Original Article
Roles of autoantibody-mediated platelet destruction in thrombocytopenic patients with HBV-related liver diseases

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Abstract: The pathophysiology of thrombocytopenia in patients with chronic liver diseases (CLD) was complex and several underlying mechanisms might act in concert. Roles of autoantibody-mediated platelet destruction in HBV-related CLD remained controversial. In this study, platelet-bound and plasma glycoprotein (GP)-specific autoantibodies in HBV-infected patients at different stages were determined by flow cytometric immunobead array and modified monoclonal antibody-specific immobilization of platelet antigen (MAIPA) technique. In thrombocytopenic group, platelet-bound GP-specific autoantibodies were detectable in 9 of the 12 chronic hepatitis B (CHB) patients, 5 of 9 Child A cirrhotic patients, and 6 of 18 Child B/C cirrhotic patients, whereas plasma GP-specific autoantibodies were positive in 7 of the 12 chronic hepatitis B (CHB) patients, 4 of 9 Child A cirrhotic patients, and 4 of 18 Child B/C cirrhotic patients. The positive frequencies of platelet-bound and plasma GP-specific autoantibodies were considerably higher in thrombocytopenic patients than that in CHB or Child A cirrhotic patients without thrombocytopenia. However, there was no correlation between platelet counts and levels of platelet-bound or plasma GP-specific autoantibodies. In vitro phagocytic assays revealed that levels of autoantibody-mediated platelet phagocytosis by macrophages in thrombocytopenic CHB patients were significantly higher than that in thrombocytopenic Child A and Child B/C cirrhotic patients, and phagocytic capacity of macrophages in thrombocytopenic Child A cirrhotic patients was also higher than that in Child B/C cirrhotic patients. These finding suggested that platelet GP-specific autoantibodies were indeed present in thrombocytopenic patients with HBV-related CLD, while autoantibody-mediated platelet phagocytosis was associated with the severity of liver cirrhosis.

Keywords: Liver cirrhosis, HBV, thrombocytopenia, glycoprotein, autoantibody

Introduction

Chronic liver diseases (CLD) affect millions of people and remain major health issues in the developing countries [1, 2]. Liver cirrhosis (LC), resulting from prolonged, widespread but patchy hepatocellular necrosis, represents a severe late stage of the long clinical course of CLD. It has been well known that patients with LC possess a significantly reduced life expectancy in contrast to non-cirrhotic ones. Thrombocytopenia, a risk factor accounting for gastrointestinal bleeding or other life-threatening haemorrhagic events, is a major hematologic disorder commonly observed in patients with LC [3]. Moreover, the degree of thrombocytopenia might be a useful prognostic marker and a diagnostic clue to the presence of esophageal varices [4-6]. Thus far, the pathogenetic mechanisms leading to thrombocytopenia in cirrhotic patients are not fully understood.

Several mechanisms have been implicated in the pathophysiology of thrombocytopenia in LC. Traditionally, sequestration of platelets by the spleen, a situation known as hypersplenism secondary to portal hypertension, has been considered as the main factor leading to the

development of thrombocytopenia in LC [7]. Nevertheless, portal decompression procedures such as portosystemic shunting do not always correct the low platelet levels in the clinical setting, [8] and thrombocytopenia might persist even after splenectomy in some patients with LC [9]. Furthermore, the portal pressure fails to show any correlation to the platelet counts in cirrhotic patients [10]. Apart from splenic sequestration, other factors including reduced activity of the hematopoietic growth factor thrombopoietin (TPO), cirrhotic coagulopathy, and cirrhotic bone marrow suppression by hepatitis virus infection or antiviral treatment with interferon (IFN)-based therapy, are also involved in LC-related thrombocytopenia [11, 12]. However, neither of the current theories could explain thrombocytopenia completely in patients with LC concerning the heterogeneity of the disease.

Hepatitis B virus (HBV) is the most common causes of LC in China [13]. Immune response induced by HBV could cause progression of liver disease from mild inflammation to severe fibrosis and liver cirrhosis. In addition, immunological derangement triggered by HBV infection could lead to the development of autoimmunity [14]. This has been implicated in the pathogenesis of multiple extra hepatic manifestations of HBV infection such as membranous glomerulonephritis, [15] cutaneous vasculitis, [16] essential mixed cryoglobulinemia, [17] polyarteritis nodosa, [16] and other forms of immune complex diseases. Moreover, an association between HBV infection and other inflammatory syndrome in diseases such as systemic lupus erythematosus (SLE), [18] rheumatoid arthritis, [19, 20] and polymyositis [21] has been suggested.

