

Severe fibrosis in hepatitis C virus-infected patients is associated with increased activity of the mannan-binding lectin (MBL)/MBL-associated serine protease 1 (MASP-1) complex

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Introduction

Hepatitis C virus (HCV) is the major aetiological agent of non-A, non-B viral hepatitis and is a significant cause of morbidity. Persistent chronic infection arises in a high percentage of patients predisposing to the development of cirrhosis and hepatocellular carcinoma [1,2]. An important factor in the pathogenesis of chronic HCV disease is liver damage sustained by the development of inflammation and tissue fibrosis. Scoring of these criteria is used as a measure of disease state and progression [3,4]. Although the mechanisms responsible for HCV persistence and disease pathogenesis are not well understood, it is likely that interactions between HCV and the host immune system play an important role.

Mannan-binding lectin (MBL) is a central component of the innate immune response and has been implicated in the defence against bacterial and viral disease. The importance

Summary

Mannan-binding lectin (MBL) binds microorganisms via interactions with glycans on the target surface. Bound MBL subsequently activates MBL-associated serine protease proenzymes (MASPs). A role for MBL in hepatitis C virus (HCV) infection had been indicated by previous studies examining MBL levels and polymorphisms in relation to disease progression and response to treatment. We undertook this study to investigate a possible relationship between disease progression and functional MBL/MASP-1 complex activity. A functional assay for MBL/MASP-1 complex activity was employed to examine serum samples from patients with chronic HCV infection, non-HCV liver disease and healthy controls. Inpatient consistency of MBL/MASP-1 complex activity levels was assessed in sequential samples from a subgroup of patients. Median values of MBL/MASP-1 complex activity were higher in sera from patients with liver disease compared with healthy controls. MBL/MASP-1 complex activity levels correlate with severity of fibrosis after adjusting for confounding factors ($P = 0.003$). MBL/MASP-1 complex activity was associated more significantly with fibrosis than was MBL concentration. The potential role of MBL/MASP-1 complex activity in disease progression is worthy of further study to investigate possible mechanistic links.

Keywords: fibrogenesis, HCV, innate immunity, mannan-binding lectin, mannan-binding lectin associated serine protease-1

of this molecule in the innate immune system relates to its multimeric structure, to its functions as an opsonin and as an adaptor for activation of MBL-associated serine proteases (MASPs). Functional MBL consists of higher order oligomers of a trimeric subunit. Each polypeptide within a subunit contains collagenous and cysteine-rich regions, which participate in the formation of trimers and larger oligomers, and lectin domains responsible for interaction with invading pathogens. Biologically functional MBL molecules are multimeric forms containing sufficient lectin domains for high avidity binding to carbohydrate patterns on pathogen surfaces [5–7]. Bound MBL enhances phagocytosis and associates with MASPs via the collagenous regions [5,6,8]. Polymorphisms in the promoter and structural regions of the MBL gene have been shown to affect oligomer formation and circulating levels of protein [7,9,10]. These mutations have been linked to increased susceptibility to disease. With regard to viral hepatitis, MBL polymorphisms

have been linked with disease progression in hepatitis B virus infection [11] and both disease progression and response to treatment in HCV infection [12–15]. These studies indicate a role for MBL in HCV disease, although the relationship between MBL/MASP complex activity and viral disease has remained unexplored.

Three MASPs have been described: MASP-1, -2 and -3. MASP-3 is an alternative splice product of the MASP-1 gene [16–18]. An alternative splice product from the MASP-2 gene, MAp19 lacks a serine protease domain, but also binds MBL [19]. There are many uncertainties concerning the exact composition of MBL-MASP complexes and the substrate specificities of the MASPs [20]. The functions of MASP-3 and MAp19 are currently unknown [16,19]. MASP-2 initiates the lectin pathway of the complement cascade via the cleavage of C4 and C2 [20]. While it has been shown that MASP-1 has some ability to cleave complement components, its contribution to complement activation is thought to be minor relative to MASP-2 [20]. MASP-1 has a thrombin-like spectrum of activity on substrates including fibrinogen and factor XIII [20–22]. Thrombin catalyses the production of fibrin from fibrinogen and acts as a hepatic stellate cell mitogen, thus contributing to fibrin deposition during fibrosis development [23–28]. MASP-1 may therefore stimulate the cross-linking of fibrin in a manner qualitatively similar to thrombin and so play a role in the development of fibrosis during HCV disease.

MBL and MASP-1 are produced and located in the liver [5,12,29,30]. The location and the proposed activity of MBL/MASP-1 complex activity in fibrin deposition indicate a potential role of MBL/MASP-1 complex activity in the development of HCV-related liver fibrosis. We have investigated serum levels of MBL and MBL/MASP-1 complex activity in patients with mild and severe chronic HCV infection defined by low and high fibrosis stage scores, respectively.

