantity of MASP-1/C1-INH complexes might be associated with different paraclinical and clinical outcomes of HAE. We measured MASP-1 serum concentrations and endogenous MASP-1/C1-INH complex levels in 128 HAE patients and 100 controls. Relatively high levels of pre-existing MASP-1/C1-INH complexes were observed in normal serum, and we found that both the serum levels of MASP-1 and the complex formation between MASP-1 and C1-INH were significantly reduced in HAE patients compared with matched controls ($p < 0.0001$). The level of MASP-1 and MASP-1/C1-INH complexes in HE patients correlated with the level of C1-INH ($p = 0.0009$ and $p = 0.0047$, respectively), the level of C4 ($p = 0.0084$ and $p < 0.0001$, respectively), and the number of attacks in the year of blood sampling ($p = 0.0075$ and $p = 0.0058$, respectively). In conclusion, we show that MASP-1/C1-INH complexes circulate in normal human blood. The levels of MASP-1 and MASP-1/C1-INH complexes are reduced in HAE patients compared with controls. Both MASP-1 and MASP-1/C1-INH complexes are related to the degree of complement C4 consumption, as well as the severity of disease. These results suggest that MASP-1 may exert a previously unrecognized role in the pathophysiology of HAE. *The Journal of Immunology*, **2015, 195: 3596–3604.**

The complement system provides effective protection mechanisms that are involved in both innate and adaptive immune defense. It is composed of three pathways, the classical pathway, the alternative pathway, and the lectin pathway (LP), which all work by initiating proteolytic cascades that detect and eliminate pathogens and endogenous Ags (1, 2). The LP includes the following pattern recognition molecules (PRMs): mannose-binding lectin (MBL), ficolins (-1, -2, -3), and collectin-10 and -11, which recognize and bind to sugar structures or acetylated compounds on microbial surfaces or altered host cells. The PRMs form complexes with the LP serine proteases MASP-1, -2, and -3 and two truncated proteins: MAP-1 (Map44) and sMAP (Map19) (3–8). It is known that MASP-2 cleaves both C4 and C2 (4), but recent studies showed that MASP-1 activates zymogen MASP-2; consequently, MASP-1 may indeed initiate activation of the LP (9–11).

One of the important soluble LP regulators is the plasma serpin C1 inhibitor (C1-INH). C1-INH belongs to the serine protease inhibitor superfamily; in addition to its involvement in the complement system, it is the principal regulator of the contact system and is able to inactivate proteolytic steps in the coagulation cascade and the kallikrein-kinin system. Proteases inactivated by C1-INH include C1r, C1s, MASP-1, and MASP-2 from the complement system; factor XII, plasma kallikrein, and factor XI from the contact/coagulation system; and plasmin and tissue plasminogen activator from the fibrinolytic system (12). A major biological role for C1-INH is regulation of the kallikrein-kinin system. Low levels and/or dysfunctional C1-INH and dysregulation of the kallikrein-kinin system are the molecular cause behind hereditary angioedema (HAE), which is characterized by increased vascular permeability and tissue edema due to cleavage of high m.w. kininogen (HMWK) and the accumulation of bradykinin peptides (12, 13). Type I HAE is characterized by mutations throughout the C1-INH gene (*SERPING1*) that lead to the formation of truncated or misfolded proteins that are not secreted efficiently, resulting in low C1-INH plasma levels. Type II HAE is characterized by mutations resulting in dysfunctional C1-INH protein and low function, despite normal levels of antigenic C1-INH. Patients generally suffer from severe attacks of swelling in the extremities, abdomen, oropharynx, and larynx, which can be fatal if not treated. The primary mediator of swelling in patients with HAE is bradykinin (13).

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All authors contributed significantly to this work and discussed the results and commented on the manuscript at all stages.

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The online version of this article contains supplemental material.

Abbreviations used in this article: AcBSA, acetylated BSA; AT, anti-thrombin; C1-INH, C1 inhibitor; HAE, hereditary angioedema; HMWK, high m.w. kininogen; LP, lectin pathway; MAP, MBL/ficolin/collectin-11–associated protein; MASP, MBL/ficolin/collectin-11–associated serine protease; MBL, mannose-binding lectin; NHS, normal human serum; pdC1-INH, plasma-derived C1-INH concentrate; PRM, pattern recognition molecule; r, recombinant.

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Recently, MASP-1 was shown to cleave HMWK into bradykinin (14); thus, we hypothesized that MASP-1 may be involved in pathophysiology of HAE. To investigate such a notion, we measured the serum concentration of MASP-1 and established ELISA-based methods to assess MASP-1/C1-INH complexes in serum samples from a cohort of 128 HAE patients during symptom-free periods, as well as 100 healthy matched controls.

Materials and Methods

Patients and controls

HAE patients. A total of 128 patients (54 men and 74 women, median age: 34 y [22–49 y]), 117 with HAE type I and 11 with HAE type II, were enrolled in 2009. Each patient was diagnosed based on the accepted clinical and laboratory criteria (positive family history, clinical symptoms of angioedema, low C1-INH level), as previously reported (15). All patients were clinically stable; semisynthetic testosterone or antifibrinolytic drugs were administered for long-term prophylaxis, whereas human plasma-derived C1-INH concentrate (pdC1-INH; Berinert; CSL Behring, Marburg, Germany) was given as an acute treatment for edematous attacks or as short-term prophylaxis. During the year of blood sampling, the subjects recorded the number and location of angioedema attacks in their patient diaries. All patients received pdC1-INH in every instance to relieve the most severe forms of edematous episodes (i.e., upper airway, abdominal, facial, genital, and severe limb edema, predominantly). Therefore, the number of vials of pdC1-INH used during the follow-up period and the annual number of episodes were regarded as objective indices of disease severity, as previously described (16) Demographics and further information on the HAE patients are shown in Table V.

Healthy controls. The control group consisted of 100 healthy adults (47 men and 53 women, median age: 33 y [range, 21–58 y]). The controls had been referred for routine medical check-up and volunteered for the study by giving informed consent. HAE patients and controls were not statistically different with regard to age. Demographics and further information on the healthy controls are shown in Table V.

Blood sampling

Serum samples, obtained from HAE patients during symptom-free periods, were stored at −70°C until processing. Peripheral blood samples also were drawn from healthy subjects, as prescribed by the study protocol approved by the Institutional Review Board of the Semmelweis University, after informed consent, in accordance with the Declaration of Helsinki. All participants provided their written informed consent to participate in the study.