It has been reported that levels of platelet-associated IgG (PAIgG) were elevated in patients with CLD, [22, 23] indicating the presence of platelet-reactive autoantibodies in these patients. Nevertheless, such increases in PAIgG were non-specific and were often found in patients with nonimmune thrombocytopenia [24, 25]. The availability of monoclonal antibodies allows the detection of true platelet autoantibodies that binds to specific platelet surface glycoprotein (GP), and platelet antigen capture techniques such as monoclonal antibody-specific immobilization of platelet antigen (MAIPA),

[26] flow cytometric immunobead assay, [27] have been developed. Platelet-specific autoantibodies mediate the removal of platelets by the splenic and hepatic reticuloendothelial system in immune thrombocytopenia (ITP) [28]. Roles of platelet-specific autoantibodies in CLD patients remain unclear. It has been observed that in LC patients of diverse etiology, frequencies of antibody-producing B cells were significantly higher [23]. A series of studies support the idea that platelet-specific autoantibodies play a pathogenetic role in the development of thrombocytopenia in LC patients [22, 23]. Nevertheless, Panzer reported that platelet-specific autoantibodies were common in patients with hepatitis C (HCV) infection irrespective of the presence of thrombocytopenia [29]. So effect of autoantibodies on platelet destruction might be varying in CLD patients of different etiology.

The precise role of platelet GP-specific autoantibodies in the pathogenesis of thrombocytopenia in HBV-related CLD has not been well established. Pradella reported that one out of fifteen patients with HBV-related cirrhosis showed serum anti-platelet antibodies, and no cases presenting PAIgG, [29] while another group observed that levels of GPIIb/IIIa antibody-producing B cells coincided with platelet-bound and plasma GPIIb/IIIa autoantibodies were significantly higher in HBV-related LC than that of healthy controls [23]. In the present study, platelet-bound and plasma GPIIb/IIIa, GPIb/IX autoantibodies were determined in thrombocytopenic patients at various stages of disease with HBV infection. Furthermore, antibody-mediated platelet phagocytosis by monocyte-derived macrophages from patients with HBV infection was also evaluated, providing new insights on immune dysregulation in the pathogenesis of thrombocytopenia in HBV-related CLD.

Material and methods

Patients and controls

A total of seventy-nine consecutive patients with HBV-related CLD (48 males and 31 females, age range 21-74 years, median 46 years) were enrolled in this study. Among thrombocytopenic group (platelet counts < 100 × 10^9/L), 12 patients were diagnosed chronic hepatitis B (CHB) and 27 patients were diag-

nosed HBV-related LC according to the Asian-Pacific consensus statement on the management of CHB based on patients' history, clinical signs and symptoms, a physical examination, laboratory test results, and ultrasonographic and/or computed tomographic imaging studies [30]. Liver biopsy was used for histological confirmation in case of diagnostic uncertainty. Additionally, 29 CHB patients and 11 LC patients without thrombocytopenia were also included. LC was classified according to modified Child-Turcotte-Pugh (CTP) score. Patients concomitant with other viral infections, fatty liver diseases, autoimmune diseases, and hepatic or extrahepatic malignancy were excluded. All patients were free from antiviral or immunosuppressive therapy at least 3 weeks before blood sampling. Enrollment took place between February 2012 and August 2015, at the Department of Gastroenterology, Shandong Provincial Hospital, Shandong University, Jinan, China. Twenty-five healthy control subjects (15 males and 10 females, age range 19-58 years, median 36 years) without any history of liver or autoimmune disease were included.

Platelet counts, transaminases, bilirubin, albumin, prothrombin time (PT), HBeAg, anti-HBe, HBsAg, anti-HBs and anti-HBc were determined by routine laboratory methods at admission. Informed consent was obtained from all patients before enrollment in the study in accordance with the Declaration of Helsinki. This study was approved by the Medical Ethical Committee of Shandong Provincial Hospital, Shandong University.

Platelet and plasma preparation

Peripheral blood was collected into vacutainer tubes containing 1.5 mg/mL of ethylenediaminetetraacetic acid (EDTA). All specimens were processed within 3 hours of collection. Blood sample were centrifuged at 150 × g for 10 minutes, and the platelet-rich plasma (PRP) was further centrifuged at 800 × g for 10 minutes to prepare the platelet pellets. Plasma samples were collected and stored at -80°C for indirect MAIPA assay. The platelet pellets were washed with 0.05 M isotonic citrate buffer (PH 6.2) for 5 times, and next 1 × 10^8/L platelets were solubilized with Tris-buffered saline containing 0.5% Triton X-100 and 0.1 mg/mL leupeptin, and next centrifuged at 1200 × g for 5 minutes. Platelet lysate were collect for flow cytometric

immunobead assays (FCI) of platelet-bound GP-specific autoantibodies.