Patients and methods

Patients

Sera from patients with chronic HCV infection were obtained from the Trent HCV cohort study [31,32]. Samples from 34 blood donors were used as healthy controls. A further control population of 77 patients with alcoholic liver disease (ALD) or non-alcoholic fatty liver disease (NAFLD) was also analysed. Patients were defined as chronically infected with HCV by testing positive for HCV antibody [third-generation enzyme-linked immunosorbent assays (ELISAs), Ortho Clinical Diagnostics, Cardiff, UK; GE Healthcare, Little Chalfont, UK or Sanofi-Aventis, Guildford, UK] and for HCV RNA [Amplicor reverse-transcription polymerase chain reaction (PCR) assay, Roche, Lewes, UK]. Diagnostic liver biopsy specimens were scored using the Ishak modified histological activity index (HAI) and 147 patients were selected on the basis of having either mild

(Ishak fibrosis stages 0–1) or severe liver disease (Ishak fibrosis stages 5–6). Serum samples were taken within 6 months of biopsy, or in an interbiopsy period where both pre- and post-sample biopsies had the same stage. Risk factors for the route of transmission of HCV infection were categorized as intravenous drug use (IVDU), blood product transfusion (BPT) and unknown risk factor. The duration of infection at the time of biopsy and age at infection were estimated where possible by using the date given for the first exposure to risk. Average weekly alcohol intake at the time of biopsy was used as a measure of alcohol consumption and given as units per week. Whether patients had ever been heavy drinkers was recorded. This was defined as consumption of more than 50 units of alcohol per week for the majority of weeks during a 6-month period at any time prior to the date of biopsy. Past or present infection with HBV was determined by anti-HBc and HBsAg status. Patients with known HIV infection were excluded from our analysis. HCV genotype and subtype were determined by restriction fragment length polymorphism or by line probe assay (Inno-LiPA HCV Kit, Innogenetics, Ghent, Belgium). Analysis of MBL/MASP-1 complex activity variation over a 2-year time-period in a subgroup of patients was performed. Four sequential samples from patients containing high ($n = 3$), medium ($n = 3$) and low ($n = 3$) MBL/MASP-1 complex activity were analysed. The patients showed no evidence of progression of fibrosis during the sampling period. All serum samples used in this study were taken prior to the commencement of treatment and stored at -80°C . Approval from the local ethics committee was obtained as was informed consent.

Methods

Functional, oligomeric MBL was quantified in an ELISA-based assay, based on that described by Arnold *et al.* [33]. Briefly, Maxisorp plates (Nunc, Roskilde, Denmark) were coated with 50 μl mannan (1 mg/ml) in 0.1 M NaHCO_3 pH 9.5. After three washes with wash buffer [20 mM HEPES, 140 mM NaCl, 5 mM CaCl_2 , 0.1% (v/v) Tween 20, pH 7.4] wells were incubated with 200 μl blocking buffer [20 mM HEPES, 140 mM NaCl, 5 mM ethylenediamine tetraacetic acid (EDTA), 0.1% (v/v) Tween-20, pH 7.4] overnight at 4°C and washes repeated. Purified MBL, together with patient and control sera, were diluted 1 : 1 in serum dilution buffer (40 mM HEPES, 2 M NaCl, 10 mM CaCl_2 , pH 7.4) and 50 μl per well added. Following incubation wells were washed three times with wash buffer. Bound MBL was detected with polyclonal rabbit anti-MBL followed by alkaline phosphatase (AP)-conjugated monoclonal anti-rabbit IgG (Sigma, Poole, UK) in wash buffer. Substrate was p -nitrophenyl phosphate (Sigma). All samples were tested in duplicate.

The activity of MASP-1 bound to MBL was assayed by a previously described method [22]. White microfluor plates (Thermolabsystems, Altrincham, UK) were coated with

Table 1. Statistical analysis for independent association of continuous variables with mild and severe liver fibrosis.

Variable (units)	<i>n</i>	Fibrosis						<i>P</i>
		Mild			Severe			
		<i>n</i>	Median	Range	<i>n</i>	Median	Range	
MBL (µg/ml)	147	89	5.0	0.0–30.8	58	9.5	0.0–26.6	0.002*
MBL/MASP-1 complex activity (units/min)	147	89	80	0.91–567	58	171	1–492	≤ 0.001*
Age at biopsy (years)	147	89	38	21–78	58	45	22–79	≤ 0.001*
Duration of infection (years)	112	77	16	3–38	35	22	10–37	0.01*
Age at infection (years)	112	77	20	3–72	35	19	5–53	0.937

Mann–Whitney *U*-test for independent association of mannan-binding lectin (MBL)/MASP-1 complex activity, age at liver biopsy, duration of and age at hepatitis C (HCV) infection with mild or severe liver fibrosis in patients with chronic HCV infection. Median and range of values for each variable given by patients with mild and severe fibrosis are shown. The number of patients included in the analysis for each variable and within mild and severe groups is indicated by *n*. Significant values ($P < 0.05$) are highlighted (*)