Complement-deficient individuals

Three individuals deficient in the complement components MASP-2, ficolin-3, and MBL were described previously (17–19).

Abs and labeling

Abs against C1-INH were raised in NMRI mice using purified C1-INH (CompTech, Tyler, TX; catalog no. A140) as Ag with three immunizations (25 μg Ag) at 14-d intervals and screened and selected as described previously (20). Two C1-INH mAbs (11-28-21 and 11-28-12) were selected and purified by conventional protein A affinity chromatography and labeled with biotin using biotin *N*-hydroxysuccinimide ester (Sigma-Aldrich; H1759), as recommended by the manufacturer. The anti–MASP-2 Ab 8B5 was from Hycult Biotech (Uden, The Netherlands; catalog no. HM2190). The two in-house Abs, MASP-1/3 Ab (mAb 8B3) and MAP-1 Ab (mAb 20C4), were described previously (7, 20).

Recombinant proteins and purified C1-INH

Recombinant human proteins MAP-1, MASP-1, MASP-2, MASP-3, MBL, and ficolin-3 were produced, as described previously (19–21), using the CHO DG44 expression system and serum-free medium (CHO-CD1; Lonza). Purified human plasma–derived C1-INH was purchased from CompTech (catalog no. A140).

MASP-1 serum level measurements

MASP-1 serum levels in the HAE patient cohort and the matched healthy controls were measured using a commercial ELISA kit (USCN Life Sciences; catalog no. SEB895Hu), as recommended by the manufacturer.

ELISA measurement of fluid-phase MASP/C1-INH complexes

MaxiSorp ELISA plates (NUNC, Roskilde, Denmark) were coated with monoclonal anti–MASP-1/3 mAb 8B3 or anti–MASP-2 8B5 at 4 μg/ml in PBS overnight at 4°C. As a control of heterophilic interference in the serum samples, other plates were coated with a monoclonal IgG Ab with no relevant specificity. The samples (from HAE patients and healthy matched controls) were diluted 1:10 and 1:50 in PBS + 0.05% Tween-20 containing 0.5% bovine serum. Plates were washed three times in PBS + 0.05% Tween-20, and the diluted samples were applied to the plates in duplicates and incubated for 2 h at room temperature. A 2-fold dilution of a pool of normal human serum (NHS; 1:5–1:320) was included on each plate. The detection Ab (biotinylated anti–C1-INH mAb 11-28-21) was applied at 2 μg/ml and incubated for 1.5 h at room temperature. Secondary detection was performed with HRP-conjugated streptavidin (GE Healthcare; catalog no. RPN1051) diluted to 1:1500 and developed with OPD/H_2O_2. The reaction was stopped by adding sulfuric acid to a final concentration of 0.5 M, and the plates were read at an OD of 490 nm.

Using the same set-up with MASP-1/C1-INH complexes, we assessed the relative endogenous complex levels in NHS, hirudin-, citrated-,

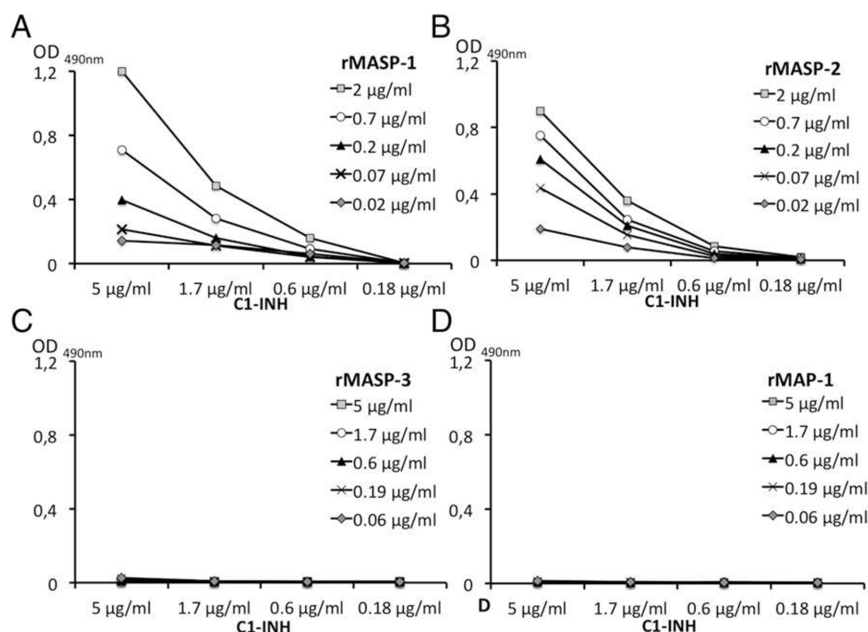

FIGURE 1. (**A**–**D**) Detection of purified C1-INH interaction with rMASPs and rMAP-1 bound to solid-phase rMBL. rMBL bound to mannan was incubated with serial dilutions of rMASP-1, rMASP-2, rMASP-3, or rMAP-1. Purified C1-INH in serial dilution (*x*-axis) was incubated on the MBL/MASP complexes and detected by a biotinylated C1-INH mAb 11-28-21. (A) rMASP-1. (B) rMASP-2. (C) rMASP-3. (D) rMAP-1.

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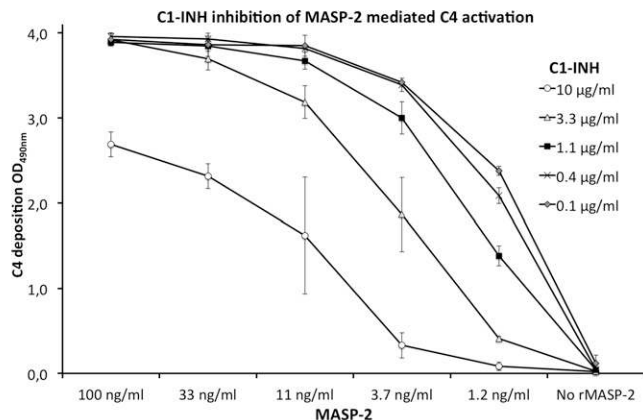

FIGURE 2. Effect of C1-INH on MASP-2–mediated C4 activation and deposition. Serial dilutions of rMASP-2 and purified C1-INH were coincubated on an rMBL/mannan activation surface. Subsequently, purified C4 was applied, and the activation and deposition of C4 were measured using polyclonal C4 Abs. The experiments were performed in triplicates, and the error bars represent SDs.