Flow cytometric immunobead assays

GPIIb/IIIa monoclonal antibody (CD41 mAb; P2 clone) and GPIb/IX monoclonal antibody (CD42 mAb; SZ2 clone) were all from immunotech, a Beckman Coulter Company. Phycoerythrin (PE)-conjugated polyclonal goat anti-human IgG antibody, PE-conjugated goat IgG isotype control were all purchased from Beckman Coulter. Microbead assays were carried out according to a previously described method [27]. Briefly, GPII/IIIa or GPIb/IX mAb-coated microbeads were incubated with platelet lysate for 2 hours, washed and incubated with PE-conjugated polyclonal goat anti-human IgG for 1 hour, washed and analyzed by FCM. The negative control consisted of normal platelet lysate (Three different samples per assay). Labeled microbeads were analyzed by BD FACS Calibur. Microbeads were gated by forward scatter/side scatter (FSC/SSC), and MFI of the microbeads was determined using CellQuestPro software. The individual autoantibody value was given as mean fluorescence intensity (MFI) ratio of individuals to the means of three negative controls.

Indirect modified MAIPA assay

Indirect modified MAIPA was carried out to determine plasma platelet GP-specific autoantibodies according to the method established by Hou et al. [31]. In brief, platelets from healthy blood group O donors were sensitized with 100 μL plasma of patients or controls, washed and solubilized in Tris-buffered saline containing 1% Triton X-100 and 0.1 mg/mL leupeptin. Microtiter plates were coated with affinity-purified goat anti-mouse IgG (Immunotech, Beckman Coulter), and next incubated with GPIIb/IIIa mAb (P2 clone) or GPIb/IX mAb (SZ2 clone) for 60 minutes at room temperature. After washing, the sensitized platelet lysate was added in duplicates to each well and incubated for 60 minutes. IgG bound to the captured GPIIb/IIIa or GPIb/IX was detected using alkaline-phosphatase-conjugated goat anti-human IgG (Fc specific; Sigma Chemical Co). *p*-Nitrophenyl-phosphate was used as the substrate, and the plates were read on an automated microtiter plate reader (Thermo-Multiskan Mk3) using dual wavelength (405 and 492 nm).

Table 1. Baseline demographic and clinical parameters in HBV-related CLD patients and health controls

| | CHB | | Cirrhosis | | | Health controls |
| | | | Child A | | Child B, C | |
	Thrombocyto-penic	Non-thrombo-cytopenic	Thrombocyto-penic	Non-thrombocy-topenic	Thrombocyto-penic	
N	12	29	9	11	18	25
Sex (M/F)	8/4	18/11	5/4	7/4	10/8	15/10
Age (years)	37 (21, 62)	45 (23, 70)	41 (35, 69)	47 (23, 74)	45.5 (26, 64)	42 (19, 68)
ALT (U/L)	85.3 ± 20.2	88.3 ± 12.7	84 ± 18.9	82.5 ± 15.6	59.3 ± 12.9	20.1 ± 6.7
TBIL (µmol/L)	19.4 ± 5.8	19.1 ± 5.3	34.9 ± 7.4	37.1 ± 6.5	47.1 ± 6.5	12.9 ± 3.3
ALB (g/L)	41.9 ± 4.1	42.4 ± 4.1	32.6 ± 4.8	32.5 ± 3.9	27.2 ± 3.6	43.8 ± 3.3
PT (s)	12.2 ± 1.5	12 ± 1.3	15.5 ± 0.8	15.6 ± 0.7	18.9 ± 2.2	11.9 ± 1.2
PC (× 10^9/L)	32.8 ± 21.2	219 ± 47.2	53.2 ± 25.1	203.3 ± 46.7	44.3 ± 16.3	226.6 ± 41.5

A positive result was defined as absorbance beyond mean + 3 SD normal controls.

Evaluation of autoantibody-mediated platelet phagocytosis by monocyte-derived macro-phages

For determination of *in vitro* platelet phagocytosis by autologous macrophages, platelets from platelet-bound GPIIb/IIIa-autoantibody-positive patients were labeled with 5-chloromethylfluorescein diacetate (CMFDA) in a previously described method [28]. In brief, peripheral blood was obtained by venipuncture into trisodium citrate tubes. PRP was prepared, and platelets were adjusted to 10^9/mL in the presence of 5 µM prostaglandin E1 (Cayman Chemical). CMFDA (GM-G; Invitrogen) was added to the platelets at a final concentration of 20 µM, incubated in the dark for 2 hours at 37°C. To evaluate the roles of plasma GP-specific auto-antibodies, CMFDA-labeled platelets from healthy blood group O donors were opsonized by plasma with detectable GPIIb/IIIa autoantibodies. Murine IgG2a anti-human major histocompatibility complex class I monoclonal antibody (W6/32, Abcam) was used as positive controls. Platelets were washed and used in phagocytosis assay.

Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Paque density gradient centrifugation, washed and further purified by centrifugation on a hypotonic Percoll gradient (1.129 g/mL; 400 × *g* for 30 minutes). Two interphases were found and the upper phase with the enriched monocytes was collected. Monocytes were cultured in RPMI 1640 medium in a 5% CO_2/95% air atmosphere at 37°C for 2 hours, and washed twice with PBS. The adherent cells were further cultured for 1 hour in Iscove modified Dulbecco medium supplemented with 10% heat-inactivated human pooled AB serum in the presence of 50 ng/mL of phorbol 12-myristate 13-acetate. The cells were then washed twice with PBS, incubated with CMFDA-labeled platelets (macrophages: platelets, 1:5), centrifuged at 200 × *g* for 1 minutes to establish contact between macrophages and platelets, and further incubated for 1 hour on ice or at 37°C. To remove free platelets, the macrophages were treated with 0.05 mM EDTA and 0.05% trypsin in PBS for 5 minutes at 37°C, followed by detachment of macrophages using PBS/5 mM EDTA at 4°C. Extracellular fluorescence was then quenched by the addition of 0.1% Trypan blue. The mixture was centrifuged at 200 × *g* for 10 minutes at 4°C, the supernatant discarded, and the macrophages were incubated with DNA stain LDS-751 (FL3; Molecular Probes). The macrophages were then washed and resuspended for flow cytometric analysis. Intracellular FL1 GM-G platelet fluorescence in the nucleated events was determined. The phagocytic index was calculated as the MFI obtained at 37°C divided by the MFI at 0°C.

Phagocytosis of autologous platelets were carried out in all thrombocytopenic patients with positive platelet-bound GPIIb/IIIa autoantibodies, and phagocytosis of allogeneic platelets opsonized by GPIIb/IIIa-autoantibody positive plasma were conducted in patients with detectable plasma GPIIb/IIIa autoantibodies.

Statistical analysis

Data of flow cytomeric immunobead assay, OD value of MAIPA and phagocytic index were

Table 2. Platelet-bound and plasma GP-specific autoantibodies in HBV-related CLD patients and health controls

| | CHB | | Cirrhosis | | | Health controls |
| | | | Child A | | Child B, C | |
	Thrombocyto-penic	Non-thrombo-cytopenic	Thrombocyto-penic	Non-thrombo-cytopenic	Thrombocyto-penic	
PB GPIIb/IIIa	75% (9/12)	6.9% (2/29)	55.6% (5/9)	9.1% (1/11)	33.3% (6/18)	0% (0/25)
PB GPIb/IX	58.3% (7/12)	3.4% (1/29)	44.4% (4/9)	0% (0/11)	27.8% (5/18)	0% (0/25)
Plasma GPIIb/IIIa	58.3% (7/12)	3.4% (1/29)	44.4% (4/9)	0% (0/11)	22.2% (4/18)	0% (0/25)
Plasma GPIb/IX	41.7% (5/12)	3.4% (1/29)	33.3% (3/9)	0% (0/11)	11.1% (2/18)	0% (0/25)

expressed as mean ± SD. Statistical significance among different groups was determined by one-way analysis of variance (ANOVA), and the difference between 2 groups was determined by Student-Newman-Keuls (SNK) test unless the data were not normally distributed, in which case the Kruskal-Wallis test and Nemenyi test were used. Spearman correlation was used for correlation analysis. Chi-square test was used to assess the positive rate of autoantibodies between different groups. *P* values < 0.05 were considered statistically significant.

Results

Demographic and clinical characteristics of patients with HBV-related CLD

Clinical details of the patients and healthy controls were shown in **Table 1**. The sex distribution and age at examination were similar between patients of different subgroups and health controls (Sex: P = 0.99, Chi-square test; Age: P = 0.492, ANOVA). Levels of alanine aminotransferase (ALT) in CHB and Child A cirrhosis patients were significantly higher than that in Child B/C cirrhosis patients and healthy controls (P < 0.001, SNK test), while levels of total bilirubin (TBIL) in CHB patients and healthy controls were considerably lower than that in patients with LC (P < 0.001, SNK test). Moreover, as the hepatic functional reserve deteriorated, levels of TBIL were elevated concomitantly. By contrast, serum albumin (ALB) levels were significantly lower in LC patients compared with CHB patients or healthy controls (P < 0.001, SNK test). Consistent with previous report, 32 statistically prolonged prothrombin time (PT) was observed in Child B/C and Child A LC patients compared with CHB patients and healthy controls (P < 0.001, SNK test), and PT in Child B/C LC patients were longer than that

in Child A cirrhosis patients (P < 0.001, SNK test). Interestingly enough, platelet counts in CHB patients with thrombocytopenia were significantly lower than that in Child A (P < 0.01, SNK test) or Child B/C thrombocytopenic LC patients (P < 0.05, SNK test).