50 µl of 1 mg/ml mannan in 0.1 M NaHCO₃ pH 9.5 for 1 h. After three washes with wash buffer wells were incubated with 200 µl blocking buffer overnight at 4°C and washes repeated. Pooled HCV-negative serum or HCV-positive patient sera (diluted 1 : 1) was diluted in serum dilution buffer and 50 µl per well added. Plates were incubated for 1 h on ice. Wells were washed twice at 37°C with high salt buffer [20 mM HEPES, 1 M NaCl, 5 mM CaCl₂, 0.1% (v/v) Tween 20, pH 7.4] and three times with wash buffer. VPR-AMC (Boc-Val-Pro-Arg-aminomethylcoumarin) substrate (Bachem, St. Helens, UK) was diluted to 0.1 mM in substrate dilution buffer (20 mM HEPES, 5 mM CaCl₂, pH 8.5) and 200 µl added per well. Excitation at 355 nm and emission at 460 nm was measured in a fluorescent plate reader to give MBL/MASP-1 complex activity in arbitrary units per minute. All samples were tested in duplicate.

Statistics

The association between fibrosis stage and each of the categorical variables was analysed using the χ^2 test. For non-normally distributed continuous variables the two groups were analysed using the Mann–Whitney *U*-test. Statistical correlation between MBL concentration and MBL/MASP-1 activity was tested by Spearman's rank test (two-tailed) and these terms were analysed separately against potential confounding factors. Binary logistic regression analysis using logarithmically transformed MBL/MASP-1 complex activity, and logarithmically transformed MBL concentration was performed to assess the effect of MBL concentration and MBL/MASP-1 complex activity on fibrosis, after adjustment for confounding factors. A crude univariate model included those factors for which significant associations with fibrosis were found by χ^2 or Mann–Whitney *U*-test as a second screening round to identify variables to be entered into the final models. Factors which were significant in the crude model were included in the two multivariate adjusted models. The relative significance of MBL concentration and MBL/MASP-1 activity in fibrosis was confirmed by including these variables in a binary logistic regression model. The

intergroup variation of MBL/MASP-1 activity was analysed by a one-way analysis of variance (ANOVA) test. In all tests a *P*-value of < 0.05 was considered statistically significant. Statistical analyses were performed using SPSS® statistical software package (version 12.0.1, Chicago, IL, USA).

Results

MBL/MASP-1 complex activity association with severe fibrosis in HCV-infected patients

A total of 147 patients with chronic hepatitis C were studied. Of these, 89 (60.5%) had mild liver fibrosis (Ishak fibrosis scores of 0 or 1) and 58 (39.5%) had severe liver fibrosis (Ishak fibrosis scores of 5 or 6). HCV patients analysed comprised 102 (69.4%) male and 45 (30.6%) female subjects. Age at the time of biopsy in this study ranged from 21 to 78 years. Serum samples within 6 months of the biopsy date or between two biopsies in the same category were assayed for MBL concentration against serial dilutions of MBL isolated from pooled human serum. The same samples were assayed for MBL/MASP-1 complex activity standardized against dilutions of a commercial pool of HCV-negative serum. MBL concentration and MBL/MASP-1 complex activity were assessed in a control group of healthy blood donors ($n = 34$). A further control group of patients with non-HCV liver diseases, namely ALD and NAFLD, was divided into those with mild fibrosis ($n = 52$) and those with severe fibrosis ($n = 25$). MBL concentrations were found to be significantly different between the mild and severe liver fibrosis groups of HCV patients ($P = 0.002$, Table 1) by the Mann–Whitney *U*-test. The median MBL concentration for the healthy control group was 2.9 µg/ml (range: 0.0–21.9 µg/ml), for the non-HCV mild fibrosis group was 1.7 µg/ml (range: 0.0–12.4 µg/ml), for the non-HCV severe group was 4.6 µg/ml (range: 0.0–20.2 µg/ml), for the HCV mild fibrosis group was 5.0 µg/ml (range: 0.0–30.8 µg/ml) and for the HCV severe fibrosis group was 9.5 µg/ml (range: 0.0–26.6 µg/ml) (Fig. 1a). Comparison of median values of MBL/MASP-1 complex activity revealed a significant