EDTA-, and heparinized plasma. Under the same conditions, we also measured the relative complex levels between MASP-1 and antithrombin (AT), substituting only the C1-INH detection Ab with a commercially available Ab against AT (Abcam; catalog no. ab79933). We also analyzed MASP-1/AT complex levels in the HAE patient cohort and controls. Data on MASP-1/AT levels are shown in Supplemental Fig. 2.

We further evaluated the presence of the PRMs MBL and ficolin-3 in these complexes with the same ELISA set-up as described above. We used NHS and two individual sera deficient in MBL or ficolin-3. After completing the incubation and washing steps, ELISA wells were eluted with reducing SDS loading buffer and analyzed by immunoblotting using mAbs to either MBL (Hyb 131-1; Bioporto, Gentofte, Denmark) or ficolin-3 (Hyb 313; in-house mAb). The immunoblot data are shown in Supplemental Fig. 3.

C1-INH interaction with ligand-bound MBL/ficolin-3/MASP complexes

Maxisorb ELISA plates were coated with 20 μg/ml mannan from *Saccharomyces cerevisiae* (Sigma-Aldrich; catalog no. M7504) or 5 μg/ml acetylated BSA [AcBSA; as described by Hein et al. (22)] in PBS overnight at 4°C. Plates were washed three times in TBST/Ca^{2+} washing/dilution buffer (10 mM Tris, 145 mM NaCl, 2 mM $CaCl_2$, 0.05% Tween-20). Recombinant (r)MBL or ficolin-3 (3 μg/ml) was added to the mannan- or AcBSA-coated plates and incubated for 1.5 h at room temperature. After washing, 3-fold dilution series of rMASP-1, rMASP-2, rMASP-3, or rMAP-1 (starting at 2 μg/ml) were applied to the plates and incubated for 1.5 h at room temperature. After washing, 3-fold dilution series of human C1-INH (starting at 5 μg/ml) were added to the plates and incubated for 1.5 h at room temperature. C1-INH binding to MBL or ficolin-3/MASP complexes was detected with the biotinylated anti–C1-INH mAb 11-28-21 and HRP-conjugated streptavidin, as described above.

The same assay compositions as described above were used to measure complex formation of serum C1-INH with ligand-bound PRM complexes. Serum samples included NHS and serum deficient in MASP-2, MBL, or ficolin-3. The serum samples were incubated as described above, and negative controls with 10 mM EDTA added to the dilution buffer were applied. After binding and activation of the PRM complexes

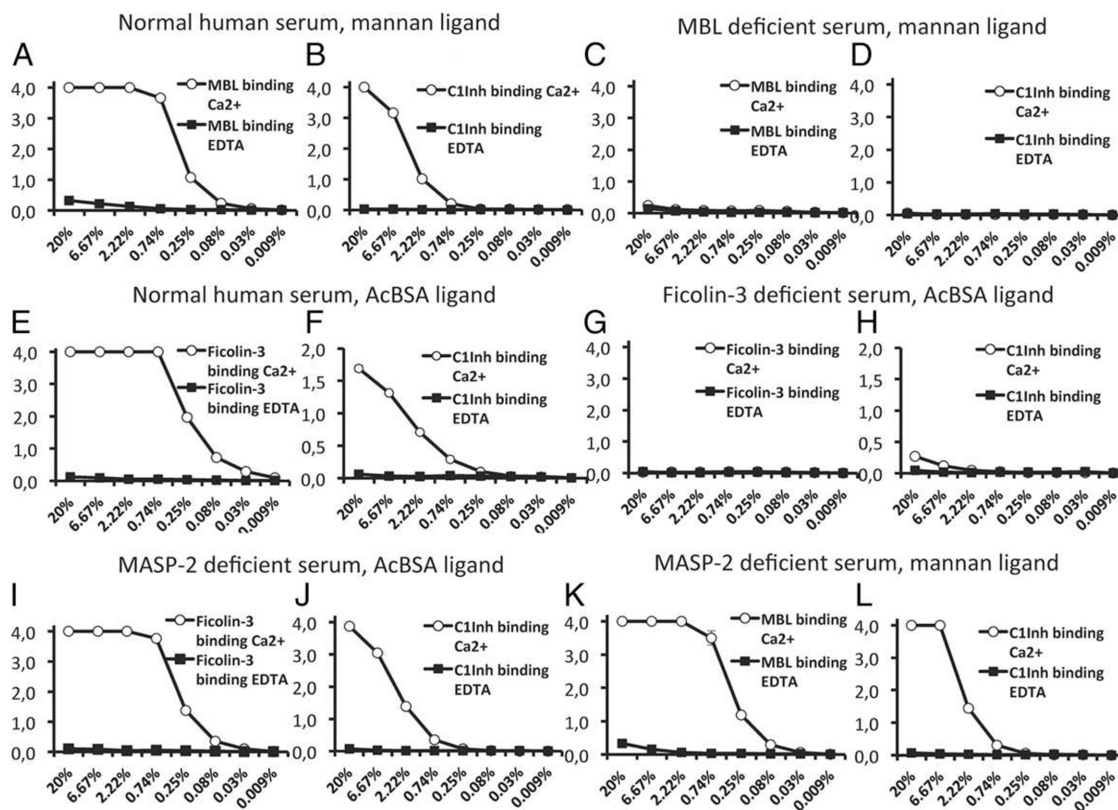

FIGURE 3. Detection of MBL/MASP/C1-INH–bound or ficolin-3/MASP/C1-INH–bound complexes on mannan or AcBSA solid-phase ligands. Detection with Abs against MBL, C1-INH, or ficolin-3. NHS or different deficient sera were diluted 3-fold starting at 20%. The experiments were performed in triplicates, and the error bars indicate SD. *Y*-axes show OD at 490 nm; *x*-axes show serum concentration (percentage). (**A**) MBL binding to mannan in NHS. (**B**) C1-INH binding to MBL complexes in NHS. (**C**) MBL binding to mannan in MBL-deficient serum. (**D**) C1-INH binding to MBL complexes in MBL-deficient serum. (**E**) Ficolin-3 binding to AcBSA in NHS. (**F**) C1-INH binding to ficolin-3 complexes in NHS. (**G**) Ficolin-3 binding to AcBSA in ficolin-3–deficient serum. (**H**) C1-INH binding to ficolin-3 complexes in ficolin-3–deficient serum. (**I**) Ficolin-3 binding to AcBSA in MASP-2–deficient serum. (**J**) C1-INH binding to ficolin-3 complexes in MASP-2–deficient serum. (**K**) MBL binding to mannan in MASP-2–deficient serum. (**L**) C1-INH binding to MBL complexes in MASP-2–deficient serum.

