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Platelet Activation in Patients with Chronic Hepatitis C

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***Objective*: To elucidate the mechanisms of thrombocytopenia in chronic hepatitis C (CHC), we investigated platelet activation in patients with chronic viral liver diseases. *Methods*: Platelet activation was evaluated with flow cytometry in twenty-five patients with chronic viral hepatitis and 11 patients with liver cirrhosis of viral etiology. Liver biopsies were carried out in all patients. *Results*: The platelet counts decreased significantly in patients with CHC and in patients with liver cirrhosis compared to controls, but not in patients with chronic hepatitis B (CHB). Patients with CHC had a significantly higher percentage of platelets positive for activation-dependent monoclonal antibodies (MoAbs), and also had a higher percentage of platelet microparticles (PMP), a marker of platelet activation, than patients with CHB. There was a significant correlation between the percentage of PMP and the levels of liver fibrosis markers, such as serum hyaluronate and N-terminal propeptide of type III procollagen (P-III-P), in CHC, suggesting the relationship between platelet activation and liver fibrosis. Platelet activation was markedly enhanced in CHC patients with high histological scores of liver fibrosis. *Conclusion*: Patients with CHC have increased platelet activation, which may contribute to the occurrence of thrombocytopenia in CHC. Liver fibrosis may play a role in activation of platelets in CHC.**

Key words : Platelet activation, Chronic hepatitis C , Liver fibrosis, Platelet microparticles

INTRODUCTION

Thrombocytopenia is a common manifestation in patients with chronic liver diseases. Possible mechanisms for thrombocytopenia proposed have been portal hypertension [1], splenomegaly [1], decreased production of thrombopoietin [2], increased platelet associated IgG [3]. However, the cause of thrombocytopenia was not clearly elucidated yet. The platelet counts are predictor of liver fibrosis. Platelet counts correlated significantly with fibrotic stage [4, 5]. In this report we investigated platelet activation and suggested platelet activation might be one of the causative factors of thrombocytopenia in patients with chronic viral hepatic diseases, especially in patients with chronic hepatitis C (CHC).

MATERIALS AND METHODS

Patients: Twenty-five patients with chronic viral hepatitis (20 cases with hepatitis C, 5 cases with hepatitis B, 21-69 years-old; 17 males and 8 females) and 11 patients with liver cirrhosis (8 cases caused by hepatitis C virus and three cases by hepatitis B virus, 49-75 years-old; 6 males and 5 females) were investigated. All the patients had been studied by liver biopsy. Patients with chronic hepatitis were classified histologically into four groups based on the staging of hepatic fibrosis using the scoring system of Desmet *et al*. [6]: (score 1;mild fibrosis, score 2; moderate fibrosis, score 3; severe fibrosis, and score 4; cirrhosis). Patients with hepatocellular carcinoma, or patients with hematological disorders such as aplastic anemia, leukemia, and idiopathic thrombocytopenic purpura,

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were excluded. Twenty-three healthy volunteers (21-80 year-old; 15 males and 8 females) were examined as controls. Both patients and the healthy controls did not take any antiplatelet drugs for ten days prior to platelet studies. Patients were not treated with steroids or interferon.

Collection of samples: Fasting venous blood was taken to plastic tubes in the morning, using a 21-gauge needle. The first 10 ml were used for routine laboratory tests including the measurement of HCV-RNA or HBV-DNA. Blood for platelet analysis and for hemostatic markers was taken to a tube containing 1/10 vol. of 3.13 % sodium citrate (Terumo, Japan).

Staining: Within 15 minutes after blood collection, 2.5 μl aliquots of the blood were added to 12 × 15-mm polystyrene tubes containing a cocktail of 10 μl each of three kinds of florescence labelled monoclonal antibodies (MoAb). One fluorescence reagent, PerCP-MoAb-CD61 was used to identify all the platelets; FITC-PAC1 and PE-MoAb-CD62P were used to detect activated platelets (three color flow cytometry). PAC-1 is a MoAb specific for fibrinogen receptor expressed on GPIIb/IIIa of platelet membranes when platelets are activated. CD62P is a glycoprotein on the α-granule membranes and is exposed on platelet surface membranes when platelets are activated. The samples were incubated for 15 minutes at room temperature in the dark, and then fixed with CELL-FIX (Becton Dickinson Biosciences, San Jose, CA) for over 2 hours in the refrigerator. To assess extent of non-specific staining by antibodies, 2.5 μl of whole blood is incubated with 0.62 μl of Mouse IgG1 PE isotype control and 10 μl of PAC-1 FITC with 10 μl of 5 mg/ml RGDS solution, and 10 μl of CD61 PerCP.