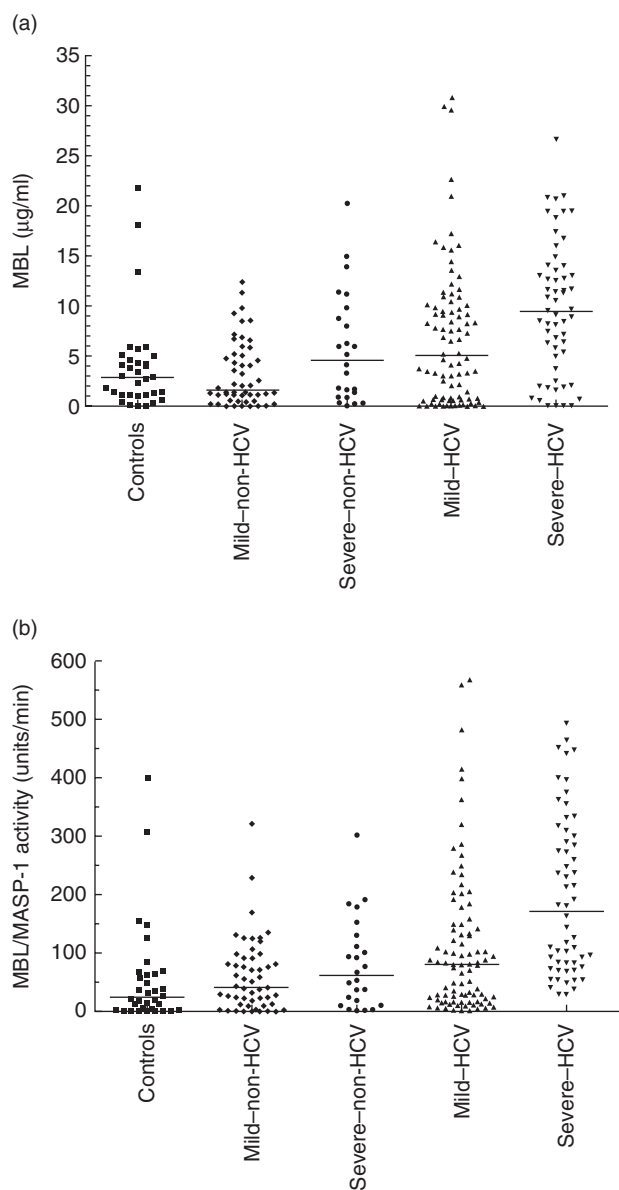


Fig. 1. Comparison of mannan-binding lectin (MBL) concentration (a) and MBL/MBL-associated serine protease-1 (MASP-1) complex activity (b) in patients with mild and severe hepatitis C (HCV)-associated liver fibrosis and healthy controls. MBL/MASP-1 complex activity determined by cleavage of synthetic fluorescent substrate, in chronically infected HCV patients with mild ($n = 89$) and severe ($n = 58$) liver fibrosis, compared with healthy blood donors ($n = 34$), non-HCV mild disease ($n = 52$) and non-HCV severe disease ($n = 25$). Units are $\mu\text{g/ml}$ for MBL and arbitrary fluorescent units per minute for MBL/MASP-1 complex activity. Horizontal bars indicate median values.

difference between the medians of mild and severe liver fibrosis groups of HCV patients ($P < 0.001$, Table 1). The MBL/MASP-1 complex activity for the healthy control group was 24.1 units/min (range: 0.0–398.4 units/min), for the non-HCV mild fibrosis group was 41.5 units/min (range: 0.0–320.5 units/min), for the non-HCV severe group was

62.0 units/min (range: 0.5–300.9 units/min), for the HCV mild fibrosis group was 80.3 units/min (range: 0.9–566.6 units/min) and for the HCV severe fibrosis group was 171.5 units/min (range: 1.1–492.4 units/min) (Fig. 1b). These results demonstrate an association of higher MBL/MASP-1 complex activity with liver disease, and an association with severity of fibrosis was particularly pronounced in the HCV-infected patients.

Identification and consideration of confounding factors

Several host factors may influence the degree of liver fibrosis in chronic infection with HCV including sex, co-infection with HIV or HBV, risk factor, ethnic group, infecting HCV genotype, alcohol intake, duration of infection, age at biopsy and age at infection with HCV. The associations between each of these factors and level of fibrosis were investigated in order to identify variables which could potentially have a confounding effect on the MBL concentration and MBL/MASP-1 complex activity and fibrosis relationships. Known HIV positive patients are excluded from the Trent Cohort Study and this variable was excluded from our analyses. No associations were found between fibrosis severity and age at infection (Table 1), HCV genotype, ethnic group, sex or past or present HBV infection (Table 2). Association between severe fibrosis and age at biopsy, duration of infection (Table 1), risk factor for HCV transmission and heavy drinking (Table 2) was identified in the cohort.