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to the solid-phase ligand surfaces, supernatants were collected and transferred to precoated ELISA plates and used to measure fluid-phase complexes composed of MASP-1/C1-INH or MASP-2/C1-INH, respectively.

Detection in the solid-phase systems was done with anti–C1-INH mAb 11-28-21, anti-MBL mAb (Hyb 131-10 and 131-11; Bioporto), and in-house anti–ficolin-3 FCN334, all applied at 2 μg/ml. The signal was obtained with HRP-conjugated polyclonal rabbit-anti-mouse Ab diluted to 1:1500 (PO260; Dako, Glostrup, Denmark) and otherwise developed as described above.

The fluid-phase complexes in the supernatants from the ligand-binding experiments above were transferred to plates coated with either anti-MASP-1/3 (mAb 8B3) or anti-MASP-2 (mAb 8B5) and detected with biotinylated anti–C1-INH mAb 11-28-21, as described above.

MBL/MASP/C1 inhibitor complex precipitation, SDS-PAGE, and immunoblotting

Maxisorb ELISA plates were coated with 20 μg/ml mannan, as described above. After blocking, NHS or MBL-deficient serum, diluted to 1:10 in TBST/Ca^{2+}, was added to the plate and incubated for 2 h at room temperature. The plate was washed three times, and the bound serum complexes were eluted from the plate by adding boiling SDS buffer directly to the wells and subsequently applied to reduced and nonreduced 4–12% SDS-PAGE and immunoblotting using the NuPAGE system, as instructed by the manufacturer (Invitrogen, Life Technologies). The membrane was probed with 0.5 μg/ml anti–C1-INH mAb 11-28-12 and HRP-conjugated streptavidin diluted 1:10,000 (GE Healthcare, catalog RPN1051). The membrane was developed with a SuperSignal West Femto Substrate Maximum Sensitivity Substrate kit (Thermo Scientific).

C4-deposition assay

The MBL/MASP-2 C4-activation and deposition assay was conducted as a pure serum-free system, essentially as described previously (7). Briefly, rMBL (0.5 μg/ml) was allowed to bind to mannan immobilized on Maxisorb ELISA plates. Two-dimensional 3-fold dilutions of rMASP-2 (starting at 0.1 μg/ml) and purified human C1-INH (starting at 10 μg/ml) were coincubated on the MBL/mannan surface, followed by washing and application of purified human C4 at 0.5 μg/ml (CompTech; catalog no. A105) and incubation for 45 min at 37°C. C4 deposition was measured with rabbit polyclonal anti-C4c and swine anti-rabbit–HRP (Dako; catalog no. Q0369 and P0217).

Cleavage of HMWK by MASP-1

We assessed the possible cleavage of HMWK by MASP-1 using a solid-phase MBL-activation assay. Briefly, ELISA plates were coated with 20 μg/ml mannan and incubated for 2 h at room temperature with 0.5 μg/ml rMBL or plasma-derived MBL complexes that contained endogenous MASPs [MO12 and MO20, respectively; Statens Serum Institute, Copenhagen, Denmark (23, 24)]. rMBL was further incubated with rMASP-1 at ~0.5 μg/ml for 1 h at room temperature. After washing, 20 μl HMWK (50 μg/ml; Merck-Millipore, Darmstadt, Germany; catalog no. 422686) was applied to each well and incubated for 4 h at 37°C. Washing and incubation buffers were TBS + 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.05% Tween-20 or TBS + 10 mM EDTA, 0.05% Tween-20, which served as negative control. After the 4-h incubation, the supernatant was harvested and analyzed by SDS-PAGE and Coomassie staining or by immunoblotting with a biotin-labeled polyclonal Ab to HMWK (Abcam; catalog no. ab79654).

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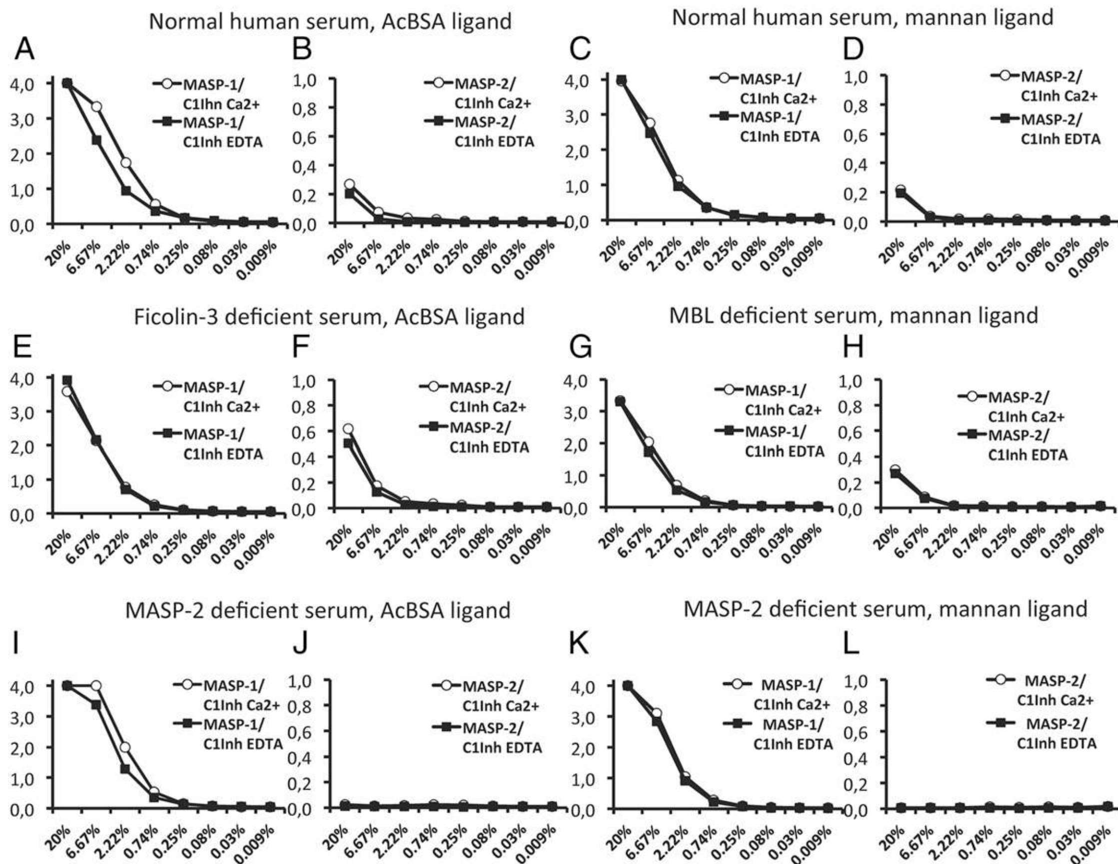

