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Platelet activation and hypercoagulability in patients with early primary biliary cholangitis compared with healthy controls

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Abstract

Background Patients with primary biliary cholangitis (PBC) who have advanced disease are hypercoagulable, with no thrombophilic factors compared to non-cholestatic cirrhotics. We investigated whether hypercoagulability is present in early-stage PBC.

Methods PBC patients with biopsy-documented early disease and healthy controls matched by sex and age were asked to participate in the study. All were evaluated using rotational thromboelastometry (ROTEM), platelet aggregation, and flow cytometry. Four ROTEM parameters were evaluated (clotting time, clotting formation time, α-angle, and maximum clot firmness [MCF]). Platelet aggregation was determined as the maximal change in light transmission after the addition of adenosine diphosphate, collagen and epinephrine. Flow cytometry was used to evaluate the expression of glycoprotein (GP) IIb, GPIIa, and P-selectin on the platelet surface.

Results We enrolled 50 individuals in the study (25 PBC patients, 25 controls). Prothrombin time and activated partial thromboplastin time did not differ significantly between PBC patients and controls (P-value not significant). In ROTEM, α-angle and MCF parameters were abnormally elevated in 9 (36%) PBC patients compared to 3 (12%) healthy controls and the difference was statistically significant (P=0.026). Platelet aggregation in PBC patients was not significantly different from controls. In flow cytometry, GPIIb and P-selectin expression was greater in PBC patients than in the control group and the difference was statistically significant (P=0.005 and P=0.006 respectively).

Conclusion In this study, we used a combination of sophisticated methods to detect evidence of platelet activation and hypercoagulability in patients with early PBC. Our findings may have important clinical implications and merit further investigation.

Keywords Rotational thromboelastometry, primary biliary cholangitis, platelet aggregation, flow cytometry, P-selectin

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Introduction

Primary biliary cholangitis (PBC) is a chronic cholestatic liver disease of unknown origin. It has a slow progressive course, and some patients may develop cirrhosis and liver failure requiring liver transplantation [1]. Previous studies have shown that patients with PBC tolerate variceal bleeding fairly well and have much more favorable survival than patients with viral or alcoholic liver disease [2,3]. Moreover, it has also been observed that PBC patients have lower blood loss during liver transplantation compared to other cirrhotics [4,5].

In addition, other investigators have reported thrombosis in the portal vein radicles of PBC patients [6]. The cause of these

observations remains unclear. A plausible explanation would be that PBC patients may have a hypercoagulable state.

Indeed, it has been previously shown that PBC patients with advanced disease are hypercoagulable, while no differences in coagulation factors or platelet counts were detected [7]. Other studies found that platelet function differs between patients with cholestatic and non-cholestatic liver disease and is stable or even hyperactive in patients with PBC [8].

However, most patients in those studies had advanced liver disease, making it impossible to determine whether hypercoagulation is an early or late manifestation in the course of the disease. Furthermore, investigation using conventional coagulation tests does not provide information on the quality of clot, or on the dynamics of its formation as well as the platelet function [9]. Therefore, we aimed to investigate coagulation and platelet function in patients with early PBC compared to healthy controls using rotational thromboelastometry (ROTEM), platelet aggregation, and flow cytometry.

Patients and methods

Patients

Consecutive patients with newly diagnosed PBC who attended the outpatient liver clinic of our Department during a 24-month period were invited to participate in the study. Patients were considered eligible if they were over 18 years of age and they fulfilled the criteria of early disease (stage I and II by Scheuer classification, normal bilirubin, no evidence of portal hypertension) [10]. We excluded patients with concomitant liver diseases, such as autoimmune hepatitis, primary sclerosing cholangitis, IgG4 cholangiopathy, viral hepatitis, metabolic disorders, alcoholic liver disease or non-alcoholic fatty liver disease (NAFLD). We also excluded patients with known coagulation and/or platelet disorders. None of the patients had a malignancy or had been receiving medication known to impair coagulation.

The study protocol conformed to the principles outlined in the Declaration of Helsinki and was approved by the Ethics Committee of the hospital. All participants gave written informed consent.

The diagnosis of PBC was made on the basis of the clinical features and biochemical findings and confirmed by finding antimitochondrial antibodies (AMA) in the serum and histological changes in the liver, typical of or compatible with this disease [11]. Each patient with PBC was matched by age (±5 years) and sex to one healthy control.

