Article

Specifically Binding of L-ficolin to *N*-glycans of HCV Envelope Glycoproteins E1 and E2 Leads to Complement Activation

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L-ficolin, one of lectin families, is a recently identified complement factor that initiates lectin pathway of complement. Little is known about its role in viral hepatitis. In the present study, we found that L-ficolin in serum from 103 patients with hepatitis C virus (HCV), were significantly higher than that in 150 healthy controls. We further found that L-ficolin expressions were significantly increased *in vitro* study by HCV JFH-1 infected human hepatocyte cell line Huh7.5.1. Investigation of the mechanisms of the L-ficolin action on HCV demonstrated that L-ficolin protein could recognize and bind to envelope glycoproteins E1 and E2 of HCV, activating the lectin complement pathway-mediated cytolytic activity in HCV-infected hepatocyte. This interaction between L-ficolin and HCV E1 and E2 glycoproteins was attributed to the *N*-glycans of E1 and E2. These findings provide new insights into the biological functions of L-ficolin in clinically important hepatic viral diseases. *Cellular & Molecular Immunology*. 2009;6(4):235-244.

Key Words: L-ficolin, hepatitis C virus, envelope glycoproteins, complement, viral hepatitis

Introduction

Hepatitis C virus (HCV) infects 170 million people worldwide, and about 80% of infected individuals develop chronic hepatitis with the risk of progression to cirrhosis and hepatocellular carcinoma (1). Up to now, there is no vaccine or effective therapy for HCV. The lack of an efficient viral culture system and a small animal infection model has hampered HCV researches (2). However, the production of infectious HCV in tissue culture was established recently from a cloned JFH-1 viral genome and has been widely applied in many labs around the world, thus, playing an important role in HCV related researches and drug discovery (3-5).

HCV is an enveloped RNA virus that is classified in the Flaviviridae family. The envelope consists of two heavily glycosylated proteins, E1 and E2, in which E1 has five potential N-glycosylated sites and E2 has eleven potential N-glycosylated sites (6). These N-glycosylation sites are well conserved, suggesting that they play essential roles in the function of these proteins. E2 is thought to initiate viral attachment, whereas E1 may be involved in virus-cell membrane fusion (7, 8).

The complement system plays an important role in innate immunity and can be activated through three different pathways namely classical, alternative, and the lectin pathways. These three pathways merge at the proteolytic activation step of C3 and activate the complement late components, C5-C9, leading to cytolytic complex (membrane attack complex) formation (9).

Lectin pathway is initiated either by mannan-binding lectin (MBL) or ficolins which are typical pattern recogning molecules and able to recognize conserved pathogen-associated molecules patterns (PAMPs) shared by large group

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Abbreviations: HCV, hepatitis C virus; PBMC, peripheral blood mononuclear cells; DC, dendritic cells; BCECF, bis-carboxyethyl-carboxyfluorescein; MBL, mannan-binding lectin; PAMP, pathogen-associated molecules pattern; GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

of microorganisms, thereby successfully defending hosts against infection. The binding of the lectin pathway recognition molecules (MBL or ficolins) to microbial carbohydrates can activate complement by a mechanism similar to the classical pathway, but using MBL associated serine proteases instead of C1r and C1s (9-11).

Three types of ficolins have recently been identified in human, L-ficolin/P35 (FCN2 or ficolin 2) (12), M-ficolin (FCN1 or ficolin 1) (13, 14), and H-ficolin/Hakata antigen (FCN3 or ficolin 3) (15-17), and two types of ficolins, ficolins A and B, have been identified in mice (18, 19). The fibrinogen-like domain of L-ficolin, forming a globular structure like the CRD of MBL, is the pattern of carbohydrate structures. L-ficolin is primarily expressed in the liver and subsequently released into the blood stream (20).

The human L-ficolin is structurally similar to the C1q, MBL and lung surfactant proteins A and D (SP-A and SP-D) (11, 20), but the ligand specificities for the ficolins and MBL differ. Whereas MBL reacts strongly with mannose and fucose, and L-ficolin binds to GlcNAc and does not bind to mannose. L-ficolin binds to GlcNAc residue next to galactose at the nonreducing terminal of the oligosaccharide (21). Several reports recently showed that L-ficolin could bind to various acetylated compounds, 1,3-β-D glucan, a molecular marker of yeast and fungal cell walls, lipopolysaccharide (LPS) on gram-negative bacterial species and Lipoteichoic Acid (LTA) on the gram-positive bacteria *Staphylococcus aureus* and *Streplococcus pneumoniae*, following the activation of complement (22-25).

The association of MBL with viral hepatitis has been investigated by several authors (26, 27); the interactions between HCV envelope glycoproteins E1 and E2 and MBL has been demonstrated *in vitro* and mutant MBL2 haplotypes have been linked to disease progression in HCV infection (26). However, the relationship between L-ficolin and viral hepatitis remains elusive.

Materials and Methods

Cell lines, bacteria and reagents

Human hepatocyte cell lines Huh7.5.1 and human hepatoma cell lines HepG2 were used in this study. *E. coli* BL21(DE3)/plysS and wild type *Salmonella typhimurium* C5 were used as described previously (28). The bacteria were harvested by centrifugation at 13,000× g and suspended in sterile PBS at the appropriate concentration. Bacteria CFU (colony forming units) were quantified from the absorbance at 600 nm. The anti-E1 or anti-E2 polyclonal antibodies were prepared as our previous publications (29, 30).

Clinical speciments

This study was approved by the local ethical committee and samples were registered as HCV patients from 2006 and 2008 in the Center for Disease Control and Prevention of Wuhan in China. All patients were previously identified as HCV-seropositive by commercial ELISA tests or as HCV-RNA positive by commercial real-time RT-PCR kits. Sera samples of patients and controls were collected from 103 HCV-positive patients (either HCV antibody positive or HCV RNA positive, and other pathogens negative) and 150 healthy donors.