Platelet-bound GP-specific autoantibodies

Nine of the 12 CHB patients with thrombocytopenia were positive for platelet-bound GPIIb/IIIa autoantibodies (75%) and 7 for GPIb/IX autoantibodies (58.3%). In CHB patients without thrombocytopenia, 2 patients were positive for platelet-bound GPIIb/IIIa autoantibodies (6.9%) and 1 for GPIb/IX autoantibodies (3.4%). Platelet-bound GPIIb/IIIa autoantibodies were detectable in 5 of 9 Child A LC patients with thrombocytopenia (55.6%), and GPIb/IX autoantibodies were positive in 4 patients (44.4%). By contrast, in non-thrombocytopenic Child A LC patients, 1 patients showed positive platelet-bound GPIIb/IIIa autoantibodies (9.1%), and nobody exhibited detectable platelet-bound GPIb/IX autoantibodies. Of the 18 Child B/C LC patients with thrombocytopenia, platelet-bound GPIIb/IIIa and GPIb/IX autoantibodies were positive in 6 and 5 patients (GPIIb/IIIa, 33.3%; GPIb/IX, 27.8%) respectively. None of the healthy controls showed detectable platelet-bound GP-specific autoantibodies (**Table 2**).

The positive rates of platelet-bound GPIIb/IIIa and GPIb/IX autoantibodies in thrombocytopenic patients were significantly higher than that in non-thrombocytopenic CHB patients (GPIIb/IIIa: *P* < 0.001; GPIb/IX: *P* < 0.001, Chi-square test) or Child A cirrhotic patients without thrombocytopenia (GPIIb/IIIa: *P* = 0.025; GPIb/IX: *P* = 0.01, Chi-square test) as well as healthy controls (GPIIb/IIIa: *P* < 0.001; GPIb/IX: *P* < 0.001, Chi-square test). It is notable that the positive rate of platelet-bound GPIIb/IIIa autoantibodies

in thrombocytopenic CHB patients was significantly higher than that in thrombocytopenic cirrhotic patients (Child A + Child B/C) (P = 0.048; Chi-square test). The positive frequency of platelet-bound GPIb/IX autoantibodies in thrombocytopenic CHB patients was also higher in comparison with thrombocytopenic LC patients (Child A + Child B/C), but this disparity did not achieve statistical significance (P = 0.143; Chi-square test). No statistical significance was found in the positive rates of platelet-bound GP-specific autoantibodies between cirrhotic Child A patients with thrombocytopenia and Child B/C LC patients (GPIIb/IIIa: P = 0.268; GPIb/IX: P = 0.386, Chi-square test). Additionally, we did not observe any significance in the positive rates of platelet-bound GP-specific autoantibodies between non-thrombocytopenic patients of CHB and Child A LC patients without thrombocytopenia (GPIIb/IIIa: P = 0.814; GPIb/IX: P = 0.515, Chi-square test).

Plasma GP-specific autoantibodies

Plasma GP-specific autoantibodies were measured by indirect modified MAIPA. Of 12 plasma samples from thrombocytopenic CHB patients, 7 had a detectable level of GPIIb/IIIa autoantibodies (58.3%) and 5 showed positive GPIb/IX autoantibodies (41.7%). Plasma GPIIb/IIIa and GPIb/IX autoantibodies were also detected in 4 and 3 child A LC patients with thrombocytopenia (GPIIb/IIIa: 44.4%; GPIb/IX: 33.3%). In Child B/C cirrhotic patients, plasma GPIIb/IIIa and GPIb/IX autoantibodies were detectable in 4 and 2 patients respectively (GPIIb/IIIa: 22.2%; GPIb/IX: 11.1%). In non-thrombocytopenic CHB patients, 1 patient showed doubly positive result of plasma GPIIb/IIIa and GPIb/IX autoantibodies (3.4%), and nobody demonstrated detectable plasma GP-specific autoantibodies in non-thrombocytopenic Child A cirrhotic patients and healthy controls (**Table 2**).

In consistence with the testing result of platelet-bound autoantibodies, the positive frequencies of plasma GPIIb/IIIa and GPIb/IX autoantibodies were remarkably higher in thrombocytopenic patients than that in non-thrombocytopenic CHB patients (GPIIb/IIIa: P = 0.001; GPIb/IX: P = 0.018, Chi-square test) or healthy controls (GPIIb/IIIa: P < 0.001; GPIb/IX: P = 0.005, Chi-square test). The positive frequency of plasma GPIIb/IIIa autoantibodies was also higher in thrombocytopenic patients than that in Child A