Study design

All patients and controls were evaluated with routine laboratory investigations including a full blood count, prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, and liver function tests.

ROTEM

Thromboelastometry was performed from fresh whole blood in all patients and controls. The time elapsed from blood collection to the beginning of thromboelastometry assays was less than 1 h. We used the ROTEM® thrombelastometer (Pentapharm GmbH, Munich, Germany), a device that estimates the overall interactive dynamic coagulation process among plasma factors, platelets, red blood cells and leukocytes [12]. Briefly, the ROTEM® analyzer evaluates the viscoelastic change properties of a pin rotating within a heated cup containing whole blood samples. An optical system detects the impedance of the rotation of the pin and the plot system describes the trace produced by viscoelastic changes associated with the fibrin polymerization. All the assays were performed according to the standard protocols supplied by the manufacturers. We used non-activated thromboelastometry (NATEM) to assess whole blood clot in the absence of activation of the clotting cascade other than recalcification and spontaneous contact activation. In this respect, NATEM is the test that better mimics the original thromboelastography in whole blood for the overview of the entire hemostatic process. The following 4 parameters were recorded: clotting time (CT, sec) is the time from the beginning of the coagulation analysis until an increase in amplitude of 2 mm. Clotting formation time (CFT, sec) corresponds to the time between an increase in amplitude of the thrombelastogram from 2-20 mm. Alpha (a) angle represents the tangent to the clotting curve through the 2 mm point. Maximum clot firmness (MCF, mm) is the maximum amplitude reached in the thrombelastogram (Fig. 1).

Light transmission aggregometry (LTA)

LTA was evaluated as previously described, using platelet-rich plasma from blood collected into buffered sodium citrate [13]. LTA was tested within 2-3 h of blood collection using a PACKs-4 aggregometer (AggRAM, Helena Biosciences, Europe) and agonists that included 2.5 μM and 5 μM adenosine diphosphate (ADP), 2 μg/mL collagen and

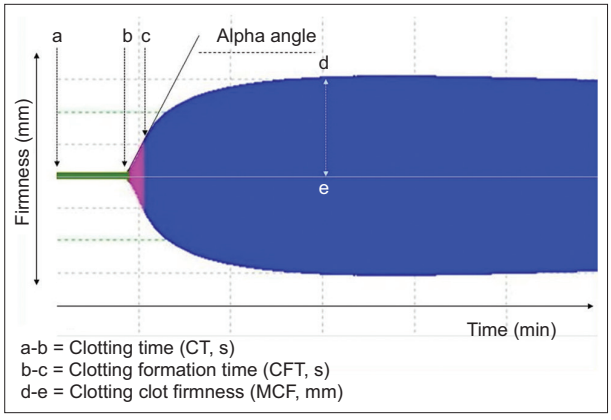

Figure 1 Thrombelastogram tracing obtained by rotational thromboelastometry, showing the different components of clot formation

5 μM epinephrine. All tracings were inspected by the same investigator, who provided an overall interpretative comment. All subjects were fasting and refrained from smoking for at least 1 h before blood collection. Blood samples were drawn with minimal or no venostasis; if a tourniquet was necessary, it was immediately released as soon as blood collection begun. Results were reported as percentage (%) of aggregation at the end of the monitoring period.

Platelet flow cytometry

We used flow cytometry to investigate the expression of glycoprotein (GP) IIb, GPIIIa and GMP140 (P-selectin) on platelet surface, in the resting state [14]. Citrated blood samples collected from the antecubital vein were incubated with different mouse monoclonal antibodies (MAb) directed against human GPIIb (CD41), GPIIIa (CD61), GMP140 (CD62P), and a negative isotypic control. Mean fluorescent intensity (MFI) was measured with a flow cytometer (BD FACSCanto™ II, BD Biosciences, Europe) after the addition of a staining reagent (polyclonal anti-mouse IgG-FITC) to the sample and calibrator tubes. Through this calibrator (coated with defined increasing numbers of MAb molecules), the MFI was converted into the absolute number of MAb molecules bound per platelet (ABC). The results were expressed in sABC (specific ABC), equivalent in this system to the number of GP molecules per platelet on the surface.

The investigators who performed the experiments were unaware of the source of each sample (PBC patient or control). Hypercoagulability was defined by values >2 standard deviations (SD) above those of healthy controls.