Measurement of L-ficolin concentrations

The sandwich Enzyme-Linked Immunosorbent Assay (ELISA) method was used to measure the concentrations of serum L-ficolin, basically according to methods as described in previous publications (31). Briefly, 96-well ELISA plates was coated with 100 μg/mL poly-L-lysine. After incubated at room temperature (RT) for one hour, removed the soluion, rinse it with water and dry. One hundred μl of inactivated *Salmonella typhimurium* C5 (1×10^6 CFU/well) was used to coat 96-well ELISA plates at 37°C for 1 h, since heat (100°C for 5 min) inactived *Salmonella typhimurium* C5 still keeps LPS which can bind with L-ficolin.

After washing three times using 0.2% Tween 20 in PBS, 100 μl fresh sera from each sample was added and incubated at 37°C for 2 h. The plates were washed three times and the nonspecific binding sites were blocked with 5% BSA overnight. Then, mouse monoclonal anti-human L-ficolin GN5 (1:1000 dilution) (HyCult Biotechnology *b.v.*) was added to each well and incubated at 37°C for 1 h. The plates were washed three times and then incubated with 100 μl labeled horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (1:1000 dilution). Color development was achieved by adding 100 μl/well of ready-to-use tetramethylbenzidine (TMB) chromogen-substrate (Sigma), and incubated for 15 min at room temperature. The reaction was stopped by adding 100 μl of 0.5 M H_2SO_4 per well and the optical density (OD) at 450 nm was measured using an ELISA reader.

HCV infection of human hepatocyte

To generate infectious HCV JFH1, *in vitro* transcribed genomic JFH1 RNA was transfected into Huh7.5.1 cells and supernatants of HCV cell culture (HCVcc) were used to infect naïve Huh7.5.1 cells as previously described (4, 32). Huh7.5.1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (HyClone) and penicillin-streptomycin.

Vector Construction

Human L-ficolin cDNA (GenBank acc. No: NM 004108) was amplified and subcloned in-frame into the *Eco*R I and *Xho*I sites of the prokaryotic expression vector pGEX-KG (Amersham Biosciences), yielding the expression plasmid pGEX-KG-Lficolin. The oligonucleotide primers were synthesized as follows: Forward primer: 5'-CAG AAT TCC TCT CCA GGC GGC AGA CAC-3', and reverse primer: 5'-GAC TCG AGC TAG GCA GGT CGC ACC TTC A-3', where the underlined sequences represent the *Eco*RI and *Xho*I sites, respectively. The sequence of the construct was confirmed by restriction enzyme digestion along with DNA sequencing analysis.

Purification of recombinant L-ficolin and preparation of polyclonal antibody against L-ficolin

pGEX-KG-L-ficolin was transformed into *E. coli* BL21(DE3)[pLysS] (Invitrogen) (28). The L-ficolin-GST fusion protein was overexpressed in *E. coli* after induction by Isopropyl β-D-1-Thiogalactopyranoside (IPTG). The expressed protein was purified by Glutathione Sepharose 4B (Amersham Biosciences) and identified by SDS-PAGE and Western blot anaysis. L-ficolin protein was obtained from L-ficolin-GST-sepharose 4B by thrombin cleavage through thrombin cutting site between GST and L-ficolin. Similarly, GST protein was overexpressed in pGEX-KG transformed *E. coli* after the induction of IPTG and purified from Glutathione sepharose 4B. The purified GST-L-ficolin, L-ficolin and GST proteins were determined by SDS-PAGE and Western blot. The purified L-ficolin-GST protein was used to immunize rabbits to generate anti-L-ficolin polyclonal antibodies.

RNA quantification by real-time fluorescence quantitative PCR

Total RNA was extracted from the cell lysates using TRIZOL (Invitrogen). The HCV primers were designed based on HCV sequence (GenBank Accession # M67463) selected within 5'-NCR of HCV genome basically according to previous method (33). RNA was quantitated by the real-time reverse transcription polymerase chain reaction using the QuantiTect^{TM} SYBR Green PCR Handbook Kit (QIAGEN) with the primers P1: 5'-CGG GAG AGC CAT AGT GGT CTG CG-3' (130 ~ 152 nt); and P2: 5'-CTC GCA AGC ACC CTA TCA GGC AGT A-3' (287 ~ 311 nt) (i.e., specific for HCV RNA), or the primers P3: 5'-GTA CAG CTC ATT CCA GGT-3' and P4: 5'-AGT TGA TGC CAT CCG CA-3' (i.e., specific for L-ficolin), or the primers GAPDH-F: 5'-ACC ACA GTC CAT GCC ATC AC-3'; GAPDH-R: 5'-TCC ACC ACC CTG TTG CTG TA-3' (i.e., specific for housekeeping gene GAPDH).

SDS-PAGE and Western blot analysis

Different doses of HCVcc were added and incubated with Huh7.5.1 cells in the six-well plate for 48 hrs at 37^{o}C in a 5% (v/v) CO_2 atmosphere. Two μM monensin (eBioscience), an inhibitor of intracellular protein transport, was added into each well for 4 h to block L-ficolin releases. The cell lysates samples were electrophoresed in 12 % SDS-polyacrylamide gels under reducing conditions. After transfer from the gels to polyvinylidene difluoride membranes (Millipore), the proteins were probed with anti-L-ficolin, anti-E1 or anti-E2 polyclonal antibodies (29, 30), and then incubated with HRP conjugated anti-rabbit IgG (1:2000 dilution). Color was developed with Nitroblue-tetrazolium and Bromo-chloro-indolyl-phosphate.

Establishment of stable cell lines

The recombinant eukaryotic expression vectors pcDNA3.1-Myc-HisB-E1 and pcDNA3.1Myc-HisB-E2 were transfected into HepG2 cells, respectively, with lipofectamineTM 2000 (Invitrogen) (29, 30). For stable transfections, G418 was added to the cell media at a final concentration of 0.6 mg/ml 48 hs post-transfection. The cell medium was changed every 2 days. After 2 weeks of selection, E1-, E2-expressing HepG2 were obtained by G418 selection, and identified by SDS-PAGE and Western blot.