Flow cytometry: Flow cytometric analysis was performed on a FACS Calibur (Becton Dickinson) with standard 488 nm excitation. Instrument setting was performed by FACSComp software (Becton Dickinson). Platelets were identified by both side scatter profiles and platelet specific antibody, MoAb-CD61, and activation-dependent antibody binding was expressed as the percentage of platelets positive for antibody. Antibody-positive cells were defined as those platelets with a fluorescence intensity $>$ 99.0 % of platelets that were determined in the presence of isotype IgG or fibrinogen receptor-blocking tetrapeptides, RGDS as negative control. Platelet microparticles (PMP) were identified by gating on anti-CD61-positive events, which reflect glycoprotein IIIa on the platelet membranes, and differentiated from normal-sized platelets by forward light scatter to that of fluorescent labelled reference beads(Becton Dickinson) of 2.0 μm in diameter [7]. Ten thousand positive platelet events were analysed, and PMP were reported as a percentage of the total platelet events [7, 8].

Ultrasonic Assessment of Spleen Size: Spleen size was estimated by ultrasonography using the method of Matunami *et al.* [9]. Briefly, using a left intercostals scan that contains the splenic hilum and the largest area of spleen, the distance between the hilar indentation and anterior cranial end (A) and length of the line perpendicular to it (B) were measured in cm. The product of A × B was used as an index of spleen size (cm^2).

Assays: Platelet counts were measured using an automated hematology analyzer (NE-8000, Sysmex, Kobe, Japan). Prothrombin time (PT) and international normalized ratio (INR) were measured using an automated analyzer (Coarex-800, Sysmex, Kobe, Japan). Serum HCV-RNA was measured by reverse transcriptase polymerase chain reaction and HBV-DNA was determined by polymerase chain reaction. Concentrations of the thrombin-antithrombin III complex (TAT) were measured by enzyme immunoassays (EIA). The prothrombin fragments F_{1+2} (F_{1+2}) were measured by ELISA. The plasmin-α^2-antiplasmin complex (PIC) was measured by LPIA. Serum hyaluronate concentration was measured by using a one-step sandwich enzyme immunoassay system. Serum N-terminal propeptide of type III procollagen (P-III-P) was measured by EIA. Type IV collagen was measured by radio immunoassay (RIA). The normal range for PT was $>$ 70 %, for INR was 0.8-1.2, for PIC was $<$ 0.8 μg/ml, for TAT was $<$ 3.0 ng/ml, for F_{1+2} was 0.4-1.4 nmol/l, for Type IV collagen was $<$ 6 ng/ml, for hyaluronate was $<$ 50 ng/ml, for P-III-P was 0.3-0.8 U/ml, respectively.

Statistical analysis: Data were analysed statistically by the SPSS for Windows software package SPSS R 6.1.2.™ (SPSS Software Gmb H, Munich, Germany). The Student's t-test or Mann Whitney U test, and differences

were considered to be significant at $p < 0.05$.

RESULTS

Platelet counts: Platelet counts, platelet activation, parameters for blood coagulation and fibrinolysis of healthy controls and patients with chronic viral hepatitis and liver cirrhosis were shown in Table 1. Compared with the normal controls ($22.72 \pm 4.27 \times 10^4/\mu l$), the platelet counts were significantly decreased in patients with CHC ($14.59 \pm 5.28 \times 10^4/\mu l$, $p < 0.001$), hepatitis C virus liver cirrhosis($10.3 \pm 7.69 \times 10^4/\mu l$, $p < 0.001$) and hepatitis B virus liver cirrhosis ($11.67 \pm 2.05 \times 10^4/\mu l$, $p < 0.001$), respectively. However, no significant decrease was observed in patients with chronic hepatitis B (CHB).

Parameters for coagulation and fibrinolysis: The coagulation markers, TAT and $F_{1+2,}$ and a fibrinolysis marker, PIC were not significantly increased in any disease conditions. There were no significant correlations between platelet counts and any coagulation or fibrionlysis markers.

Platelet activation: We examined the relation between thrombocytopenia and platelet activation in chronic liver diseases. Three different parameters (CD62P, PAC-1 and PMP) were used for monitoring platelet activation. The patients with CHC showed significantly higher percentage of platelets positive for CD62P (9.35 ± 9.84 %, vs. 1.44 ± 1.67 %, $p < 0.001$), PAC-1 (44.65 ± 27.24 %, vs. 17.81 ± 11.00 %, $p < 0.001$) and PMP (4.54 ± 3.86 %, vs. 1.63 ± 2.04 % $p < 0.001$) than normal controls, respectively (Table 1). The patients with CHB, however, did not have significantly higher percentage of platelets positive for activation markers.

The relationship between platelet activation and HBV-DNA or HCV-RNA: The three parameters for platelet activation were not correlated with viral load of HBV-DNA or HCV-RNA. (data not shown).