Those factors found to be associated significantly with degree of fibrosis were used in a crude univariate logistic regression model as a second screening round for confounding factors. HCV genotype was also included in a crude model, as this factor appeared close to a significant association with liver fibrosis by χ^2 test. Logarithmically transformed MBL concentration and MBL/MASP-1 complex activity was used in all logistic regression analysis. Having previously been a heavy drinker was not associated significantly with severe liver fibrosis in the crude model and was excluded from further analysis. Risk factor, HCV genotype, age at biopsy and duration of infection were associated significantly with severe liver fibrosis and used as covariates with MBL concentration and MBL/MASP-1 complex activity in two multivariate adjusted models. MBL concentration and MBL/MASP-1 complex activity were not included with potential confounders in the same multivariate model due to their strong positive correlation, confirmed by two-tailed Spearman's rank test ($\rho = 0.782$, $P = 0.01$). Crude and adjusted values for MBL concentration, MBL/MASP-1 complex activity, risk factor, HCV genotype, previous heavy drinking, age at biopsy and duration of infection are given in Table 3. The values for MBL concentration and MBL/MASP-1 complex activity in the adjusted models differ from those in the crude models. Higher MBL concentration remains significantly associated with severe fibrosis after adjusting for confounding factors ($P = 0.011$, OR 3.48

Table 2. Statistical analysis for independent association of categorical variables with mild and severe liver fibrosis.

Variable (<i>n</i>)	Category (<i>n</i>)	Proportion (%)		<i>P</i>
		Mild	Severe	
Risk (144)	IVDU (74)	58.0	41.1	0.020*
	BPT (32)	26.1	16.1	
	Unknown (38)	15.9	42.9	
Heavy drinker (110)	Yes (56)	43.1	62.2	0.048*
	No (54)	56.9	37.7	
HCV genotype (129)	1 (52)	45.5	32.7	0.051
	2, 4 or 5 (19)	18.2	9.6	
	3 (58)	36.4	57.7	
Ethnic group (139)	White (124)	92.8	83.5	0.082
	Other (15)	7.2	16.6	
Sex (147)	Male (102)	67.4	72.4	0.422
	Female (45)	32.6	27.6	
Anti-HBc (123)	Yes (52)	40.8	44.7	0.733
	No (71)	59.2	55.3	

χ^2 analysis for independent association of risk factor for acquisition of hepatitis C (HCV), history of heavy drinking, infecting HCV genotype, ethnic group, sex and past/present HBV infection with mild or severe liver fibrosis in patients with chronic HCV infection. Risk factors included intravenous drug use (IVDU) and blood product transfusion (BPT). Patients known to be heavy drinkers previous to the date of biopsy were defined as those having drunk more than 50 units a week for most weeks over 6 months. The proportions of patients with mild and severe disease within categories for each variable are given as percentage mild/severe (%). The numbers of patients included in the analyses for each variable and within each category are shown (*n*). Significant values ($P < 0.05$) are highlighted (*).

per log₁₀ units, 95% CI 1.33–9.15). Higher MBL/MASP-1 complex activity also remains significantly associated with severe fibrosis after adjusting for confounding factors ($P = 0.003$, OR 4.73 per log₁₀ units/min, 95% CI 1.67–13.98).

Duration of infection ($P = 0.018$, OR 1.092 per year, 95% CI 1.015–1.174) and infection with HCV genotype 1 ($P = 0.041$, OR 0.294, 95% CI 0.91–0.95) remain associated in the adjusted MBL model. Duration of infection ($P = 0.003$, OR

Table 3. Binary logistic regression analysis of mannan-binding lectin (MBL) concentration, MBL/MBL-associated serine protease-1 (MASP-1) complex activity and potential confounding factors in patients with mild and severe liver fibrosis.

Variable	Unadjusted				MBL (adjusted)				MBL/MASP-1 activity (adjusted)				
	OR	95% CI		<i>P</i>	OR	95% CI		<i>P</i>	OR	95% CI		<i>P</i>	
		Lower	Upper			Lower	Upper			Lower	Upper		
MBL concentration	1.781	1.106	2.867	0.018*	3.486	1.329	9.146	0.011*	NI	NI	NI	NI	
MBL/MASP-1 activity	4.670	2.140	10.150	$\leq 0.001^*$	NI	NI	NI	NI	4.730	1.667	13.980	0.003*	
Age at biopsy	0.459	1.036	1.112	$\leq 0.001^*$	1.063	0.999	1.131	0.054	1.059	0.996	1.125	0.067	
Duration of infection	0.459	1.034	1.153	0.002*	1.092	1.015	1.174	0.018*	1.081	1.008	1.160	0.003*	
Risk factor	a	Ref	Ref	Ref	0.003*	Ref	Ref	Ref	0.342	Ref	Ref	Ref	0.250
	b	0.268	0.118	0.611	0.002*	0.217	0.028	1.703	0.146	0.176	0.022	1.361	0.096
	c	0.228	0.083	0.629	0.004*	0.273	0.028	2.690	0.266	0.195	0.020	1.897	0.159
HCV genotype	a	Ref	Ref	Ref	0.044*	Ref	Ref	Ref	0.068	Ref	Ref	Ref	0.055
	b	0.437	0.201	0.952	0.037*	0.294	0.091	0.950	0.041*	0.283	0.087	0.925	0.037*
	c	0.321	0.102	1.011	0.052	0.269	0.056	1.293	0.101	0.237	0.047	1.205	0.083
Previous heavy drinker	2.176	1.000	4.725	0.050	NI	NI	NI	NI	NI	NI	NI	NI	