In order to detect the cell surface expression of the E1, E2 proteins on E1-, E2-HepG2, 10^6 cells were incubated with rabbit anti-E1 or E2 polyclonal antibody (1 : 1000) in PBS respectively, at 37^{o}C for 2 hs. The cells were washed 3 times and incubated with 1 μl goat PE-anti-rabbit IgG (eBioscience) in PBS at room temperature for 30 min. The stained cells were analyzed using a Beckman Coulter EPICS ALTRA II flow cytometer. All results were statistically analyzed.

GST-pull down analysis

Cell lysates from 10^6 E1-, E2-HepG2 cells preincubated with 50 μM 1-deoxynojirimycin (DNM, an *N*-glycosylation inhibitor) (Sigma) for 48 h, were mixed with L-ficolin-GST-Sepharose 4B at 4^{o}C with rotation for 2 hs. The cell lysates were then incubated with L-ficolin-GST-Sepharose 4B or GST-Sepharose 4B at 4^{o}C with rotation for 2 hs. The samples were centrifuged at 1000 g for 5 min, and pellets were washed three times with PBS. The pellets were dissolved in 2× SDS-PAGE sample buffer and subsequently used for SDS-PAGE and Western blot analysis with anti-E1 or -E2 antisera.

In addition, the cell lysates of 1×10^6 E2-HepG2 , or HCVcc-Huh7.5.1 cells (1×10^4 copies of HCVcc incubated with 1×10^6 Huh7.5 cells for 48 h) were boiled and digested with PNGase F (Glyko, Prozyme, CA) at 37^{o}C overnight according to the manufacturer's recommendations, and then incubated with L-ficolin-GST-Sepharose 4B at 4^{o}C with rotation for 2 hs. The samples were centrifuged at 1000 g for 5 min, and pellets were washed three times with PBS. The pellets were dissolved in 2× SDS-PAGE sample buffer and subsequently used for SDS-PAGE and Western blot analysis with anti-E2 or anti-L-ficolin polyclonal antibodies.

Binding assay by flow cytometry analysis

To analyze the binding interaction between L-ficolin protein and E1-, E2-HepG2 or HepG2 cells, 10^6 of E1-, E2-HepG2 or HepG2 cells were incubated with different amounts of recombinant L-ficolin-GST, L-ficolin or GST control proteins at room temperature for 2 hrs. Each concentration was run in triplicate. Cells were then washed with PBS 3 times. Rabbit anti-GST-L-ficolin polyclonal antibody (1:100) in PBS was added and incubated at 37 $^{\circ}$C for 1 h. Cells were washed again 3 times. Subsequently, goat anti rabbit FITC-IgG antibody was added and then incubated at 37 ^{o}C for 30 min. The stained cells were examined by flow cytometry. All results were statistically analyzed.

Complement C4 activation and deposition assay

Lectin pathway activation was quantified using the C4 deposition assay developed by Petersen et al (34). MBL and ficolin-depleted serum was generated by incubating fresh

Table 1. Comparative results of serum L-ficolin levels in the samples of 103 HCV patients and 150 healthy donors

Characteristic	Healthy donors[A] (n = 150)	HCV patients[B]			*p* value (B *vs* A)
		CIH (n=59)	CAH (n=23)	LC (n=21)	
Sex					
Female	75	26	10	11	
Male	75	33	13	10	
Median Age (yr)	45	45	54	55	
L-ficolin (μg/ml)	4.67 ± 0.19	5.7 ± 0.32	7.4 ± 0.28	8.2 ± 0.55	< 0.05

CIH, Chronic inactive HCV; CAH, Chronic active HCV; LC, Liver cirrhosis. The statistical analyses were performed by using the Student's *t*-test. Differences were considered to be statistically significant for *p*-value < 0.05.

human serum with D-Mannose-agarose beads (Sigma) and N-Acetyl-D-glucosamine-agarose beads (Sigma).

C4c deposition on the E1-, E2-HepG2 cells was measured as following method: 96-well microtiter plates were pretreated with 100 μg/ml poly-L-lysine at RT for 1 h, and then washed with TBS and dried. E1- or E2-HepG2 cells (10^4 cells/well) were cultured in the microtiter plates for 6 hs and then fixed by adding 125 μl/well of 4 % formalin at RT for 15 min. Wells were washed with TBS-Ca^{2+}/Tween 20 buffer and then blocked with TBS/Ca^{2+} buffer containing 0.1 % BSA at 37°C for 2 h. After washing, human serum and purified recombinant L-ficolin protein were added and incubated in buffer with 20 mM Tris-Cl, 1 M NaCl, 10 mM $CaCl_2$, 0.05 % Triton X-100, 0.1 % BSA, pH 7.4. After incubation at 4°C overnight the plates were washed with PBS thoroughly and 0.1 μg of purified human C4 protein (Diagnostic Biosystems) was added and incubated at 37°C for 1.5 h. The plates were washed again before adding and incubating with FITC-conjugated rabbit anti human C4c (Diagnostic Biosystems) (1:100 dilution) at room temperature for 30 min. After wash with PBS, the absorbance was measured at 485 nm using a microplate reader.

