

## Platelet Activation in Patients with Alcoholic Liver Disease

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**Objective:** To elucidate the mechanisms of thrombocytopenia in alcoholic liver diseases, we investigated activation status of platelets in patients with alcoholic fatty liver (AI-FL), alcoholic liver cirrhosis (AI-LC) or hepatitis-C liver cirrhosis C (C-LC).

**Methods:** Platelet activation was evaluated by flow cytometry using monoclonal antibodies against P-selectin (CD62P) and the fibrinogen receptor (PAC-1), both specific for platelet activation, and anti-CD61 antibody for the presence of microparticles (PMP) in seven patients with AI-FL, thirteen patients with AI-LC and, as a non-alcoholic liver disease control, nine patients with C-LC. As a normal control, seventeen healthy subjects without liver dysfunction were also evaluated.

**Results:** Compared with the healthy controls, the platelet count was significantly decreased in patients with alcoholic liver diseases or C-LC. Ten days after discontinuation of alcohol intake, the platelet count was significantly higher in both the AI-FL and AI-LC groups than that measured on admission. There was an inverse correlation between the platelet count and PMP, a marker of platelet activation. The AI-FL, AI-LC and C-LC groups showed significantly higher percentages of platelets positive for CD62P than the healthy controls. The PAC-1 positivity was increased only in the C-LC group. PMP were significantly increased in the AI-FL, AI-LC and C-LC groups compared to that in the healthy group. In the AI-LC group, PMP were significantly decreased 10 days after discontinuation of alcohol intake from that measured on admission.

**Conclusion:** Patients with alcoholic liver diseases have increased platelet activation, which may contribute to the occurrence of thrombocytopenia. The formation of PMP might be one of the important factors of thrombocytopenia in alcoholic liver diseases.

**Key words :** Platelet activation, Alcoholic liver disease, Platelet microparticles

### INTRODUCTION

In patients with chronic liver diseases, thrombocytopenia occurs at a high incidence depending on the severity of the disease and is one of the pathological characteristics of important clinical significance, especially in patients with liver cirrhosis. Mechanisms have been proposed for the decreased platelets, including destruction of the storage pool of platelets by hypersplenism [1, 2], megakaryopoiesis in the bone marrow, a reduction in the production of thrombopoietin, a platelet-specific growth factor [3-5], increased

platelet-associated IgG [6], structural vulnerability due to abnormalities in lipid or protein components of the platelet membrane [7, 8], and a reduction in the platelet lifespan as a result of the structural weakening [9].

Thrombocytopenia has also been observed in patients with alcoholic liver diseases in association with the aggravation and progression of the disease, and the reduced platelet count has been reported to be improved by discontinuation of alcohol intake [10]. From this finding, a direct effect of alcohol on the bone marrow, besides the above mechanisms, has been proposed [11, 12].

The lifespan of platelets is about 10 days and, under normal conditions, circulating platelets exist in the resting form with a stable surface membrane structure. When tissue damage or inflammation occurs, platelets stick to the lesions of the blood vessels and adhere to the exposed endothelial tissues. Upon adhesion, platelets are activated to release substances, such as  $\beta$ -thromboglobulin ( $\beta$ -TG) and platelet factor 4 (PF4), through which a conformation change is induced. Following the structural change of platelets, prothrombinase-complexes are formed and cause platelet aggregation, leading to the formation of hemostatic thrombi along with fibrin.

To date, only few clinical studies have examined the function of platelets using activated platelets in chronic liver diseases. We have reported that platelets were activated in patients with liver cirrhosis secondary to HCV infection [13]. In the present study, we examined activation status of platelets in patients with chronic alcoholic liver diseases. Activated platelets were detected in patients with fatty liver or liver cirrhosis by flow cytometric evaluation of platelet activation-specific molecules, CD62P and PAC-1. In addition, platelet counts and platelet activation were compared between the two time points, namely immediately after admission and 10 days after discontinuation of alcohol intake. Platelet activation and disease state were also compared between patients with alcoholic liver diseases and normal subjects or, as a control of non-alcoholic liver disease, those with liver cirrhosis secondary to HCV infection. The relationships between the reduced platelet count and the pathology of alcoholic liver diseases are discussed.