Binary logistic regression was performed on MBL concentration and MBL/MASP-1 complex activity in chronically infected hepatitis C (HCV) patients with mild and severe liver fibrosis. The outcomes of crude and adjusted ($n = 96$; mild = 65, severe = 31) models are shown. Adjusted models include infecting HCV genotype, risk factor for HCV infection, age at biopsy and duration of infection as confounding factors. Risk factor (a) unknown, (b) intravenous drug use and (c) blood product transfusion. HCV genotype (a) genotype 3; (b) genotype 1; (c) genotypes 2, 4 and 5. Logarithmically transformed MBL concentration and MBL/MASP-1 complex activity were used in all logistic regression analyses. Confidence intervals (CI) are stated and odds ratios (OR) shown. Significant values ($P < 0.05$) are highlighted (*). NI = not included. The adjusted values indicate significant association between fibrosis and MBL concentration, duration of infection and HCV genotype and association between fibrosis and MBL/MASP-1 complex activity, duration of infection and HCV genotype. For every log increase in MBL concentration the chance of having severe *versus* mild fibrosis is increased by 3.49 (OR) and for every log increase in MBL/MASP-1 complex activity the chance of having severe *versus* mild fibrosis is increased by 4.73 (OR).

1.081 per year, 95% CI 1.008–1.160) and infection with HCV genotype 1 ($P = 0.037$, OR 0.283, 95% CI 0.087–0.925) also remain associated significantly in the adjusted MBL/MASP-1 activity model. To investigate the interaction between MBL concentration and MBL/MASP-1 complex activity and duration of infection the variables were multiplied and included in a logistic regression model. The associated regression coefficient was close to 0 in both cases (data not shown) and it was concluded that MBL concentration and MBL/MASP-1 complex activity were independent of duration of infection. The greater significance of MBL/MASP-1 complex activity relative to MBL concentration in the development of liver fibrosis is indicated by higher OR and lower P -values for MBL/MASP-1 complex activity in both models. This was confirmed, as MBL/MASP-1 activity remained significant ($P = 0.001$, OR 7.26 per \log_{10} units, 95% 2.355–22.404) after adjustment for MBL concentration whereas significant association between MBL activity and fibrosis was lost ($P = 0.272$, OR 0.654 per \log_{10} units, 95% CI 0.307–1.396).

Analysis of correlation between MBL concentration and MBL/MASP-1 complex activity

MBL serum concentrations for each patient or control were compared with the corresponding MBL/MASP-1 complex activity to give a specific activity in terms of units/min/ μg of MBL. Specific activities of MBL/MASP-1 complexes differed markedly between individual patients. Grouping subjects based on healthy controls, non-HCV and HCV disease indicated a gradual rise in specific activity with group mean activities of 7.1 ± 2.6 units/min/ μg for controls, 12.6 ± 1.1 units/min/ μg for non-HCV liver disease patients and 16.0 ± 0.8 units/min/ μg for patients with HCV (Fig. 2). These results are consistent with the trends observed for increases in MBL and MBL/MASP-1 complex activity across the three groups.

Sequential analysis of MBL/MASP-1 complex activity levels

MBL/MASP-1 complex activity levels could be influenced by several genetic or environmental factors. We analysed four sequential serum samples from nine patients, taken over a period of between 13 and 31 months, during which time their biopsies showed no change in fibrosis. Patients were categorized into groups with low, medium or high MBL/MASP-1 complex activity. Inpatient mean activities of MBL/MASP-1 for each of the nine patients analysed showed little variation in activities across the four time-points (Fig. 3). Statistical analysis was performed using within-group modelling. Samples from patients with low MBL/MASP-1 activity showed little variation across time-points with an activity of 5.2 ± 1.6 units/min [mean \pm standard error (s.e.)]. Small variations in MBL/MASP-1 activity levels were also observed in the medium activity group, with a