Statistical analysis

All information and laboratory results were entered into a database and double-checked for accuracy before analysis. Categorical variables were reported as counts (percentage) and continuous variables as means (±SD) or median and interquartile range. Quantitative variables were compared between patients and controls using the Wilcoxon matched pairs test. Categorical data were analyzed using the χ^2-test with Yate's correction or Fisher's exact test, as appropriate. The relationships between PT and CT values, as well as between bilirubin or albumin and MCF values, were evaluated using Spearman's rank correlation coefficient. Missing data attributed completely at random were handled by pairwise deletion. Only 2-tailed probabilities were used for testing statistical significance. All statistical analyses were performed using SPSS 22.0 for Windows (SPSS, Chicago, IL, USA). A P-value of less than 0.05 was considered statistically significant.

Results

Thirty-two PBC patients with early disease were considered eligible for the study. Seven patients were excluded because of coexistent autoimmune hepatitis (n=2), chronic hepatitis B (n=2), NAFLD (n=1), or because they were under treatment with antiplatelet drugs (n=2; one with a history of venous thromboembolism and the other with a previous transient ischemic attack). Therefore, 25 PBC patients (mean age: 58.2±11.3, 24 female) were included in the analysis. AMA were detected in all patients. The duration of the disease was 8.9±3.5 months. The majority of patients were asymptomatic, while 2 suffered from pruritus, 3 reported fatigue, and 2 had both of those symptoms. Three patients had previously been diagnosed with autoimmune thyroiditis and 1 with sicca syndrome. Twenty-one patients (87.5%) had started treatment with ursodeoxycholic acid within the previous 6 months. The 25 PBC patients were matched with 25 healthy controls. There were no differences in demographic and laboratory data between patients and controls, apart from alkaline phosphatase levels, which were higher in PBC patients to a statistically significant degree. Data are presented in Table 1.

Four different parameters (CT, CFT, α-angle, and MCF) were estimated with ROTEM. Mean values of CT, CFT, MCF, and α-angle did not differ significantly in PBC patients compared to controls although the difference approached statistical significance for α-angle and MCF comparisons (Table 2). However, α-angle and MCF parameters were abnormally greater in 9 (36%) PBC patients compared to 3 (12%) healthy controls and the difference was statistically significant (P=0.026).

Platelet aggregation measurements were within normal range in all PBC patients. No statistical difference was found in

Table 1 Baseline characteristics of PBC patients and healthy controls

Characteristics	PBC (n=25)	Controls (n=25)	P-value*
Patients (n)	25	25	
Age (years)	58.2±11.3	57.1±12.2	
Female	24	24	
BMI (kg/m^2)	27.4±3.1	27.9±3.4	NS
Smoking (Y/N)	8/17	11/14	NS
PT (sec)	12.7±0.6	12.6±0.4	NS
INR	1.02±0.06	0.99±0.05	NS
PTT (sec)	38±4	37±4	NS
Fibrinogen (mg/dL)	392±90	298±58	NS
Platelets (10^9/mm^3)	238±82	243±67	NS
Bilirubin (mg/dL)	0.5±0.2	0.4±0.2	NS
Albumin (g/dL)	3.9±0.3	4.0±0.3	NS
ALP (IU/L)	190±160	79±21	<0.05
AST (IU/L)	29±8	22±7	NS
ALT (IU/L)	30±8	20±6	NS
UDCA treatment	21	0	

Values are expressed as mean±SD

* P-value comparing PBC patients and healthy controls

PBC, primary biliary cholangitis; BMI, body mass index; PT, prothrombin time; INR, international normalized ratio; PTT, partial thromboplastin time; ALP, alkaline phosphatase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; UDCA, ursodeoxycholic acid

PBC patients compared to controls in the use of ADP 2.5 μM, ADP 5 μM, collagen, and epinephrine (Table 2).

In flow cytometry, GPIIb expression on platelet surface was greater in PBC patients than in the control group and the difference was statistically significant (P=0.005; Fig. 2). No significant difference was detected in GPIIIa expression in PBC patients compared to matched controls (P=0.5). P-selectin was expressed on platelet surface in 17 (68%) patients and 6 (24%) healthy controls. The difference was statistically significant (P=0.006).