C4c deposition on the HCVcc coated plate was measured as below method: 96-well plates were coated with HCVcc infectious particles (3×10^5 virus in 180 μl/well) in carbonate buffer, pH 9.6, or cell culture media, for 7 h at 4°C and blocked with 1 % BSA at 4°C overnight. L-ficolin protein was added into the coated plate in the presence of human serum and C4 protein, The plates were washed and incubating with FITC-conjugated rabbit anti human C4c

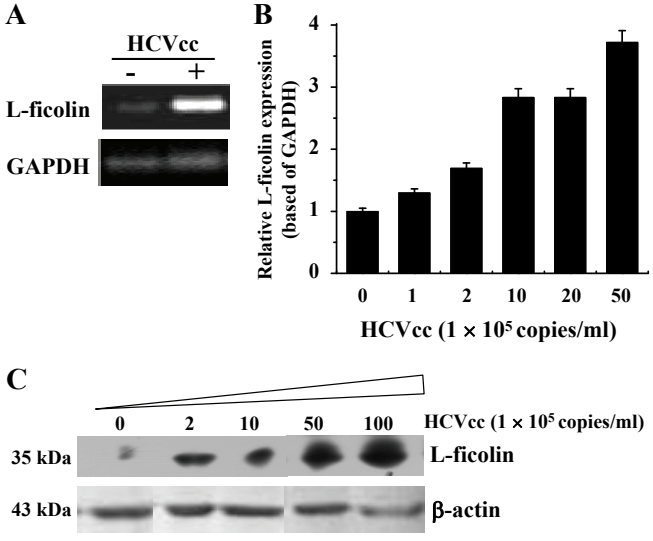

Figure 1. Increased L-ficolin expression in Huh7.5.1 cells after HCVcc infection *in vitro*. (A) Increased L-ficolin mRNA expression was observed *in vitro* study by HCVcc infected Huh7.5.1 cells by RT-PCR. (B) Statistical analysis of the L-ficolin mRNA increased in HCVcc infected Huh7.5.1 cells by real-time PCR. (C) Increased L-ficolin protein expression was observed *in vitro* study by HCVcc infected Huh7.5.1 cells by Western blot with anti-L-ficolin polyclonal antibody. β-actin was used as internal control.

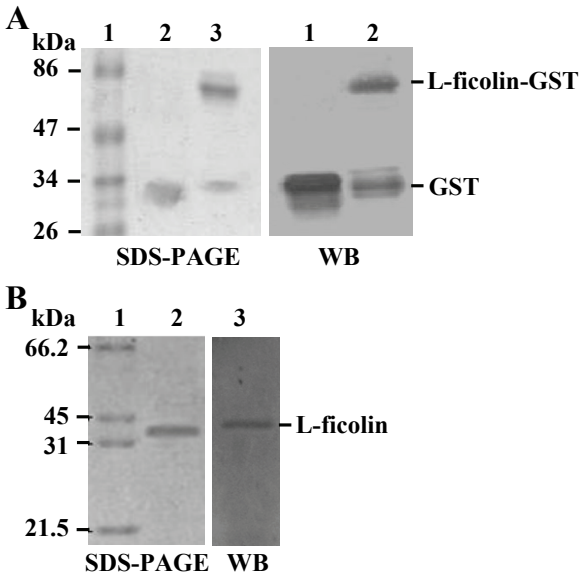

Figure 2. Identification of recombinant L-ficolin protein. (A) The purified GST-L-ficolin and GST proteins were analyzed by SDS-PAGE and Western blot with anti-L-ficolin-GST polyclonal antibody. (B) The purified L-ficolin protein was analyzed by SDS-PAGE and Western blot.