The relationship between platelet counts, platelet activation and biological parameters for liver fibrosis in patients with chronic liver diseases: Platelet counts had inverse correlation with serum hyaluronate ($r = -0.55$, $p < 0.05$), and P-III-P ($r = -0.52$, $p < 0.05$), (data not shown). There were significant positive correlation between PMP and hyaluronate ($r = 0.64$, $p < 0.01$), and also positive correlation between PMP and P-III-P ($r = 0.53$, $p < 0.05$), (Fig. 1).

The relationship between histological score for liver fibrosis and platelet activation, splenomegaly or serum parameters for liver fibrosis in patients with CHC: The percentage of PMP was significantly increased in patients with fibrosis score 3 (9.36 ± 4.20 %) than those with the fibrosis score 1 (1.77 ± 0.61 %, $p < 0.05$). The hyaluronate was also significantly increased in patients with score 3 (994.33 ± 996.9

Table 1 Platelet counts, platelet activation, parameters of coagulatin and fibrionlysis in patients with liver diseases.

	Control	CHB	CHC	LCB	LCC
	n = 23	n = 5	n = 20	n = 3	n = 8
Platelet Counts	22.72 ± 4.27	21.44 ± 7.72	14.59 ± 5.28 *	11.67 ± 2.05 *	10.3 ± 7.69 *
CD62P (%)	1.44 ± 1.67	2.45 ± 1.05	9.35 ± 9.84 *	2.07 ± 1.5	5.83 ± 4.37
PAC-1 (%)	17.81 ± 11.00	28.66 ± 12.98	44.65 ± 27.24 *	42.33 ± 23.44	40.59 ± 21.92
PMP (%)	1.63 ± 2.04	3.24 ± 2.03	4.54 ± 3.86 *	2.50 ± 0.173	3.81 ± 3.20
PT %	> 70	85.0 ± 4.58	91.85 ± 5.15	81.0 ± 8.49	78.5 ± 81.7
INR	0.8-1.2	1.12 ± 0.05	1.06 ± 0.07	1.17 ± 0.1	1.21 ± 0.11
PIC (μg/ml)	< 0.8	1.70 ± 1.75	0.84 ± 0.60	0.53 ± 0.15	0.77 ± 0.31
TAT (ng/ml)	< 3.0	5.2 ± 6.93	1.32 ± 0.22	1.30 ± 0.36	1.63 ± 0.55
F_{1+2} (nmol/L)	0.4-1.4	0.92 ± 0.27	0.78 ± 0.18	0.52 ± 0.15	0.87 ± 0.25

Values are expressed mean ± SD

* Statistically significant compared with controls * $p < 0.001$

CHB: chronic hepatitis B, CHC: chronic hepatitis C, LCB: liver cirrhosis B, LCC: liver cirrhosis C

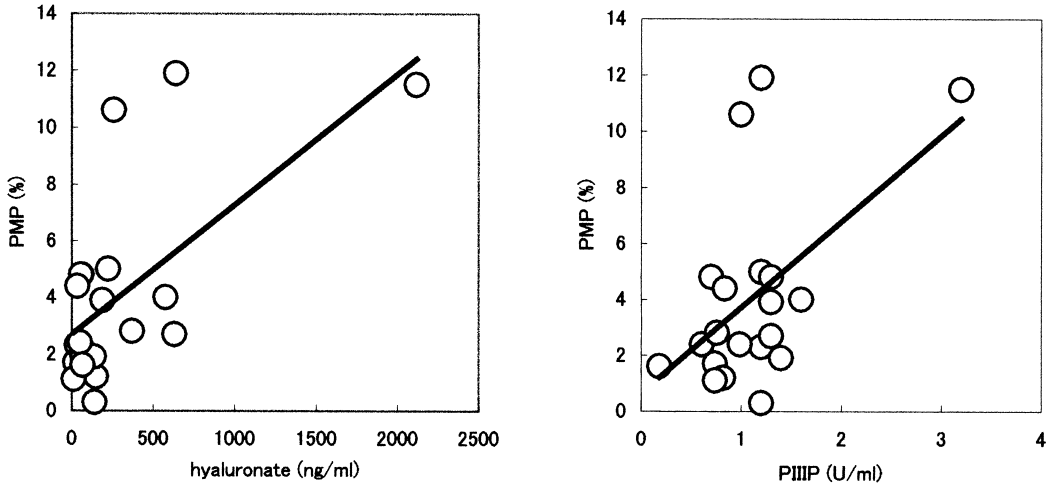

Fig. 1 Relationship between PMP and serum fibrosis parameters (hyaluronate and P-III-P). There are significant correlations between PMP and fibrosis parameters. (hyaluronate; $r = 0.64$, $p < 0.01$, P-III-P; $r = 0.53$, $p < 0.05$)

Table 2 Histological scores for liver fibrosis and platelet counts, platelet activation markers, fibrosis parameters and spleen size in patients with chronic hepatitis C.