## SUBJECTS AND METHODS

### Study Subjects

The study enrolled a total of 20 male patients with alcoholic liver diseases, who were hospitalized for close observation and treatment. Patient who have complications of diabetes mellitus or a history of thromboembolic diseases such as cardiac and cerebral infarctions were excluded from this study, because these diseases were reported to affect platelet activation [14, 15]. The study subjects included 7 cases of alcoholic fatty liver (mean age:  $51.1 \pm 11.0$  years) and 13 cases of alcoholic liver cirrhosis (mean age:  $56.1 \pm$

10.6 years). In addition, 9 male patients with hepatitis-C liver cirrhosis without a history of alcohol drinking (mean age:  $69.3 \pm 8.6$  years) were used as a control of non-alcoholic liver disease. As a normal control, 17 healthy male subjects without liver abnormalities (mean age:  $50.7 \pm 18.9$  years) were also enrolled.

### Diagnosis of Alcoholic Liver Diseases

According to the diagnostic criteria proposed for Japanese by Takada *et al.* [16], subjects were considered to have alcoholic liver diseases when they were habitual drinkers of *Sake* taking more than 3-go ( $3 \times 0.18$  liters) per day (corresponding to more than 60 g/day of ethanol), and showed abnormalities on liver function tests, but were negative for blood HBV and HCV. The subjects studied here were classified into two groups: the fatty liver group, and the liver cirrhosis group.

Patients were diagnosed to have alcoholic fatty liver (AI-FL) when they showed a body mass index of not more than 22 and, besides bright liver, hepatorenal contrast and deep attenuation on echography, or showed pathological findings of fatty liver, i.e. fatty metamorphosis in more than 30% of hepatocytes on liver biopsies.

Patients were diagnosed to have alcoholic liver cirrhosis (AI-LC) when abnormal findings were manifested on echography or abdominal CT, such as marginal dullness and irregular nodularity of the liver, splenomegaly and ascites.

### Diagnosis of Hepatitis-C Liver Cirrhosis

Patients were diagnosed to have hepatitis-C liver cirrhosis (C-LC) when clinical findings or blood data indicative of positive HCV RNA and chronic liver dysfunction were found, besides the characteristic findings, such as a reduction in albumin, an AST/ALT ratio of over 1, and an increase in  $\gamma$ -globulin, along with the echographic or abdominal CT findings as described above for AI-LC. Patients with hepatocellular carcinoma were excluded from the present the study.

### Collection of Blood Samples

Blood sample collection was performed by the 2-syringe method via the cubital vein under a fasting condition in the early morning. The blood sample for platelet analyses was collected in a test tube containing 1/10

volume of 3.14% sodium citrate, while that for platelet counting was collected in a blood-collecting tube containing EDTA-2K as detailed elsewhere [13, 17].

### Measurement of Platelet Counts

Platelet counts were measured using an automated hematology analyzer (XE-2100; Sysmex, Kobe, Japan).

### Detection of Platelet Activation

To quantitate activated platelets, we used fluorescein isothiocyanate (FITC)-labeled anti-PAC-1 antibody (25 µg/mL) (hereafter abbreviated as PAC-1), which recognizes the structure of the fibrinogen receptor expressed on GPIIb/IIIa molecules, phycoerythrin (PE)-labeled anti-CD62P monoclonal antibody (MoAb) (1.5 µg/mL) (hereafter abbreviated as CD62P), which recognizes P-selectin, a glycoprotein of  $\alpha$ -granules expressed on the platelet surface upon platelet activation, and peridinin chlorophyll protein (PerCP)-labeled anti-CD61 MoAb (6.0 µg/mL) (hereafter abbreviated as CD61), which is an anti-GPIIIa antibody that recognizes all types of platelets. All antibodies mentioned above were purchased from Becton Dickinson.

Ten micro litter each of the respective antibodies were mixed to make a 30 µL mixture of an antibody-cocktail, to which 2.5 µL of a whole blood sample containing citric acid was added. The mixture was incubated at room temperature in the dark for 15 minutes. Cells then were fixed by adding paraformaldehyde to a final 1% and kept in a cool and dark place for 2 hours.

Using the FACS Calibur Flow Cytometer (Becton Dickinson), flow cytometric analysis was performed with argon laser (output: 15 mW; excitation wavelength: 488 nm) by fixing and capturing 10,000 platelets positive for CD61 using forward light scatter.

As negative controls for CD62P and PAC-1, 0.63 µL of PE-labeled mouse IgG (Becton Dickinson) and 10 µL of 5 mg/mL tetrapeptide, arginine-glycine-aspartic acid-serine (RGDS) (Sigma Corp.) were added together with PAC-1 to block the fibrinogen receptors expressed on GPIIb/IIIa molecules.