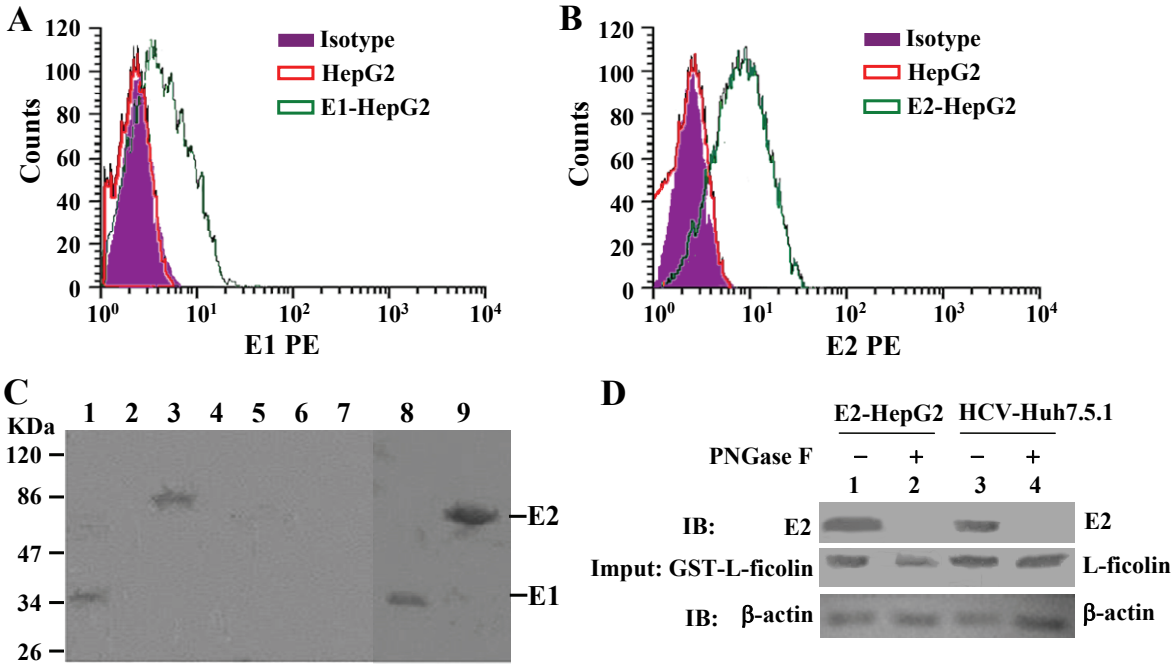

Figure 3. Interaction between L-ficolin and HCV E1/E2 glycoproteins is attributed to the N-glycans of E1/E2. (A, B) Flow cytometric analysis of E1-surface expressing cells (A) and E2- surface expressing cells (B) with anti-E1, or anti-E2 rabbit poly- clonal antibodies and PE-anti-rabbit IgG, respectively. (C) N-glycans of HCV E1 and E2 interacted with L-ficolin-GST protein by GST-pull down and Western blot analysis with HCV positive sera. Lane 1, E1-HepG2 + L-ficolin-GST; Lane 2, E1-HepG2 + GST; Lane 3, E2-HepG2 + L-ficolin-GST; Lane 4, E2-HepG2 + GST; Lane, 5HepG2 + L-ficolin-GST; Lane 6, E1-HepG2 + L-ficolin-GST + DNM; Lane 7, E2-HepG2 + L-ficolin-GST + DNM; Lane 8, E1-HepG2; Lane 9, E2-HepG2. (D) E2-HepG2 cells and HCV (JFH-1)-HuH7.5.1 cells were treated by PNGaseF digestion, respectively. Non-glycosylated E2 proteins from E2-HepG2 cells or HCV (JFH-1)-HuH7.5.1 cells by PNGaseF pretreatment did not interact with L-ficolin by GST-pull down and Western blot analysis with anti-E2 or anti-L-ficolin polyclonal antibodies.

(1:100 dilution) at RT for 30 min. After wash with PBS, the absorbance was measured at 485 nm using a microplate reader.

Measurement of Complement Mediated Cytolytic Activity

Complement lectin mediated cytolytic activity by the bis-carboxyethyl-carboxyfluorescein (BCECF) release assay was performed basically as previously described (35). The classic complement pathway activation was blocked by diluting buffer with hypertonic (1 M) NaCl. The endogenous L-ficolin and MBL of healthy donor serum were excluded by affinity chromatography on GlcNAc-agarose (Sigma-Aldrich) and MBL-agarose. Huh7.5.1 cells (1×10^4) were applied as the target cells and cultured in 96-well plates pretreated with 100 μg/ml poly-L-lysine and 100 μl HCVcc supernatant (5×10^4 HCV infectious particle) was added to each well and incubated at 37°C for 24 h. Then, 100 μl of 2 mg/L BCECF-AM was added to each well and incubated in a humid box at 37°C for 30 min. The supernatants were discarded and cells were washed three times with buffer (10 mM Tris-HCl, 140 mM NaCl, 5 mM $CaCl_2$). Recombinant L-ficolin protein or GST protein and fresh MBL and ficolin-depleted donor serum (100 μl) were added to each well and incubated in a humid box at 37°C for 30 min. The supernatant was collected and the fluorescence value (FI_{sup}) was measured at 503 nm. Cell lysis buffer (50 mmol/L Tris-HCl, 0.3 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L dithiotheitol and 1 mol/L phenylmethylsulphonyl fluoride) was added to each well to measure the residual fluorescence (FI_{lys}) at 503 nm. The rate of cell lysis mediated by complement lectin in the serum was calculated according to the formula: BCECF release rate or cytolytic activity (%) = $FI_{sup} / (FI_{sup} + FI_{lys}) \times 100$ %.

Data analysis

The data were analyzed by student's *t* test using Origin 6.0 and SPSS15.0 software.

Results

Significantly increased serum L-ficolin concentration in HCV-infected patients

Serum L-ficolin concentrations were examined by a sandwich ELISA method using samples from 103 HCV infected patients, and 150 healthy controls. Serum L-ficolin levels in HCV patients were significantly higher than those in healthy controls (HCV patients *vs* Healthy, $p < 0.05$, *t*-test) (Table 1). The mean level of L-ficolin in HCV patients (including chronic inactive-HCV patients, chronic active-HCV infected patients and liver cirrhosis HCV patients) was