FIGURE 4. Detection of MASP-1/C1-INH or MASP-2/C1-INH complexes in the fluid phase after incubation on the mannan or AcBSA ligand surfaces from Fig. 3. The fluid-phase complexes from NHS or different deficient sera were captured with a MASP-1/-3 or MASP-2 mAb and detected with anti–C1-INH mAb. The experiments were performed in triplicates, and the error bars represent SDs. *Y*-axes show OD at 490 nm; *x*-axes show serum concentration (percentage). (**A**) MASP-1/C1-INH, NHS/AcBSA ligand. (**B**) MASP-2/C1-INH, NHS/AcBSA ligand. (**C**) MASP-1/C1-INH, NHS/mannan ligand. (**D**) MASP-2/C1-INH, NHS/mannan ligand. (**E**) MASP-1/C1-INH, ficolin-3–deficient serum/AcBSA ligand. (**F**) MASP-2/C1-INH, ficolin-3–deficient serum/AcBSA ligand. (**G**) MASP-1/C1-INH, MBL-deficient serum/mannan ligand. (**H**) MASP-2/C1-INH, MBL-deficient serum/mannan ligand. (**I**) MASP-1/C1-INH, MASP-2–deficient serum/AcBSA ligand. (**J**) MASP-2/C1-INH, MASP-2–deficient serum/AcBSA ligand. (**K**) MASP-1/C1-INH, MASP-2–deficient serum/mannan ligand. (**L**) MASP-2/C1-INH, MASP-2–deficient serum/mannan ligand.

Statistical analysis

Statistical calculations were performed with Prism 6.0 (GraphPad Software, San Diego, CA). Spearman's rank correlation coefficients were calculated to estimate the relationship between variables, whereas the χ^2 test was used to compare categorical variables. All statistical analyses were two-tailed, and $p < 0.05$ was considered to represent a statistically significant difference or correlation.

Results

C1-INH interaction with MASPs and MAP-1

We addressed the interaction of C1-INH with the different MASP variants and the truncated variant MAP-1. We used rMBL bound to solid-phase mannan as a ligand partner for rMASP-1, rMASP-2, rMASP-3, and rMAP-1. Using this pure, serum-free system, we found a clear dose-dependent interaction between C1-INH and MASP-1 and MASP-2 (Fig. 1). No obvious interaction was observed between MAP-1 and MASP-3, supporting previous findings that MASP-3 does not interact with C1-INH, as well as demonstrating the specificity by the lack of interaction with MAP-1 (25). We further analyzed the C1-INH protease inhibitor influence on MASP-2–dependent C4 activation in this pure system. We found a clear dose-dependent inhibitory effect of C1-INH on C4 activation and deposition mediated by MASP-2 (Fig. 2), because it was shown before under different settings (26). To induce a significant inhibition of C4 activation, we needed to add a much higher molar concentration of C1-INH compared with MASP-2. However, the physiological plasma concentration of C1-INH is also ~500 times higher than MASP-2, and similar molar inhibition ranges were reported by other investigators (27, 28).

Formation of complexes between C1-INH and ligand-bound MBL and ficolin-3 in serum

To further investigate and validate the observation above, we used different serum systems to examine endogenous C1-INH complex formation with MBL/MASP or ficolin-3/MASP binding to respective ligands. Concentration-dependent binding of MBL and endogenous C1-INH was observed for NHS on the mannan matrix with a physiological calcium concentration, whereas the binding of both components was eliminated when incubated with EDTA (Fig. 3A, 3B). No obvious MBL or C1-INH binding was seen with the same set-up using MBL-deficient serum, suggesting that C1-INH binding to the surface is completely dependent on the presence of MBL/MASP complexes in this ELISA set-up (Fig. 3C, 3D). We substantiated this further by examining NHS and serum deficient in ficolin-3 on an AcBSA-binding surface. Dose-dependent binding was observed for ficolin-3 and C1-INH in NHS, and the binding was abolished with EDTA (Fig. 3E, 3F). In contrast, no significant binding was evident for the ficolin-3–deficient serum (Fig. 3G, 3H). Using pure systems (Fig. 1), we observed equivalent binding of C1-INH to both rMASP-1 and rMASP-2. The serum concentration, in general, is considered to be much higher for MASP-1 than for MASP-2 (3, 29, 30). We detected binding of C1-INH to both MBL and ficolin-3 complexes in MASP-2–deficient serum and, in general, observed C1-INH–binding curves that were comparable to NHS for both assays

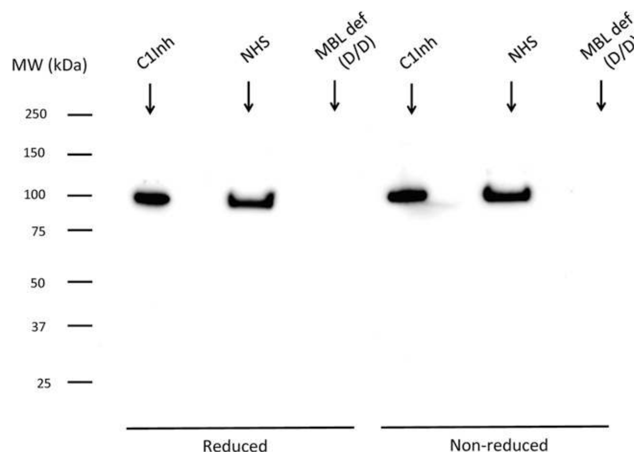

FIGURE 5. Analysis of precipitated MBL/MASP/C1-INH complexes from NHS and MBL-deficient serum. Using solid-phase mannan, serum MBL/MASP complexes were precipitated, and the samples were subjected to reducing and nonreducing SDS-PAGE and Western blotting using the mAb 11-28-12.