The positive rates of PAC-1 and CD62P were presented as the percentage of platelets emitting fluorescence stronger than that emitted by the negative controls. PMP were identified by gating on CD61-positive events,

which reflect glycoprotein IIIa on the platelet membranes, and differentiated from normal-sized platelets by forward light scatter to that of fluorescence-labelled reference beads (Becton Dickinson) of 2.0 µm in diameter. Ten thousand CD61-positive platelet events were analysed, and PMP were reported as a percentage of the total platelet count. For the analyses of the data obtained, the software of BD Application (Becton Dickinson) was used [13, 17-19]. Since the true value of blood PMP could not be determined by the flow cytometric method employed in this study, a tentative peripheral blood PMP concentration was calculated for each sample using the following equation:

$$\begin{aligned} & \text{PMP concentration (count/}\mu\text{L)} \\ & = \text{PMP (\%)} \times \text{Platelet count (}\times 10^4/\mu\text{L)} \end{aligned}$$

### Statistical Analysis

Data analysis was performed using the SPSS for Windows software package (SPSS Software GmbH, Munich, Germany). The mean values and correlation coefficients were analyzed by the Mann-Whitney test. Significant difference was set at  $P < 5\%$ .

## RESULTS

### Blood Biochemical Findings

The patients with alcoholic liver diseases showed the following biochemical findings. With regard to AST and ALT, an increase in AST was noted on admission. In particular, the increase in AST was more notable in the AI-LC group than that in ALT. However, both AST and ALT were markedly decreased 10 days after the discontinuation of alcohol intake, with no difference from the on-admission values. These parameters returned to almost normal levels in the AI-FL group.

An increase in  $\gamma$ -GTP was noted on admission in the alcoholic liver disease groups, with a most marked increase seen in the AI-FL group.  $\gamma$ -GTP level was greatly improved 10 days after the discontinuation of alcohol intake in both the AI-FL and AI-LC groups. However, the values were significantly higher in the two groups than that in the C-LC group ( $P < 0.001$ ).

A marked decrease in albumin was noted also on admission in the AI-FL group as well as in the AI-LC group, which was not improved 10 days after the discontinuation of alcohol intake.

A marked increase in bilirubin was noted

**Table 1** Laboratory data of patients with AI-FL, AI-LC or C-LC.

	Alcoholic FL		Alcoholic LC		Liver cirrhosis C
	Day1 n=7	Day10	Day1	Day10	n=9
Platelet Counts ( $\times 10^4/\mu\text{L}$ )	15.5 $\pm$ 7.8*	29.0 $\pm$ 13.2	12.0 $\pm$ 4.9***	18.4 $\pm$ 10.2	10.9 $\pm$ 7.2***
Alb (g/dL)	3.3 $\pm$ 0.6	3.4 $\pm$ 0.4	3.2 $\pm$ 0.6	3.4 $\pm$ 0.4	3.8 $\pm$ 0.3
T-Bil (mg/dL)	1.7 $\pm$ 0.8	0.9 $\pm$ 0.5	6.5 $\pm$ 4.9	4.5 $\pm$ 5.5	0.8 $\pm$ 0.2
AST (IU/L)	264 $\pm$ 228	60 $\pm$ 36	568 $\pm$ 1560	85 $\pm$ 43	58 $\pm$ 25
ALT (IU/L)	99 $\pm$ 59	47 $\pm$ 14	233 $\pm$ 564	75 $\pm$ 70	47 $\pm$ 23
$\gamma$ -GTP (IU/L)	988 $\pm$ 1090	534 $\pm$ 598	585 $\pm$ 357	310 $\pm$ 218	41 $\pm$ 18

Values are expressed as mean  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  vs. healthy control subjects. Alb, albumin; T-Bil, total bilirubin.