cirrhotic patients without thrombocytopenia (P = 0.021, Chi-square test), whereas no statistical difference was found in detection rate of plasma GPIb/IX autoantibodies between thrombocytopenic patients and non-thrombocytopenic Child A LC patients (P = 0.092, Chi-square test). Additionally, the positive rates of plasma GPIIb/IIIa and GPIb/IX autoantibodies in thrombocytopenic CHB patients were not different from that in thrombocytopenic LC patients of Child A (GPIIb/IIIa: P = 0.67; GPIb/IX: P = 0.697, Chi-square test) or Child B/C (GPIIb/IIIa: P = 0.063; GPIb/IX: P = 0.084, Chi-square test). There was no difference in the positive frequencies of plasma GPIIb/IIIa and GPIb/IX autoantibodies between cirrhotic Child A patients with thrombocytopenia and Child B/C LC patients (GPIIb/IIIa: P = 0.375; GPIb/IX: P = 0.295, Chi-square test), nor was there between non-thrombocytopenic CHB patients and Child A LC patients without thrombocytopenia (GPIIb/IIIa: P = 0.99; GPIb/IX: P = 0.99, Chi-square test).

Correlation of GP-specific autoantibodies with platelet counts in thrombocytopenic patients

To evaluate weather platelet autoantibodies were related to platelet counts, correlation analysis was performed between values of platelet counts and MFI determined by FCI or ΔOD of MAIPA in thrombocytopenic patients. The data demonstrated that detectable platelet-bound GPIIb/IIIa or GPIb/IX autoantibodies were not associated with platelet counts in thrombocytopenic CHB patients (GPIIb/IIIa: r = -0.344, P = 0.274; GPIb/IX: r = -0.495, P = 0.102; Spearman correlation analysis), or Child A (GPIIb/IIIa: r = -0.46, P = 0.213; GPIb/IX: r = -0.549, P = 0.092; Spearman correlation analysis) and Child B/C cirrhotic patients with thrombocytopenia (GPIIb/IIIa: r = -0.26, P = 0.298; GPIb/IX: r = -0.056, P = 0.824; Spearman correlation analysis). In addition, no correlation was observed between platelet counts and MAIPA ΔOD of GPIIb/IIIa or GPIb/IX in thrombocytopenic patients (CHB GPIIb/IIIa: r = -0.537, P = 0.072; CHB GPIb/IX: r = -0.189, P = 0.555; Child A GPIIb/IIIa: r = -0.159, P = 0.683; Child A GPIb/IX: r = -0.259, P = 0.5; Child B/C GPIIb/IIIa: r = -0.074, P = 0.769; Child B/C GPIb/IX: r = -0.077, P = 0.763; Spearman correlation analysis), suggesting the presence of plasma GP-specific autoantibodies was also not related to platelet counts in thrombocytopenic patients. (**Figure 1**).
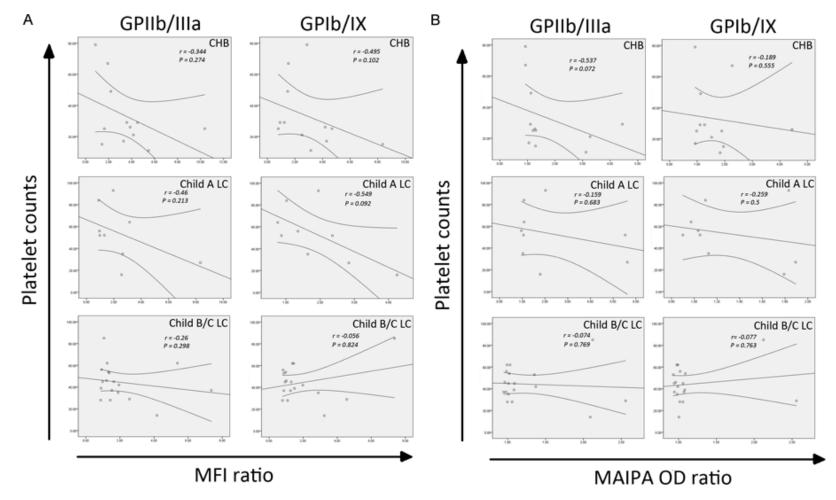
Figure 1. Correlation between platelet counts and MFI determined by FCI or ΔOD of MAIPA in thrombocytopenic patients. A. Detectable platelet-bound GPIIb/IIIa or GPIb/IX autoantibodies were not associated with platelet counts in thrombocytopenic patients of CHB, Child A or Child B/C. B. No correlation was observed between platelet counts and ΔOD ratio of GPIIb/IIIa or GPIb/IX in thrombocytopenic patients.
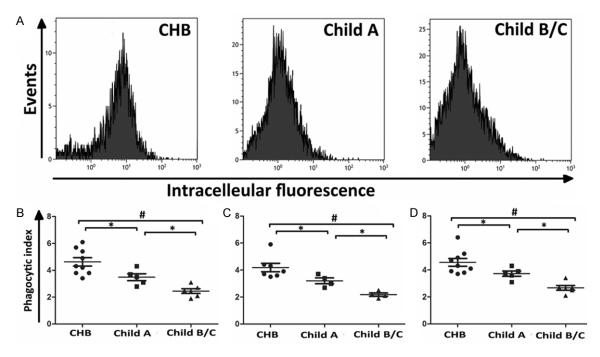
Figure 2. Autoantibody-mediated platelet phagocytosis by macrophages. Monocyte-derived macrophages were incubated with autologous platelets or opsonized allogenic platelets from healthy blood group O donors, and analyzed on a flow cytometry. A. Representative histogram of macrophage intracellular fluorescence from thrombocytopenic CHB, Child A and Child B/C patients. B. Phagocytosis of autologous platelets by macrophages in thrombocytopenic CHB, Child A and Child B/C patients with detectable platelet-bound GPIIb/IIIa autoantibodies. C. Phagocytosis of GPIIb/IIIa-autoantibody-opsonized allogenic platelets by macrophages in thrombocytopenic CHB, Child A and Child B/C patients with detectable plasma GPIIb/IIIa. D. Phagocytosis of murine-mAb-opsonized allogenic platelets by macrophages in thrombocytopenic CHB, Child A and Child B/C patients with detectable platelet-bound GPIIb/IIIa autoantibodies.