PT and APTT were not significantly different between PBC and controls (P=NS). PT was significantly correlated with CT values (P=0.005). No correlation was found between MCF and bilirubin (P=0.114) or albumin (P=0.235).

Discussion

There is now a growing body of evidence that patients with chronic liver disease appear to have a hypercoagulable profile, which may in itself be implicated in the progression of fibrosis and the development of complications [15,16]. These findings seem to be more profound in patients with cholestatic liver disease. However, in all published studies, the authors included patients with different cholestatic diseases (PBC, primary sclerosing cholangitis [PSC], overlap syndrome) as well as cirrhotic and non-cirrhotic populations, so it cannot be determined whether hypercoagulation is an early or late phenomenon in the course of the disease [7,8]. In our study, we only recruited patients with early PBC and we investigated potential abnormalities in platelet function and coagulation using routine coagulation tests as well as thromboelastometry, LTA, and platelet flow cytometry, comparing them with an equal number of healthy controls of the same age and sex.

We did not find any differences in routine coagulation tests between our patients and healthy controls. In a previous study, elevated fibrinogen levels were found in patients with non-cirrhotic PBC/PSC and were attributed to an acute phase reaction related to cholestasis [8]. In our study, although PBC patients had higher fibrinogen levels, the difference did not reach statistical significance.

We used ROTEM to evaluate the interactions between plasma clotting factors, platelets and other blood cells, as it is more sensitive than routine assays in detecting hypercoagulability. The main advantage of ROTEM compared to classic thromboelastography (TEG) is that is very robust and not susceptible to variations or mechanical shocks [17]. We found abnormal values of α-angle and MCF in a substantial proportion of our early PBC patients. A-angle denotes the speed at which a solid clot forms and is mainly a function of platelets and plasma component on platelet surface. A high α-angle indicates hypercoagulability. MCF represents the absolute strength of clot and is associated with the dynamic properties of fibrin and platelet bonding via GPIIb/IIIa receptors [18]. Previous studies that used classic TEG had detected hypercoagulability in patients with cholestatic liver disease. However, in the study conducted by Ben-Ari *et al*, most of the patients had advanced liver disease, which may have influenced the results [7]. Pihusch *et al* also found hypercoagulability using classic TEG, but they included patients with PBC as well as patients with PSC [8]. In our study, we evaluated a homogeneous population of patients with early PBC, proved histologically; therefore, our results are independent of any bias attributed to heterogeneity of the study cohort.

We also investigated for the first time platelet function in early PBC patients using LTA [19]. We did not find any abnormalities in platelet aggregation in our population using different activators (ADP, collagen, epinephrine), nor were there any differences between PBC patients and healthy controls. This is not surprising, as platelet aggregation is affected by platelet count, usually normal in patients with early PBC. In line with our results, Pihusch *et al* used a different methodology (PFA-100) and found no abnormalities of platelet aggregation in patients with cholestatic liver disease, independently of the stage of disease [8]. The authors suggested that platelet function might compensate for low platelet numbers in end-stage cholestatic liver disease.

Table 2 Rotational thromboelastometry and platelet aggregation parameters in PBC patients and controls

Parameters	PBC (n=25)	Controls (n=25)	P-value*
CT	509±107 (357-831)	483±104 (310-737)	0.346
CFT	116±44 (14-220)	133±48 (78-240)	0.209
α-angle	71±6 (63-82)	69±5 (61-80)	0.086
MCF	78±9 (65-96)	74±7 (64-90)	0.067
ADP 2.5 μM	70±26 (25-104)	62±25 (15-94)	0.223
ADP 5 μM	77±18 (27-95)	78±20 (25-104)	0.910
Collagen	73±13 (41-96)	66±18 (6-103)	0.123
Epinephrine	79±17 (19-94)	72±24 (12-100)	0.201

Values are expressed as mean±SD, range

*P-value comparing PBC patients and healthy controls

PBC, primary biliary cholangitis; CT, clotting time; CFT, clotting formation time; MCF, maximum clot firmness; ADP, adenosine diphosphate