**Table 2** Summary of Platelet Count and Platelet Activation.

	Control	Alcoholic FL		Alcoholic LC		Liver cirrhosis C
	n=17	Day1 n=7	Day10	Day1	Day10	n=9
Age	50.7 $\pm$ 18.9	51.1 $\pm$ 11.0		56.08 $\pm$ 10.6		69.33 $\pm$ 8.6
Platelet Counts ( $\times 10^4/\mu\text{L}$ )	21.9 $\pm$ 4.1	15.5 $\pm$ 7.8*	29.0 $\pm$ 13.2	12.0 $\pm$ 4.9***	18.4 $\pm$ 10.2	10.9 $\pm$ 7.2***
CD62P (%)	0.85 $\pm$ 0.73	9.24 $\pm$ 7.21***	9.34 $\pm$ 4.89	5.94 $\pm$ 4.30***	5.02 $\pm$ 3.79	5.32 $\pm$ 4.24***
PAC-1 (%)	23.21 $\pm$ 16.85	38.28 $\pm$ 21.86	45.98 $\pm$ 16.27	28.24 $\pm$ 9.61	25.86 $\pm$ 13.35	42.49 $\pm$ 15.56*
PMP (%)	1.72 $\pm$ 1.31	3.73 $\pm$ 2.20***	1.67 $\pm$ 0.99	11.15 $\pm$ 4.82***	5.89 $\pm$ 4.44	8.39 $\pm$ 5.79***
Conc PMP ( $\mu\text{L}$ )	3770 $\pm$ 736	4980 $\pm$ 821	3900 $\pm$ 517	6740 $\pm$ 875***	7350 $\pm$ 688**	6800 $\pm$ 1690

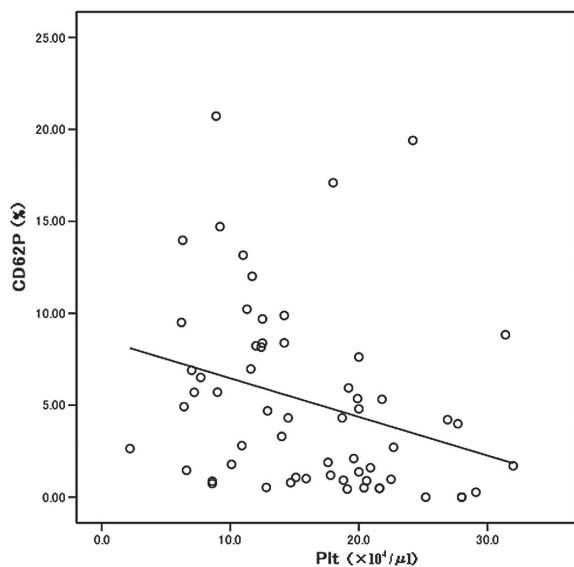
Values are expressed mean  $\pm$  SD. \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$  vs. healthy controls.

in the AI-LC group on admission. Ten days after the discontinuation of alcohol intake, bilirubin returned to almost a normal level in the AI-FL group, while that in the AI-LC group decreased slightly, yet, remained at a high level (Table 1).

No correlations were observed between the respective blood biochemical data such as albumin, bilirubin, AST, ALT or  $\gamma$ -GTP and the platelet count, CD62P and PAC-1 positivities or PMP, which were measured around the same time as the blood biochemistry.

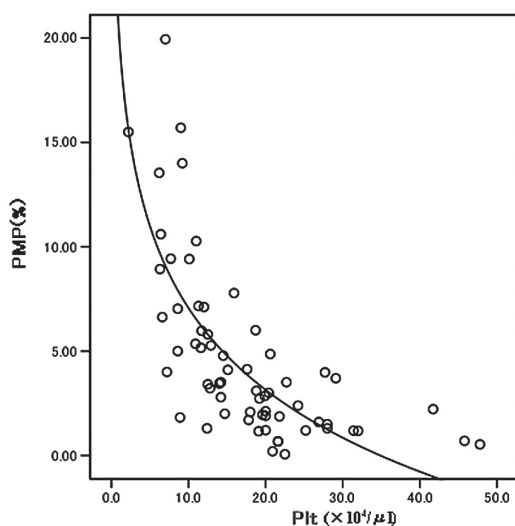
### Platelet Counts

The platelet count was significantly decreased in the AI-FL and AI-LC groups (15.5 $\pm$ 7.8 and 12.0 $\pm$ 4.9 $\times 10^4/\mu\text{L}$  vs. 21.9 $\pm$ 4.1 $\times 10^4/\mu\text{L}$  in the healthy subjects;  $P < 0.05$  and  $P < 0.001$ ). Ten days after discontinuation of alcohol intake, the platelet counts were significantly elevated in both the AI-FL and AI-LC groups compared to those measured on admission at 29.0 $\pm$ 13.2 $\times 10^4/\mu\text{L}$  ( $P < 0.05$ ) and 18.4 $\pm$ 10.2 $\times 10^4/\mu\text{L}$  ( $P < 0.05$ ), respectively. In the C-LC group, a non-alcoholic



**Fig. 1** Correlation between Platelet Count and CD62P Positivity.

An inverse correlation was observed between platelet count (Plt) and CD62P positivity in patients with alcoholic liver diseases ( $y = -0.23x + 18.0$ ;  $r = -0.47$ ;  $n = 66$ ;  $P < 0.01$ ).