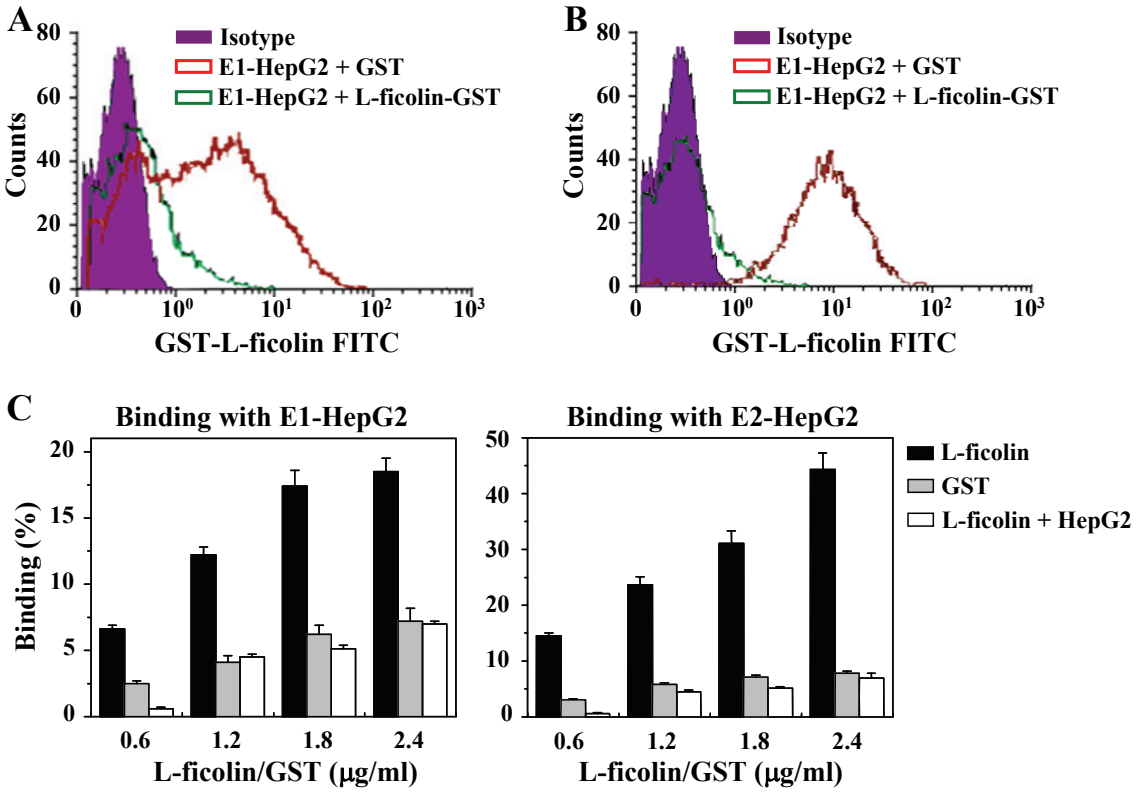

Figure 4. Interaction between L-ficolin and E1/E2 glycoproteins is L-ficolin dose-dependent. (A, B) Representive data of interaction between L-ficolin-GST, GST proteins with E1, E2-HepG2 cells by flow cytometric analysis. (C) Interaction between L-ficolin, GST proteins with E1, E2-HepG2 cells or control HepG2 cells by flow cytometric analysis. Experiments were repeated for six times. Data are represented as mean ± SEM.

7.2 μg/ml, while that of healthy controls was 4.67 μg/ml. Our results for L-ficolin concentrations of healthy donors were similar to previous report that the median concentration of L-ficolin in blood donor sera was found to be 3.7 μg/ml, with most values below 6.0 μg/ml (31). In addition, we observed that the L-ficolin levels correlated with the severity of fibrosis and the active state of HCV infection as follows: HCV infected liver cirrhosis patients > chronic active HCV-patients > chronic inactive- HCV patients (Table 1). These data demonstrate that L-ficolin activity plays a potential role in disease progression.

HCV infection causes increased expression of L-ficolin in human hepatocytes in vitro

Previous researchers have reported that L-ficolins are serum proteins mainly synthesized in the livers (9, 11). To test the effect of HCV infection on L-ficolin expression in human hepatocytes, we infected Huh7.5.1 cells with HCVcc (JFH-1) *in vitro,* then we measured L-ficolin expression by RT-PCR and Western blot analysis. The results have shown highly increased L-ficolin mRNA expression in HCVcc infected Huh7.5.1 cells compared to that in uninfected control Huh7.5.1 cells by RT-PCR analysis (Figure 1A). Both real-time PCR (Figure 1B) and Western blot (Figure 1C) analysis demonstrated HCVcc infection caused the increased L-ficolin expression of Huh7.5.1 cells in an HCV dose-dependent manner.

L-ficolin recognizes and binds to the N-glycans of viral E1 and E2 glycoproteins

Next, we tried to determine whether L-ficolin binds to HCV. Recombinant L-ficolin-GST, L-ficolin or GST proteins were expressed in *E. coli* BL21DE3 and purified by Glutathione Sepharose 4B and identified by SDS-PAGE and Western blot anaysis (Figure 2). A 64 kDa L-ficolin-GST fusion protein and a 29 kDa GST protein were observed and the sizes of the expressed proteins were consistent with their predicated molecular masses (Figure 2A). A 35KDa L-ficolin recombinant protein was purified and identified as its predicted size as well (Figure 2B).

In order to test whether L-ficolin protein recognizes and binds cell surface-express E1, or E2 glycoprotein, E1 and E2 surface stable expressing HepG2 cell lines were established and identified by flow cytometry, respectively (Figures 3A and 3B).

Physical associations between L-ficolin and glycosylated E1 or E2 proteins were determined by GST-pull down assay. E1- and E2-HepG2 cell lysates were incubated with a Sepharose 4B-GST-L-ficolin complex or Sepharose 4B-GST, pulled down with glutathione sepharose 4B beads and

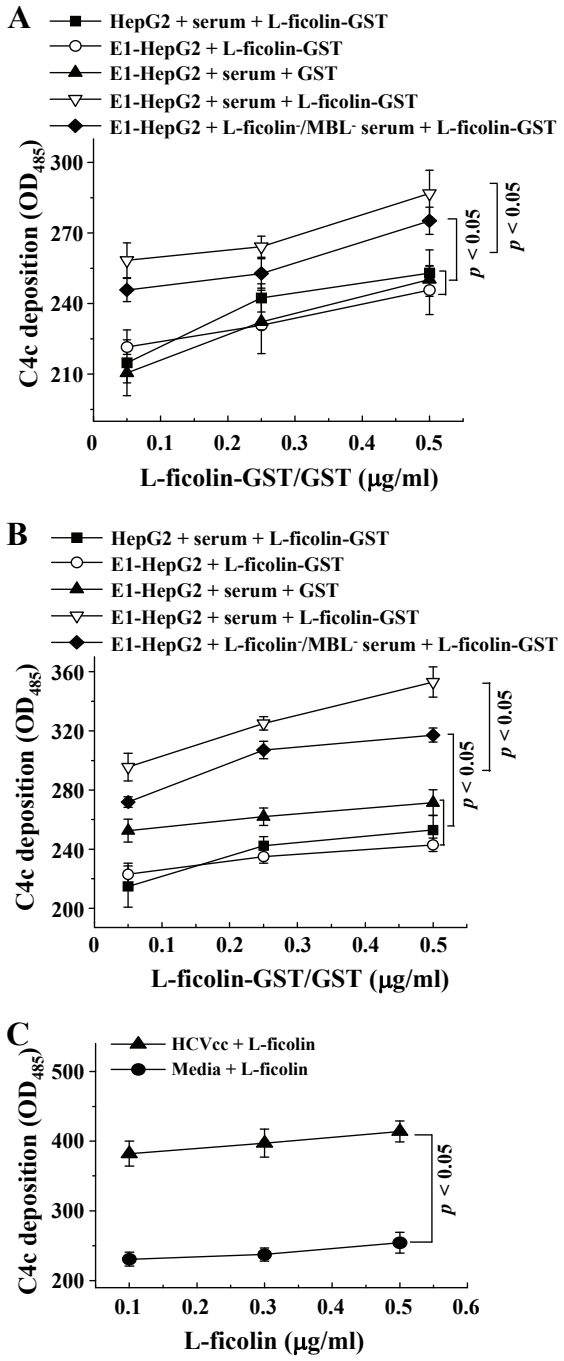

Figure 5. Binding of L-ficolin with E1-, E2-expressing cells leads to activation of complement lectin pathway. L-ficolin- GST or GST proteins were incubated with E1-HepG2 (A), E2-HepG2 (B) or HepG2 cells in the presence of human serum and C4 protein, C4c depositions on the cells were measured as described in the Materials and Methods. (C) Comparison of C4c depositions on the HCVcc or media coated plate in the presence of human serum and C4 protein after addition of recombinant L-ficolin protein.