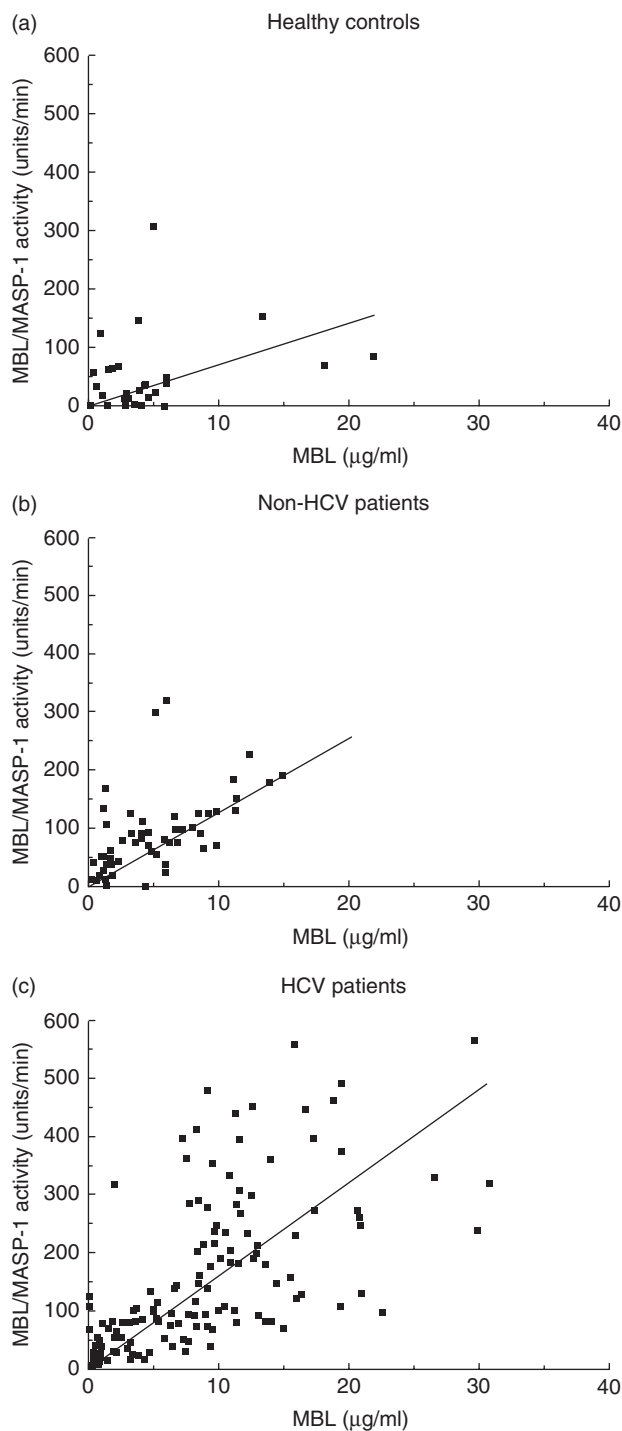


Fig. 2. Comparison of intrasubject mannan-binding lectin (MBL) concentration and MBL/MBL-associated serine protease-1 (MASP-1) complex activity. (a) Healthy controls ($n = 34$), (b) non-hepatitis C (HCV) liver disease ($n = 77$) and (c) HCV patients ($n = 147$) were compared. Linear regression analysis identified mean specific activities within the groups as 7.1 ± 2.6 units/min/ μg for blood donors, 12.6 ± 1.1 units/min/ μg for non-HCV liver disease patients and 16.0 ± 0.8 units/min/ μg for patients with HCV.

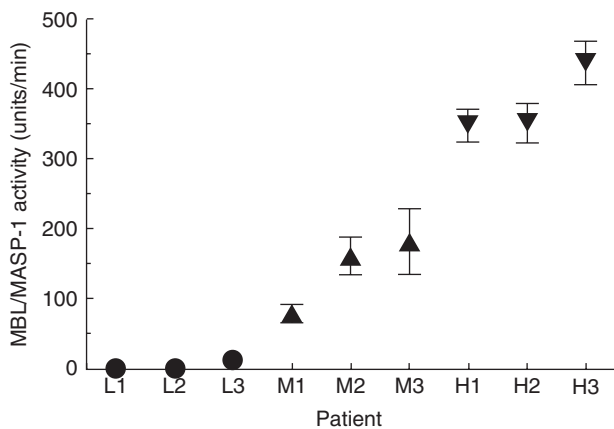


Fig. 3. Comparison of variation in mean mannan-binding lectin/MBL-associated serine protease-1 (MASP-1) (MBL/MASP-1) activity levels in patients identified as having low, medium and high activity. Four sequential serum samples from patients identified as having low (L), medium (M) and high (H) MBL/MASP-1 complex activity were analysed by the MBL/MASP-1 activity assay. The mean activity is marked as a closed circle, with standard error defined by vertical bars. These groups were identified as having significant differences in MBL/MASP-1 complex activity by the analysis of variance test, with an *F*-value of 96.2 ($P < 0.001$).

value of 149 ± 27 units/min and the high activity group, with a value of 376 ± 19 units/min. A one-way ANOVA test was performed to determine the significance of differences in MBL/MASP-1 complex activity levels between each group. The three groups were shown to be distinct with an *F*-value of 96.2 ($P < 0.001$).