**Fig. 2** Correlation between Platelet Count and PMP in Patients with Alcoholic Liver Diseases.

An inverse correlation was observed between platelet count (Plt) and PMP in patients with alcoholic liver diseases ( $r = -0.69$ ;  $n = 66$ ;  $P < 0.001$ ).

liver disease control, the platelet count was significantly decreased to  $10.9 \pm 7.1 \times 10^4/\mu\text{L}$  ( $P < 0.001$ ) compared to that in the healthy controls. No improvements were noted during the 10 days of the hospitalization period (Table 2).

### Correlation between Platelet Activation and Platelet Counts

In patients with alcoholic liver diseases, an inverse correlation was observed between platelet counts and CD62P positivity rate ( $r = -0.47$ ;  $P < 0.01$ ) (Fig. 1). The platelet counts

were also inversely correlated with the PAC-1 positivity. An inverse correlation was also observed between platelet counts and PMP measured by flow cytometry and expressed as percentages ( $r = -0.69$ ;  $P < 0.001$ ) (Fig. 2). Thus, %PMP was apparently increased with the decrease in platelet counts (Fig. 2).

### Platelet Activation

The positivity of CD62P, a marker of platelet activation, was  $0.85 \pm 0.73\%$  in the healthy subjects, while the values in the AL-FL, AL-LC and C-LC groups were  $9.24 \pm$

7.21% ( $P < 0.001$ ),  $5.94 \pm 4.30\%$  ( $P < 0.001$ ) and  $5.32 \pm 4.24\%$  ( $P < 0.001$ ), respectively, demonstrating significantly increased platelet activation in patients with either alcoholic or non-alcoholic liver diseases, with a most marked increase noted in the Al-FL group. Furthermore, the CD62P positivity tended to increase slightly in the Al-FL group after discontinuation of alcohol intake, while it slightly decreased in the Al-LC group ( $5.89 \pm 4.44\%$ )(Table 2).

The PAC-1 positivity tended to be increased in both the Al-FL and Al-LC groups compared to that in the healthy group. In the Al-FL group, there was a tendency to increase 10 days after discontinuation of alcohol intake. Furthermore, the PAC-1 positivity was significantly increased in the C-LC group compared to that in the healthy subjects ( $42.49 \pm 15.56\%$ ;  $P < 0.01$ ). On admission, the PAC-1 positivity in the Al-LC group was lower than that in the C-LC group, and this relationship remained the same 10 days after discontinuation of alcohol intake (Table 2).

PMP in the Al-FL, Al-LC and C-LC groups were  $3.73 \pm 2.20\%$ ,  $11.02 \pm 4.90\%$  and  $8.39 \pm 5.97\%$ , respectively, all significantly higher than that in the healthy group ( $P < 0.001$ ). In the Al-LC group, PMP significantly decreased to  $5.89 \pm 4.44\%$  ( $P < 0.01$ ) 10 days after discontinuation of alcohol intake, compared to that measured on admission (Table 2). Both on admission and 10 days after discontinuation of alcohol intake, the PMP concentration in the peripheral blood was found to be significantly increased in the Al-LC group compared to that of the healthy subjects (Table 2). The PMP concentration at 10 days after discontinuation of alcohol intake tended to be slightly increased compared to that at on admission. On the other hand, the PMP concentration appeared to be slightly decreased at 10 days after discontinuation of alcohol intake in the Al-FL group.

## DISCUSSION

The results of the present study revealed that CD62P and PAC-1 positivities and PMP increased in the Al-FL, Al-LC and C-LC groups compared to those in the healthy subjects, indicating an elevation in platelet activation. Ten days after discontinuation of alcohol intake, the platelet count was significantly increased in both the Al-FL and Al-

LC groups, compared to that measured on admission. In particular, the increase found in the Al-FL group was marked. An inverse correlation was observed between platelet activation and the platelet count, indicating that the platelet count decreased with an increase in platelet activation. This finding strongly suggests that the consumption of platelets *via* platelet activation is a cause of the decrease in platelets.