subjected to SDS-PAGE and Western blot analysis. The results confirmed the recognition of glycosylated E1- or E2 by L-ficolin (Figure 3C, Lanes 1 and 3). However, we could not detect any binding between L-ficolin and the E1- or E2 protein when E1- or E2-HepG2 cells were pretreated with the *N*-glycosylation inhibitor 1-deoxynojirimycin (DNM) for 48 h (Figure 3C, Lanes 6 and 7).

Furthermore, E2-surface-expressing HepG2 cells or HCVcc (JFH-1) infected HuH7.5.1 cells were lysed and then incubated in the presence or absence of N-glycanase (Peptide-N-Glycosidase F, PNGaseF). Subsequently cell lysates were incubated with the Sepharose 4B-GST-L-ficolin complex, pulled down with glutathione sepharose 4B beads as mentioned above. Western blot analysis using anti-E2 or anti-L-ficolin antibodies showed that glycosylated E2 protein from E2-HepG2 or HCV (JFH-1) could bind to L-ficolin (Figure 3D, Lanes 1 and 3), while the non-glycosylated E2 protein in the presence of PNGaseF did not bind (Figure 3D, Lanes 2 and 4).

We have also found that non-glycosylated recombinant E1 and E2 proteins purified from *E. coli* could not bind to L-ficolin expressing HepG2 stable cell lines cells (data not shown).

Taken together, above results demonstrate that interaction between L-ficolin and HCV E1 and E2 glycoproteins was attributed to the N-glycans of E1 and E2. The non-glycosylated E1 and E2 proteins did not bind to L-ficolin.

The association between L-ficolin and E1/E2 glycoproteins was in a dose-dependent manner

The recombinant L-ficolin-GST, L-ficolin or GST proteins were incubated with E1-, E2-HepG2 or HepG2 cells, respectively. Binding of proteins with the cells was detected by flow cytometry analysis as described in Materials and Methods. As shown in Figure 4, binding percentages of L-ficolin-GST with E1- and E2-HepG2 cells were significantly higher than GST with E1- and E2-HepG2 cells (Figures 4A, 4B and 4C), or L-ficolin with control HepG2 cells (Figure 4C), and binding affinities of L-ficolin with E1- and E2-HepG2 cells were L-ficolin dose dependent (Figure 4C).

Binding of L-ficolin to E1- and E2-expressing cells triggers activation of the complement lectin pathway

Next, we examined whether the binding of L-ficolin protein to E1- or E2-HepG2 cells in presence of MSAPs supplied from serum would lead to lectin pathway activation and C4 deposition on HepG2 cells.

Activation of lectin pathway was measured by C4 deposition assays as described in Materials and Methods, in which the classical complement pathway was blocked by the use of 1 M NaCl. This high ionic strength buffer can block the binding of C1q to immune complexes and disrupt the C1 complex. In addition, C4 is not involved in the alternative complement pathway.

As shown in Figure 5A, in the presence of serum, the highest levels of C4c deposition were observed after mixing L-ficolin with E1, E2-HepG2 cells. The levels of complement activation in the GST-L-ficolin plus E1-, E2-HepG2 cells groups were much higher than that of the control protein GST plus E1-, E2-HepG2 cells groups, or parental HepG2 cells group ($p < 0.05$, Figures 5A and 5B).