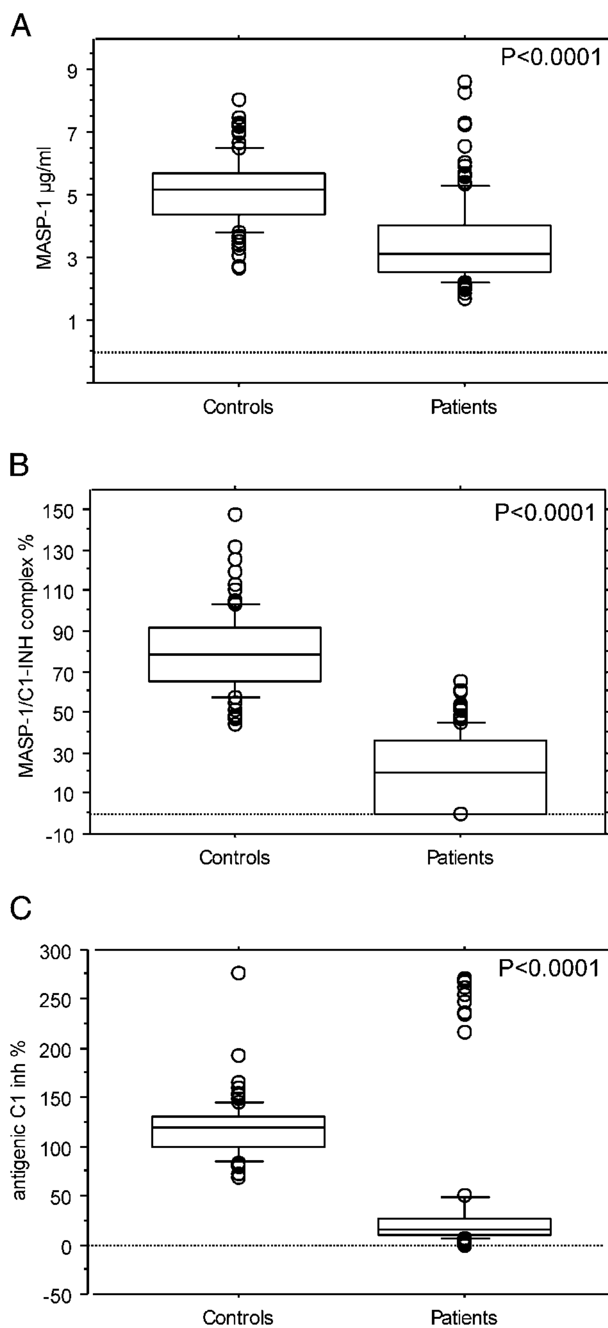

FIGURE 6. Measurements of MASP-1 and C1-INH Ag levels and MASP-1/C1-INH complex levels in 128 HAE patients and 100 matched, healthy controls. (**A**) Ag levels and distribution of MASP-1. (**B**) MASP-1/C1-INH complex levels and distribution. (**C**) Ag levels and distribution of C1-INH.

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Table I. Correlation between MASP-1 concentrations and other selected complement parameters in the HAE patient cohort and the matched healthy control group

MASP-1 Ag Versus	Healthy Controls		HAE Patients	
	ρ	p Value	ρ	p Value
Antigenic C1-INH concentration	+0.156	0.1210	+0.308	0.0009
Functional C1-INH percentage activity	−0.038	0.7067	+0.201	0.0319
MASP-1/C1-INH complex	−0.018	0.8531	+0.250	0.0044
C4 level	+0.237	0.0176	+0.245	0.0084
C3 level	+0.122	0.2245	+0.248	0.0053

(Fig. 3I–L), suggesting that MASP-1 plays a dominant role in C1-INH/serine protease complex formation.

Fluid-phase complexes of C1-INH with MASP-1 and MASP-2

After showing interactions between C1-INH and MASP-1 and MASP-2 in solid-phase bound activation complexes, we wanted to address whether these complexes exist naturally in the circulation and/or whether they are formed during in vitro activation. Therefore, we established two assays based on an in-house monoclonal MASP-1/-3 Ab 8B3 (20) and a commercially available MASP-2 Ab 8B5 as capture reagents and, for detection, a newly developed mAb raised against C1-INH (mAb 11-28-21). This time we assessed the fluid-phase complexes in the different serum types described above after application to mannan or AcBSA activation surfaces, with and without calcium chelation. We found measurable levels of MASP-2/C1-INH in the NHS, MBL-deficient, and ficolin-3–deficient serum (Fig. 4B, 4D, 4F, 4H) and no detectable signal in the MASP-2–deficient serum (Fig. 4I, 4L). In general, we obtained higher signals from the MASP-1/C1-INH complexes (Fig. 4A, 4C, 4E, 4G, 4I, 4K) compared with the MASP-2/C1-INH complexes, and these complexes appeared to be independent of deficiencies in MBL or ficolin-3. Furthermore, we did not find significantly elevated fluid-phase complex levels in the solid-phase activated samples. However, we observed substantial additional C1-INH binding to MBL/MASP or ficolin-3/MASP in the solid phase. This added quantity appears to be independent of the pre-existing MASP/C1-INH complexes in the fluid phase.

Coprecipitation of C1-INH with MBL/MASP complexes

To further validate the observations above, we precipitated MBL/MASP complexes using solid-phase mannan. The samples were subsequently analyzed by reduced and nonreduced SDS-PAGE and immunoblotting using yet another newly developed Ab raised against C1-INH (mAb 11-28-12). A very distinct pattern was seen on the Western blot where NHS-derived MBL/MASP complexes clearly coprecipitated C1-INH (Fig. 5). In contrast to this, no C1-INH coprecipitation was observed when using MBL-deficient serum. Equivalent results were obtained with polyclonal Abs against C1-INH (data not shown). When we analyzed the captured complexes from the MASP-1/C1-INH assay, we found that PRMs (MBL and ficolin-3) were present in these complexes as well (Supplemental Fig. 3).

Establishing a MASP-1/C1-INH assay

Taking the above observations into account, we decided to proceed with an assay measuring fluid-phase complexes containing MASP-1 and C1-INH. We assessed the relative complex formation between C1-INH and MASP-1 in serum and different types of plasma. We observed very similar dose-dependent dilution curves of the complexes in serum, hirudin, EDTA, and citrated plasma. Much lower signals and a different dilution curve were observed in heparinized plasma (Supplemental Fig. 1). AT is known to interact with MASP-1, and the affinity is increased by heparin (31, 32). Therefore, we measured MASP-1/AT complexes and found elevated levels in heparinized plasma compared with the other plasma types (Supplemental Fig. 1).

MASP-1 serum levels in HAE patients and healthy controls

The serum Ag levels of MASP-1 were measured in a patient cohort diagnosed with HAE and a matched, healthy control group using a commercially available assay. The two groups were from the same ethnic population and were matched with respect to age and gender. We found significantly lower MASP-1 Ag levels in the HAE patients compared with the healthy controls ($p < 0.0001$) (Fig. 6A). We quantified the concentrations of complement components, C4, C3, total antigenic C1-INH, and the functional activity level of C1-INH.