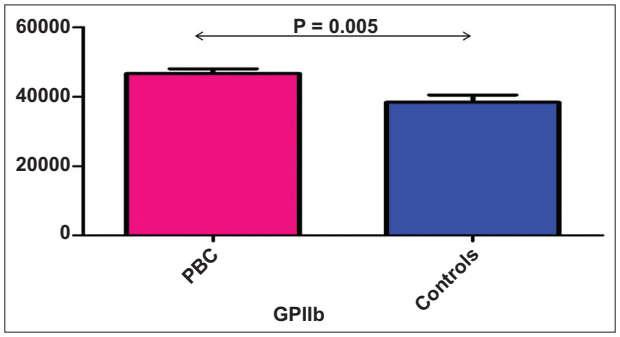

Figure 2 GPIIb expression on platelet surface at the resting state in early PBC patients and in matched controls (results are expressed as number of GP molecules per platelet on the surface). The difference between PBC patients and controls was statistically significant (P=0.005). *PBC, primary biliary cholangitis; GP, glycoprotein*

Finally, we evaluated platelet activation using flow cytometry to detect the expression of GPIIb (CD41), GPIIIa (CD61), and P-selectin (CD62P) on the platelet surface in the resting state [20]. We found greater expression of GPIIb in the platelets of patients with early PBC compared to healthy controls, and the difference was statistically significant. Glycoprotein IIb, part of the highly expressed GPIIb/IIIa receptor, is in a low affinity state under resting conditions. Following platelet activation, GPIIb/IIIa undergoes a conformational change, which increases its affinity for fibrinogen leading to platelet-platelet adhesion and aggregation [21]. We also found a greater number of PBC patients who expressed P-selectin on the platelet surface and the difference was statistically significant. P-selectin is a membrane glycoprotein expressed by platelets after fusion of a-granules to the cell membrane following cell activation. In the resting state, P-selectin is virtually absent from the surface of the platelets and therefore is considered a reliable marker of platelet activation [22]. P-selectin promotes platelet aggregation through platelet-fibrin and platelet-platelet binding [23]. In the only previous study that used flow cytometry for the investigation of platelet function, the surface expression of GPIIb and P-selectin was similar in patients with cholestatic and non-cholestatic liver disease, while an increased membrane density of glycoprotein Ib (CD42b) and ligand-induced binding sites was detected in cholestatic patients, suggesting pre-activated circulating platelets [8]. Those results cannot directly be compared with our findings, as they investigated a mixed population including cirrhotic and non-cirrhotic PSC and PBC patients.

At present, it is well recognized that a hypercoagulation syndrome might be involved in patients with chronic liver disease, manifested as macrovascular thrombosis or as micro-thrombotic complications within the liver parenchyma, with a typically more indolent course [24]. Occlusion of intrahepatic vasculature may lead to localized ischemia and infarction, a process described as parenchymal extinction [25]. Previous studies have shown an increased incidence of thrombosis in portal vein radicles of PBC patients [6]. Recently, 2 publications emphasized the significance of peripheral venous thromboembolic disease and portal vein thrombosis in cirrhosis [26,27]. Importantly, Villa *et al* have recently reported that a 12-month course of a low-molecular-weight heparin was safe and effective in preventing portal vein thrombosis in patients with cirrhosis, and appeared to delay the occurrence of hepatic decompensation and to improve survival [28]. We should also take into consideration that thrombosis in patients with chronic liver disease might become an emerging issue, owing to their increasing life expectancy, which exposes them much more than in the past to risk factors such as tumors, surgery, reduced physical activity and prolonged hospitalization.

In conclusion, we investigated several coagulation parameters in patients with early PBC and we found indices of hypercoagulability due to increased platelet activation. Future studies will probably clarify the underlying pathophysiological mechanisms and will identify appropriate means for early diagnosis and effective treatment to prevent complications and improve prognosis.

Summary Box

What is already known:

- Patients who have primary biliary cholangitis (PBC) with advanced disease are hypercoagulable, while no differences were detected in coagulation factors or platelet counts
- It is not clear whether this is a late or early manifestation of the disease
- Rotational thromboelastometry (ROTEM), platelet aggregation, and flow cytometry are sophisticated methods that provide information about the quality of clot, the dynamics of its formation as well as platelet function

What the new findings are:

- Patients with early PBC showed elevated ROTEM parameters, α-angle and maximum clot firmness, that reflected hypercoagulability
- These patients also showed platelet activation on flow cytometry, as reflected by increased expression of glycoprotein IIb and P-selectin on platelet surface
- Hypercoagulability might be an early manifestation in the course of PBC

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