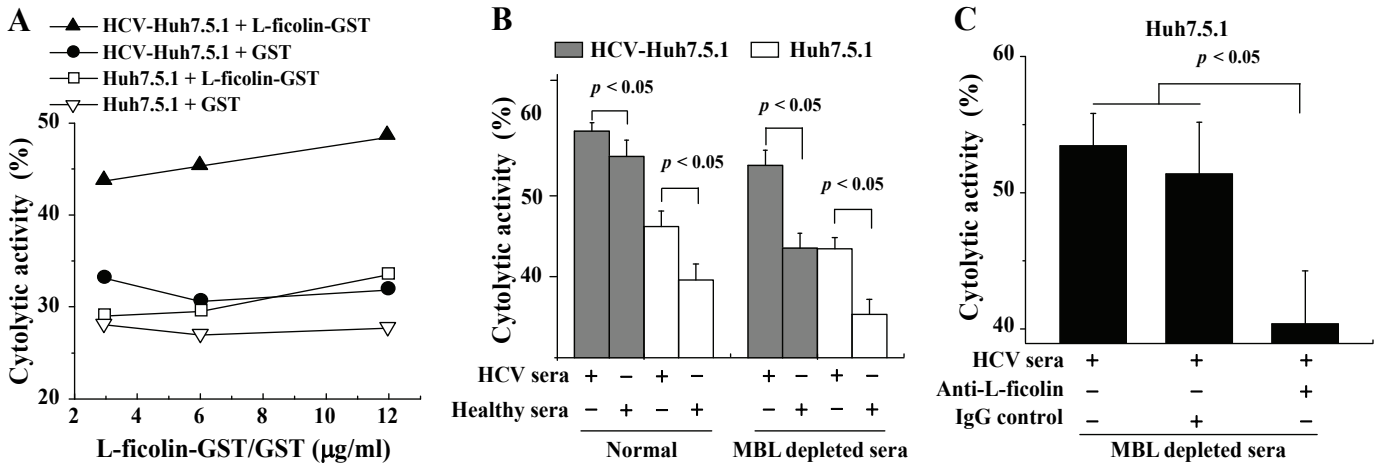

Figure 6. L-ficolin stimulates cytolytic activity in HCV-infected hepatocytes. (A) HCV-Huh7.5.1 or Huh7.5.1 cells were labeled with BCECF, then L-ficolin-GST or GST proteins were incubated with the cells in the presence of human serum. BCECF release rates from the cells were calculated as described in the Materials and Methods. (B) Comparison of the cytolytic activity between the sera of HCV patients or healthy donors. HCV-Huh7.5.1 or Huh7.5.1 cells were labeled with BCECF, and then the sera of HCV patients or healthy donors were added and incubated with the cells in the presence of normal or MBL-depleted sera. BCECF release rates from the cells were calculated. (C) The cytolytic activity of the sera of HCV patients was blocked by anti-L-ficolin antibody. Huh7.5.1 cells were labeled with BCECF, and then the sera of HCV patients were added and incubated with the cells in the presence or absence of anti-L-ficolin antibody. BCECF release rate from the cells were calculated.

Activation of lectin pathway was in a L-ficolin dose dependent manner (Figures 5A and 5B).

We further found that L-ficolin triggered C4c deposition on HCV particles coated plate. As shown in Figure 5C, in the presence of serum, much higher levels of C4c deposition on HCVcc coated plate were observed when additon of L-ficolin protein comparing to cell culture media coated plate. Activation of lectin pathway was proportional to L-ficolin protein dose as well (Figure 5C).

Complement lectin L-ficolin mediates cytolytic activity

Binding of L-ficolin to E1- or E2-expressing cells triggers activation of complement lectin pathway, which eventually leads to the formation of membrane attack complex (MAC) and cell lysis. We measured L-ficolin mediated cytolytic activity in HCV-infected hepatocyte using the BCECF release assay as described in Materials and Methods.

We observed that the cytolytic activity assessed by the alive cells fluorescence dye BCECF release rate was increased in HCV-infected Huh7.5.1 cells when GST-L-ficolin was added and was in a L-ficolin dose dependent manner (Figure 6A). While, much lower cytolytic activities were detected in Huh7.5.1 cells plus GST-L-ficolin, or in GST plus infected- and uninfected-Huh7.5.1 cells groups (Figure 6A). These data indicate that L-ficolin protein could recognize and bind to glycoproteins E1 and E2 of HCV envelope, activating the lectin complement pathway-mediated cytolytic activity in HCV-infected hepatocyte.

We further detected whether sera samples of HCV patients had higher complemet mediated cytolytic activities than those of healthy donors. Our data demonstrated that the sera of HCV patients had the highest cytolytic activity to HCVcc infected Huh7.5.1 cells among all groups (Figure 6B). The sequence of cytolytic activity was: HCV patients group with HCV-Huh7.5.1 > healthy control group with HCV-Huh7.5.1, or HCV patients with uninfected Huh7.5.1 cell group > healthy control with uninfected Huh7.5.1 cells group (Figure 6B). Our data also showed that MBL-depleted sera of HCV patients had lower cytolytic activity than those non-depleted sera groups, but still exhibited the higher cytolytic activity compared to the MBL-depleted healthy control sera group (Figure 6B).

Morever, MBL-depleted sera samples of HCV patients preblocked with L-ficolin antibody had much decreased cytolytic activity than same sera incubated with IgG control antibody (Figure 6C). These data indicate that the increased L-ficolin activity in the sera of HCV patients are due to the increased levels of L-ficolin in the sera of HCV patients, and the sera of HCV patients had increased L-ficolin mediated cytolytic activity than that of healthy donors.

Discussion

L-ficolin is a pattern-recognizing receptor that specifically binds to carbohydrate-based pathogen-associated molecular patterns (PAMPs) on clinically important bacteria or fungi, e.g. various acetylated compounds, 1, 3-β-D glucan, LPS and LTA, following the activation of complement. However, which kind of PAMPs of viruses recognized by this lectin remains elusive. Our data are the first demonstration that up-regulated serum L-ficolin specifically binds to *N*-glycans of E1 and E2 glycoproteins of HCV envelope, subsequently activates lectin complement mediated cytolytic activities in

HCV infected hepatocytes, which illustrates that *N*-glycans are possibly PAMPs of HCV recognized by the innate immune molecule L-ficolin.

In the present study, we also found that serum L-ficolin was increased in HCV patients. We also observed that serum L-ficolin levels correlated with the severity of fibrosis and the active state of HCV infection (Table 1). These data illustrate that L-ficolin activity may be closely related to disease progression. An increased MBL concentration in HCV patients in comparison to controls and a link to more severe hepatitis has been shown previously (36). MBL/MASP-1 complex activities were closely related to disease progression (37). Our results show an association between L-ficolin and fibrosis development in HCV-infected patients that is similar to that of MBL/MASP-1 complex activity. Measurement of serum L-ficolin concentrations in HCV patients might hold a potential as a diagnostic molecular marker for disease progression in HCV infection.

In summary, our findings provide new insights that the increased L-ficolin protein not only can recognize and bind to glycoproteins E1 and E2 of HCV envelope, but also activate the complement lectin pathway-mediated cytolytic activity in HCV-infected hepatocytes. We speculate that the overexpression of L-ficolin might increase liver injury when liver cells infected with HCV, and play a potential role in disease progression. The overexpression of L-ficolin might contribute to liver pathological changes. These findings provide a new insight into the biological functions of L-ficolin in clinically important liver disease.

Since L-ficolin is a lectin which can recognize microbial carbohydrates, we speculate that it might also recognize other viruses with heavily glycosylated envelope proteins, such as human immunodeficiency virus (HIV). Whether L-ficolin recognizes other viruses and their biological functions and clinical significances are needed to be further investigated.

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