We observed a significant, positive correlation between MASP-1 serum levels and C1-INH concentration, as well as MASP-1/C1-INH complex formation ($\rho = +0.308$, $p = 0.0009$ and $\rho = +0.250$, $p = 0.0044$, respectively), in the HAE patient cohort, whereas no correlation was seen in the control group (Table I). Furthermore, positive correlations were observed between MASP-1 and C3 and C4 in the HAE patients, whereas only C4 correlated with MASP-1 in controls (Table I). Interestingly, the MASP-1 concentration showed a negative correlation with the number of s.c. attacks and the total number of attacks within the year of blood sampling ($\rho = -0.238$, $p = 0.0109$ and $\rho = -0.249$, $p = 0.0075$) (Table II).

MASP-1/C1-INH levels in patients with HAE and healthy controls

We used the MASP-1/C1-INH assay described above to assess the relative serum complex formation in the same HAE and control groups. Comparing the two groups, we observed a clear difference in MASP-1/C1-INH complex formation. Much higher complex levels were found in the control group than in the HAE cohort ($p < 0.0001$) (Fig. 6B). The same trend was found when we compared MASP-1/AT complex levels in the two groups (Supplemental Fig. 2).

Table II. Correlation between MASP-1 concentration and disease severity markers in the HAE patient cohort

MASP-1 Ag Versus	ρ	p Value
Total number of attacks (in the year of blood sampling)	−0.249	0.0075
No. s.c. attacks (in the year of blood sampling)	−0.238	0.0109
No. abdominal attacks (in the year of blood sampling)	−0.138	0.1419
No. upper airway attacks (in the year of blood sampling)	−0.165	0.0799
C1-INH concentrate consumption (for treatment of severe attacks)	−0.162	0.0718

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Table III. Correlations between MASP-1/C1-INH complex levels and other selected complement parameters in the HAE patient cohort and the matched healthy control group

MASP-1/C1-INH Complex Versus	Healthy Controls		HAE Patients	
	ρ	*p* Value	ρ	*p* Value
Antigenic C1-INH concentration	+0.223	0.028	+0.263	0.0047
Functional C1-INH percentage activity	+0.0242	0.81	+0.296	0.0024
MASP-1/C1-INH Complex	−0.018	0.8451	+0.250	0.0044
C4 level	+0.0516	0.62	+0.396	<0.0001
C3 level	+0.185	0.070	+0.137	0.13

A significant, but weak, correlation was seen in both groups with the relative MASP-1/C1-INH complex level and C1-INH antigenic level (controls: ρ = +0.223, $p = 0.0278$ and HAE: ρ = +0.263, $p = 0.00323$), but we did not find a correlation with the functional C1-INH measurements in the control group (Table III). In contrast, a correlation was identified in the HAE group between the MASP-1/C1-INH level and the functional C1-INH activity measurements (ρ = +0.296, $p < 0.0024$). Furthermore, no correlation was seen between MASP-1/C1-INH and C3 or C4 in the control group, and the same was evident for the HAE-C3 levels. However, a very clear correlation was found between the C4 levels and MASP-1/C1-INH complexes (rho: 0.396, $p < 0.0001$) in the HAE group (Table III). Additionally, and similar to the MASP-1 concentration, we found that the number of s.c. attacks and the total number of attacks correlated negatively with the MASP-1/C1-INH complex levels (ρ = −0.252, $p = 0.0042$ and ρ = −0.244, $p = 0.0058$, respectively). This correlation was also seen with upper airway attacks (ρ = −0.247, $p = 0.0051$) (Table IV). Furthermore, the consumption of C1-INH in patients who received it exogenously for treatment of severe attacks also correlated negatively with MASP-1/C1-INH levels (Table IV). Additional relevant information regarding the HAE patients and healthy controls are shown in Table V.

It was shown previously that MASP-1 is able to cleave HMWK (14). We also addressed this using a solid-phase MBL/mannan set-up and both recombinant proteins (MBL and MASP-1) and MBL/MASP complexes purified from human plasma. In both cases MASP-1 was able to cleave kininogen (Fig. 7), which confirmed the previous findings suggesting a link between the contact system and the lectin complement pathway.

Discussion

The relationship between C1-INH deficiency and HAE is well established. It is also known that C1-INH engages at several levels in regulation of the complement, coagulation, and contact systems. In contrast, the biological functions of MASP-1 are far less characterized. However, a recent study showed that MASP-1 may operate in the contact system by cleaving HMWK to bradykinin (14), and it has been known for some time that C1-INH interacts with activated MASP-1 at a 1:1 molar ratio (33). Thus, we hypothesized that MASP-1 and MASP-1/C1-INH complex formation might be relevant to the pathophysiology of HAE.

First, we addressed the basic interactions of C1-INH with all three human MASPs and the recently described LP inhibitor MAP-1, as specificity control. When evaluated in pure systems using MBL ligand-binding complexes, only MASP-1 and MASP-2 displayed interaction properties with C1-INH, whereas, as expected, MASP-3 and MAP-1 did not. Other investigators saw similar results in different settings for MASPs (25). We were able to detect both pre-existing, endogenous MASP-1/C1-INH and MASP-2/C1-INH complexes in the fluid phase in serum. At the same time, we detected de novo MASP-1/C1-INH and MASP-2/C1-INH complex formation generated on solid-phase MBL or ficolin ligands, which did not lead to an increase or decrease in the amount of fluid-phase complexes measured in the same experiments. Combined with C1-INH inhibition on MASP-2–mediated C4 activation, it indicates that C1-INH inhibits the MASP activity irreversibly by interacting with the protease site and not by dissociation of the MASPs from the LP complexes, as might be anticipated from C1-INH interaction with the C1q-C1r/C1s complex (34). In contrast, the finding of substantial amounts of fluid-phase MASP-1/C1-INH complexes suggests that these might be generated continuously in the circulation or on surfaces by mechanisms that we were not able to mimic in vitro.