Autoantibody-mediated platelet phagocytosis by autologous monocyte-derived macrophages

To evaluate the role of GP-autoantibodies in the process of platelet destruction, monocyte-derived macrophages from thrombocytopenic subjects with positive platelet-bound GPIIb/IIIa autoantibodies were incubated with autologous CMFDA-labeled platelets for phagocytosis assay. Macrophages from thrombocytopenic CHB patients showed significantly higher ability for phagocytosis of autologous platelets (4.6 ± 0.9) compared with Child A (3.5 ± 0.6, $P < 0.05$, SNK test) and Child B/C cirrhotic patients with thrombocytopenia (2.5 ± 0.4, $P < 0.001$, SNK test). In addition, autologous platelet phagocytosis by macrophages from thrombocytopenic Child A cirrhotic patients (3.5 ± 0.6) was also higher than that from Child B/C cirrhotic patients with thrombocytopenia (2.5 ± 0.4, $P < 0.05$, SNK test) (**Figure 2B**).

To further identify whether plasma GP-specific autoantibodies could mediate platelet destruc-

tion, platelets from healthy blood group O donors were opsonized by plasma with detectable GPIIb/IIIa autoantibodies and used in phagocytosis experiment. In consistence with the results of autologous platelet phagocytosis assay, phagocytic capacity of macrophages from thrombocytopenic CHB patients (4.2 ± 0.8) were remarkably enhanced in comparison with that from Child A (3.2 ± 0.4, $P < 0.05$, SNK test) and Child B/C cirrhotic patients with thrombocytopenia (2.2 ± 0.3, $P < 0.001$, SNK test), and macrophages from thrombocytopenic Child A cirrhotic patients (3.2 ± 0.4) had higher phagocytic capacity than that from Child B/C cirrhotic patients with thrombocytopenia (2.2 ± 0.3, $P < 0.05$, SNK test) (**Figure 2C**).

In order to identify whether the reduction in platelet phagocytosis in thrombocytopenic LC patients was due to weakened phagocytic ability of macrophages, phagocytosis assay was also carried out using blood group O platelets opsonized by murine IgG2a anti-human major histocompatibility complex class I monoclonal

antibody. Our results showed that phagocytic index of macrophages in thrombocytopenic CHB patients (4.6 ± 0.9) was considerably elevated compared with that in Child A (3.7 ± 0.4, $P < 0.05$, SNK test) and Child B/C cirrhotic patients with thrombocytopenia (2.7 ± 0.4, $P < 0.001$, SNK test). Aside from that, phagocytic index of macrophages in thrombocytopenic Child A cirrhotic patients (3.7 ± 0.4) was higher compared to Child B/C cirrhotic patients with thrombocytopenia (2.7 ± 0.4, $P < 0.05$, SNK test) (**Figure 2D**).

Discussion

CLD have several impacts on distribution, survival and production of hematopoietic elements. Thrombocytopenia is probably the most common and prevalent hematological abnormality that can be found in patients affected by CLD [33]. In china, HBV remains the leading cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC), and. 25% of these patients will finally die of cirrhosis-associated complications [34]. Behnava et al. reported that the prevalence of thrombocytopenia was 17.7% in CHB patients and 10.6% in HBV inactive carriers [35]. HBV-infected patients complicated with thrombocytopenia increased the risk of bleeding which might raise morbidity and mortality [36, 37]. It has been well-known that infection with HBV could cause profound changes in the host immune response, including the occurrence of platelet autoantibodies [14]. However, roles of autoantibody-mediated platelet destruction in the pathophysiological process of HBV-related CLD of different stages still remain obscure to date.