Discussion

The development of liver fibrosis in HCV-infected patients remains poorly understood. A previous report examining an HCV outbreak in a plasmapheresis centre identified a wide pattern of disease progression in a group of patients infected at the same time with the same virus strain [34]. This sustains the idea that host factors are involved critically in the development of fibrosis. In the present study we have identified that the serum level of MBL and the enzyme activity of MBL/MASP-1 complexes was raised in HCV patients with severe fibrosis compared to those with mild fibrosis, other non-HCV liver disease or healthy controls. We analysed the relationship of factors likely to affect liver fibrosis for independent association with the development of severe fibrosis. Within our cohort, age at biopsy, duration of infection, risk factor for the transmission of HCV infection and having previously been a heavy drinker were all associated independently with severe liver fibrosis. The relationship of these factors to severe disease is not evident in all cohorts, but indicates that our cohort is not unusual [31,32,34]. We determined that individually the association of MBL concentration and MBL/MASP-1 complex activity with HCV-related

severe fibrosis remained significant in light of potentially confounding factors. MBL/MASP-1 complex activity was found to be associated more significantly with severe disease than MBL concentration.

Increased MBL concentration in HCV patients in comparison to controls and a link to more severe hepatitis has been shown previously, although statistical significance was not reached. In our study, the difference in MBL levels for mild *versus* severe fibrosis in HCV patients was statistically significant, while levels in the severe HCV group only were significantly in excess of control levels of MBL. We also observed increased levels of MBL in non-HCV liver disease patients with severe fibrosis compared with mild fibrosis, although this difference was not statistically significant.

The correlation between MBL/MASP-1 complex activity and fibrosis stage scoring presented in this study could result from alteration of the hepatic gene expression profile seen during HCV disease [35,36] or inherent host genetic factors affecting MBL or MASP-1. Increased activity of the MBL/MASP-1 complex may be due to factors affecting MBL levels, oligomeric structure or MASP-1 regulation. While other investigators have determined serum levels of MBL and MBL polymorphisms in relation to viral hepatitis [12–15], we subsequently analysed the enzyme activity of the MBL/MASP-1 complex to determine a functional link with disease. Synthesis of both MBL and MASP-1 is liver-specific and both promoter sequences contain control elements which may be affected by the altered environment of the liver during HCV infection. Indeed, MBL/MASP-1 complex activity is increased in both our liver patient groups compared to controls.

The observed different specific activities for MBL/MASP-1 complexes in different groups suggests that MASP-1 regulation, independent of MBL regulation, might be responsible for the increased MBL/MASP-1 complex activity observed in HCV liver disease and non-HCV liver disease compared with healthy controls. In these instances MASP-1 may compete successfully against the other MASPs for MBL binding. This is consistent with the observation of variable occupancy of MBL molecules described recently [37].

Liver fibrosis is the main complication of chronic liver disease. A role of the coagulation cascade in the development of fibrosis has been suggested. Activation of coagulation relies on the cleavage of prothrombin to thrombin and subsequent conversion of fibrinogen to fibrin. Accumulation of fibrin in the liver during acute and chronic liver injury has been observed [26–28]. Thrombin itself may influence fibrinogenesis through generation of a fibrin clot inducing local hypoxia, thought to be a fibrotic co-factor, or via the stimulation of hepatic stellate cell proliferation and collagen synthesis [23–25]. Thrombotic risk factors including anti-thrombin III, protein C, plasminogen deficiency, Factor V Leiden polymorphisms (conferring activated protein C resistance), have been found to be associated with the extent of fibrosis in chronically infected HCV patients [38,39]. MASP-1 has a reactivity profile similar to thrombin and may

therefore cleave factor XIII and fibrinogen for fibrin cross-linking and deposition in a similar manner [20,22]. This function of MASP-1 may be relevant to the innate immune system in the development of the fibrin clot, where localized coagulation helps prevent the spread of microorganisms within the host [40]. Fibrin deposition may also encourage opsonization [41,42]. MASP-1 acts on fibrinogen to release fibrinopeptides A and B [21], which are potent chemoattractants for neutrophils, fibroblasts and monocytes [43].

This study is not able to distinguish between the possibilities that either raised MBL/MASP-1 complex activity is causative in fibrogenesis or whether severe fibrosis is associated with increased production of MBL or MASP-1. While MBL/MASP-1 complex activity levels are higher in each group of patients with severe liver disease compared with their mild disease counterparts, it is interesting to note that this effect is more marked in the HCV patient groups than non-HCV controls. There is clearly an overlap in MBL/MASP-1 complex activity between mild and severe liver disease groups. It will be of interest to determine whether MASP-1 levels in patients with mild disease are predictive or not of further disease progression. Such a longitudinal study is currently under way and may help us to resolve the cause/effect dichotomy.

In conclusion, our results show an association between MBL/MASP-1 complex activity and fibrosis development in HCV-infected patients. These results are intriguing, as the synthetic function of the liver decreases with progression to cirrhosis, yet we observe increased levels of a functional protein complex. This initial observation is being pursued to determine whether there is a causal link between MBL/MASP-1 activity and liver fibrosis in HCV patients.

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