	Score 1 n = 3	Score 2 n = 12	Score3 n = 5
Platelet Counts	15.6 ± 4.2	15.5 ± 5.0	11.7 ± 6.3
CD62P (%)	7.29 ± 8.1	10.1 ± 12.1	6.84 ± 2.48
PAC-1 (%)	35.9 ± 28.8	44.7 ± 30.0	49.80 ± 23.4
PMP (%)	1.77 ± 0.61	3.22 ± 2.17	9.36 ± 4.20 *
Type IV Collagen (ng/ml)	5.60 ± 1.13	5.18 ± 1.81	6.60 ± 0.85
P-III-P (U/ml)	0.97 ± 0.33	1.08 ± 0.28	1.87 ± 1.15
Hyaluronate (ng/ml)	37.33 ± 28.0	109.71 ± 58.51	994.33 ± 996.90 *
Spleen Index (cm^2)	40.33 ± 5.51	40.83 ± 10.53	41.50 ± 9.80

Values are expressed mean ± SD
* Statistically significant compared with score1 $P < 0.05$

ng/ml) than in patients with score 1 (37.33 ± 28.0 ng/ml, $p < 0.05$). There were no significant differences in type IV collagen and P-III-P levels. There were no significant differences in spleen size among patients with the three scores of fibrosis (Table 2).

DISCUSSION

Thrombocytopenia is a common complication of chronic liver diseases. However, the mechanisms involved in the thrombocytopenia are not fully elucidated. Our study demonstrated that thrombocytopenia and platelet activation coexisted in chronic liver diseases; especially in patients with CHC.

As the causes of platelet activation, we paid attention to the fibrosis of liver in chronic liver diseases. The markers of liver fibrosis, such as serum hyaluronate and P-III-P increased as PMP increased. The results suggested that activation of platelets correlated well with liver fibrosis. The fibrotic process of sinusoid may damage and activate the platelets through the changes of vasculature in the liver. PMP are considered to be fragments of platelet membranes

and granules with the size of 0.02-0.1 μm in diameter, and are known to increase in number when platelets are activated by stimulation such as high shear stress.

PMP are generated by high shear stress in diseased arteries and arterioles [10]. Many clinical disorders are associated with increased PMP levels; the levels of PMP increased in idiopathic thrombocytopenic purpura (ITP) [11], thrombotic thrombocytopenic purpura (TTP), acute myocardial infarction, hemolytic uremic syndrome [12]. diabetes mellitus [8, 12, 13], transient ischemic attacks (TIA) [14], cerebral infarction [7].

Murawaki reported that the measurement of platelet counts should be valuable in the assessment of fibrosis of the liver, because the thrombocytpenia is related to the degree of liver fibrosis in patients with CHC [4, 5]. And Adnolfi described that hepatic fibrosis seemed to be an independent factor, which contributes to thrombocytopenia in chronic viral hepatitis, however, the mechanisms were not known [15]. We obtained the results that the percentage of PMP increased when the stages of the liver fibrosis progressed. PMP showed significant positive correlation with hyarulonate and P-III-P. In 1963, Schaffner and Popper reported narrowing of hepatic sinusoid accompanied the progression of hepatic fibrosis [16]. Most of circulating hyaluronate is commonly degraded by hepatic sinusoidal endothelial cells (SECs), and formation of narrowing of hepatic sinusoid [17–20]. The serum hyaluronate concentration allows the evaluation of morphological and functional changes of SECs. The high serum hyaluronate levels, $>$ 200 ng/ml, may suggest the damage of SECs, and the development of fibrosis in sinusoid [21]. In our data, serum hyaluronate concentration was 900 ng/ml in patients with histological liver fibrosis, score 3. Wanless prepared the liver fibrosis models in New Zealand White Rabbit and found that many hepatic venules became narrow, and the platelet counts were decreased [22]. In our study, increased percentage of PMP might be caused by high shear stress of blood vessels in fibrotic liver.

The reason why PMP was not increased and platelet counts were not decreased in patients with CHB was not clear. CHB might present with different pathophysiology from CHC. Liver fibrosis is significantly higher in CHC patients than in CHB. In CHB, most cases had seroconversion from hepatitis e antigen to hepatitis e antibody, and progression of fibrosis tended to stop. On the contrary, in CHC, the virus could replicate continuously even after chronic hepatitis progressed, leading to persisting inflammatory reactions and active fibrosis throughout its natural course [23].

Thrombocytopenia may result from heightened coagulation pathway such as disseminated intravascular coagulation (DIC). In our study, however, there was no data suggesting the enhanced coagulation or fibrinolysis.

CONCLUSION

Patients with CHC demonstrate increased platelet activation, which may contribute to the mechanisms of thrombocytopenia in these patients. Liver fibrosis may play a role in activation of platelets in CHC.

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