Our present data showed that platelet-bound and plasma GP-specific autoantibodies were detectable in different stages of thrombocytopenic patients with HBV-related CLD. Of note, the positive frequencies of GP-specific autoantibodies in thrombocytopenic CHB patients were higher than that in thrombocytopenic LC patients. We also observed that platelet counts in thrombocytopenic CHB patients were statistically lower than that in thrombocytopenic LC patients. As hypersplenism and TPO reduction was supposed to be absent in thrombocytopenic CHB, [12] platelet count decrease in thrombocytopenic CHB could most probably attributed to immune-mediated platelet destruction. Indeed, the detection rates of GP-specific auto-

antibodies in thrombocytopenic CHB patients were comparable to these reported in immune thrombocytopenia (ITP), [27] suggesting the coexistence of that two disorders. Surprisingly, GP-specific autoantibodies were also detected in 1 non-thrombocytopenic CHB patients. Previous observations with even higher positive frequencies of GP-specific autoantibodies have been reported in non-thrombocytopenic HCV-infected patients [29]. It was speculated that these patients with GP-specific autoantibodies were able to maintain a status of compensated thrombocytolysis. Thrombocytopenia only occurred when platelet destruction exceeded compensatory platelet production by marrow megakaryocytes.

Reasons for thrombocytopenia in HBV-related LC remain the subject of debate, and multiple factors, including splenic sequestration, reduced production of TPO as well as bone marrow suppression have been proposed. It was well demonstrated a decade ago that frequencies of circulating anti-GPIIb/IIIa antibody-producing B cells in HBV-related LC patients were significantly higher than in the healthy controls [23]. Consistently, the levels of platelet-bound anti-GPIIb/IIIa antibodies were also elevated remarkably in HBV-related LC patients and showed a positive correlation with the frequencies of circulating anti-GPIIb/IIIa antibody-producing B cells [23]. In line with that, our data indicated that not only GPIIb/IIIa autoantibodies but also GPIb/IX autoantibodies on platelet surface or in plasma were elevated in thrombocytopenic HBV-related LC patients, but we did not observe any correlation between platelet counts and levels of GP-specific autoantibodies.

It has been well established that function of macrophage Fcγ receptors (FcγRs) was impaired in cirrhotic patients, [38] and type I activation of macrophages (M1) which leading to T-helper type 1 (Th1) responses and enhanced phagocytic ability was defective in LC patients with HBV infection [39]. In accordance with these observations, our results showed that phagocytosis function of macrophages was associated with the disease severity of thrombocytopenic CLD. More specifically, phagocytic ability was the highest in CHB patients, lower in those with Child A LC and lowest in those with Child B/C LC. Moreover, discrepancies in the ability of phagocytosing opsonized platelets could attribute to macrophages themselves

instead of properties of GP-specific autoantibodies, as the same phagocytic difference was also found when murine IgG2a anti-human major histocompatibility complex class I monoclonal antibody was used as the opsonizing antibody. Consequently, it could be speculated that the defects in phagocytic ability of macrophages could reduce autoantibody-mediated platelet destruction to a certain extent in LC patients.

GPIb/IX autoantibodies were recently demonstrated to be associated with Fc-independent platelet clearance and resistance to intravenous immunoglobulin G (IVIg) therapy in ITP [40, 41]. Accordingly, phagocytosis experiments were conducted only in GPIIb/IIIa-autoantibody-positive thrombocytopenic patients. Mechanism behind GPIb/IX-autoantibody-mediated platelet destruction in LC has not been addressed. Ni's group observed in ITP that GPIb/IX autoantibodies could trigger platelet activation and neuraminidase translocation to the cell membrane, leading to platelet desialylation and hepatic platelet clearance [42, 43]. Whether a similar process happens in GPIb/IX-autoantibody-positive LC patients still awaits further investigation.

In summary, our present study further confirmed the presence of platelet GP-specific autoantibodies in HBV-related CLD, and we demonstrated for the first time that platelet phagocytosis was enhanced in autoantibody-positive thrombocytopenic CLD patients. Therefore immune-mediated platelet destruction indeed took part in the development of thrombocytopenia in HBV-related CLD. Combination of immunosuppressive agents with antiviral drugs might provide therapeutic benefits for thrombocytopenic CLD.

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Disclosure of conflict of interest

None.

Authors' contribution

Liu XG- designed research, performed research, analyzed data and wrote the paper; Li T- performed research and analyzed data; Feng Q- performed research and analyzed data; Liu Y- performed research; Liu Y- performed research and analyzed data; Wang YM- performed research and analyzed data; Li GS- performed research, Zhu YY- performed research and analyzed data; and Hou M- designed research, performed research, analyzed data and wrote the paper.

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