We established assays to measure specific fluid-phase complexes formed between C1-INH and MASP-1 or MASP-2 in serum and found that MASP-2/C1-INH complexes were only detected in very high serum concentrations compared with MASP-1/C1-INH. This could be a result of the much lower serum concentration of

Table IV. Correlations between MASP-1/C1-INH complex levels and disease severity markers in the HAE patient cohort

MASP-1/C1-INH Complex Versus	ρ	*p* Value
Total number of attacks (in the year of blood sampling)	−0.244	0.0058
No. s.c. attacks (in the year of blood sampling)	−0.252	0.0042
No. abdominal attacks (in the year of blood sampling)	−0.134	0.135
No. upper airway attacks (in the year of blood sampling)	−0.247	0.0051
C1-INH concentrate consumption (for the treatment of severe attacks)	−0.286	0.0011

Table V. Demographics and additional information on the healthy controls and HAE patients

Demographics	Healthy Controls	HAE Patients
Gender distribution	47 men, 53 women	54 men, 74 women
Age at blood sampling (y)[a]	33 (21–58)	34 (22–49)
Age at first symptoms (y)[a]	NA	10 (5–18)
Age at HAE diagnosis (y)[a]	NA	21 (8–36)
HAE type		
HAE type I	NA	117
HAE type II	NA	11
Long-term prophylactic treatment with attenuated androgen or antifibrinolytics	0 subjects	58 subjects
Total number of attacks in the year of blood sampling[a]	NA	4 (1.13–10.13)

[a]Data are median (25–75% percentiles).

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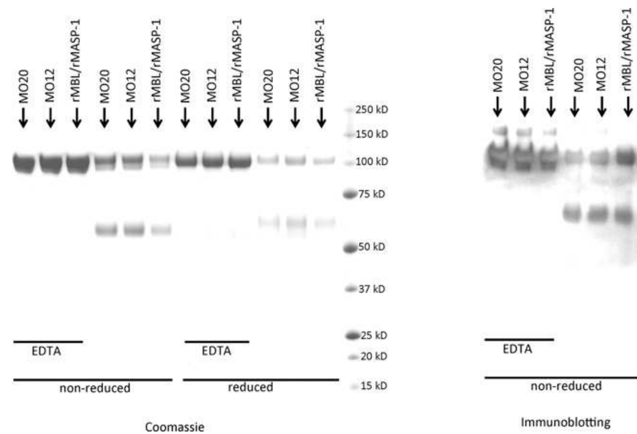

FIGURE 7. Cleavage of HMWK by rMASP-1 or plasma-derived MBL/MASP complexes (MO12 and MO20). Reduced and nonreduced SDS-PAGE/Coomassie staining (*left panel*). Nonreduced immunoblotting with polyclonal HMWK Abs (*right panel*).

MASP-2 (estimated mean, 0.5 mg/l) (29) compared with MASP-1 (different reports estimate means in the range of 7–25 mg/l) (3, 30) or the fact that the commercially available MASP-2 Ab is less applicable as an assay Ab compared with the MASP-1 Ab used in this study.

When we assessed the relative amount of MASP-1/C1-INH complex in different plasma types, we noted a profound difference with respect to heparinized plasma, for which we measured much less MASP-1/C1-INH complexes compared with NHS, hirudin, citrated, and EDTA plasma. It is well known that heparin is a stimulating factor for AT (31, 32), and we observed a more prominent MASP-1/AT complex formation in heparinized plasma, illustrating that C1-INH and AT share the same binding sites on MASPs.

Because of the putative involvement of MASP-1 in the kallikrein-kinin system, we assessed the serum levels of MASP-1 and the MASP-1/C1-INH complex content in a cohort of patients diagnosed with HAE and a matched, healthy control group. The HAE patients, who were in remission at the time of blood sampling, and the control group were also evaluated for several other parameters, and levels of relevant complement components were assessed. We found that MASP-1 serum levels and MASP-1/C1-INH levels were significantly reduced in the HAE cohort compared with the control group. In particular, the finding of significantly lower MASP-1 serum levels in HAE patients was surprising; we propose that ongoing contact activation results in MASP-1 activation and subsequent turnover and consumption that are reflected in this patient group. In addition, MASP-1/C1-INH complex formation was significantly lower in HAE patients, which might reflect both the MASP-1 Ag level or activation state and the C1-INH level and function. This reflects a different situation from what was shown recently for the classical pathway serine proteases C1r/s complex formation with C1-INH (16), which could indicate fundamental differences in the importance of the classical and lectin C1-INH complexes in HAE. Furthermore, positive correlations were observed between MASP-1 and C3 and C4 in HAE patients, whereas only C4 correlated with MASP-1 in the controls. Interestingly, MASP-1 concentration showed a negative correlation with the number of s.c. attacks and total number of attacks within the year of blood sampling, emphasizing a possible direct role for MASP-1 in the pathophysiology of HAE.

The antigenic level of MASP-1 and C1-INH correlated highly in HAE patients, but not in the controls, which could indicate that they follow a common path during activation and subsequent systemic clearance when C1-INH capacity is limited. A difference between the levels of MASP-1 and MASP-1/C1-INH was observed in relation to C4 and C3; both parameters correlated with C4, but only MASP-1 correlated with C3 in HAE patients. In general, C3 is not considered a relevant marker in HAE, but C4 plasma concentration and turnover are used as important parameters of disease status in HAE and are used as surrogate prognostic markers (35). Thus, the importance of this finding awaits further study.

Interestingly, an inverse correlation was seen between the total number of attacks and both MASP-1 Ag concentration and MASP-/C1-INH complex level. Additionally, we observed an inverse correlation between MASP-1/C1-INH level and C1-INH consumption where the patients with severe attacks were treated with exogenously added C1-INH.

These results indicate a hitherto unrecognized role for MASP-1 in the pathophysiology of HAE. Based on these findings, we hypothesize that C1-INH deficiency leads to uncontrolled activation and consumption of MASP-1, which could lead to further release of bradykinin from HMWK. This release could be an aggravating factor in HAE or even a central component in the disease pathophysiology, and it should be investigated thoroughly in future studies. In any case, it suggests that measurements of MASP-1 and the use of the novel MASP-1/C1-INH assay might be valuable tools in the evaluation of HAE.

In conclusion, we investigated the interaction properties and functional characteristics of C1-INH in relation to serine proteases from the LP. We show that substantial amounts of MASP-1/C1-INH complexes exist in NHS and that the levels of both MASP-1 and MASP-1/C1-INH complexes in HAE patients are reduced and correlate with the degree of complement C4 consumption and with several indicators of disease severity. These data indicate a connection between MASP-1 and HAE and suggest that the LP serine proteases may play a previously unrecognized role in the pathophysiology of HAE. We propose that MASP-1 Ag and MASP-1/C1-INH complex measurements should be additional analyses to consider in relation to HAE.

Disclosures

The authors have no financial conflicts of interest.

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