CD62P, a membrane glycoprotein, is present in the membrane of  $\alpha$ -granules and Weibel-Palade body of endothelial cells. When these cells are stimulated, P-selectin migrates to the cell surface in association with the granular release, thereby P-selectin becomes recognizable by the CD62P MoAb. In the present study, the CD62P positivity increased to the same extent in the Al-FL, Al-LC and C-LC groups compared to that in the healthy subjects. It has been reported that granular release is suppressed by ethanol [20]. In the present study, we found no clear decrease in granular release from the on-admission values 10 days after discontinuation of alcohol intake, a finding inconsistent with the previous reports [20].

PAC-1 is a monoclonal antibody against GPIIb/IIIa. It shows almost no binding affinity to the resting form of GPIIb/IIIa, whereas it recognizes the binding site of fibrinogen on activated GPIIb/IIIa molecules, in which a structural modification has occurred. The PAC-1 positivity was increased in the Al-FL group compared to that in the healthy subjects and, characteristically, it remained significantly increased even after discontinuation of alcohol intake. On the other hand, in patients with liver cirrhosis, a marked increase in the PAC-1 positivity was noted in the C-LC group, whereas the change seen in the Al-LC group was slight compared to that in the Al-FL group.  $\text{TXA}_2$  activates GPIIb/IIIa and is increased in association with the progression of the disease state of C-LC [21].  $\text{TXA}_2$  secretion is reported to be suppressed by alcohol [22]. In the present study, the PAC-1 positivity was found to be lower in the Al-LC group than that in the C-LC group, which might be accounted for by the difference in  $\text{TXA}_2$  secretion. The finding that the Al-FL group showed a higher PAC-1 positivity suggests a difference in the mechanism by the disease stage.

PMP are released upon platelet activation

and include membrane fragments that are generated by mechanical cell destruction. Their particle size is 0.02 to 0.1  $\mu\text{m}$  in diameter, and they are produced by stimulation, such as the high shear stress present in arteries or arteriola. When platelets are activated, phosphatidylserine migrates from the cytoplasmic layer to the extracellular layer of the membrane. Since blood coagulation factors strongly bind to phosphatidylserine, blood coagulation is enhanced under the condition of concentrated coagulation factors on the platelet surface. In particular, PMP have many binding sites for factor V, therefore, is the main body of the platelet procoagulant activity [21].

Thus far, PMP are reported to be increased under some conditions, such as idiopathic thrombocytopenic purpura [24], thrombotic thrombocytopenic purpura [25], diabetes mellitus [14], acute myocardial infarction, hemolytic uremic syndrome [24], transient ischemic attack and cerebral infarction [15]. In the present study, a correlation was observed between the increase in PMP and the decrease in the platelet count in the AI-FL and AI-LC groups. Also, PMP was found to be decreased during the period of 10 days after discontinuation of alcohol intake, when platelet counts increased. This finding may be a reflection of the sequential changes of pathologic platelet activation that occur in the presence or absence of ethanol in alcoholic liver diseases. In order to elucidate the above phenomenon, studies on true plasma or peripheral blood concentrations of PMP (count/ $\mu\text{L}$ ) in liver diseases may be necessary. However, it was technically impossible by the present flow cytometric method using whole blood. Therefore, a tentative concentration of PMP was calculated as described in the Method. PMP concentrations were found to be increased more dramatically in cirrhosis than in AI-FL. Interestingly, it was high in the C-LC group as well as in the AI-LC group, despite that the platelet count markedly decreased in the former. This finding may indicate that effects of pathologic conditions relating to cirrhotic changes, other than the direct effects of alcohol, may be involved in the PMP elevation in the blood.

It has been reported that blood alcohol induces metabolic disorders in platelet membrane lipids [8]. The correlation between the metabolic disorders and PMP abnormality

require further study. PMP are in an inverse correlation with the platelet count, indicating that the platelet count decreased, while platelets were in an activated state. Therefore, the formation of PMP might be suppressed, while platelet count increased 10 days after discontinuation of alcohol intake, thereby the newly generated platelets were not activated in the absence of any effect of alcohol. To elucidate the mechanism of platelet abnormalities, which play an important role in the pathology of alcoholic liver diseases, further studies are necessary on the effects of alcohol on the metabolism of arachidonic acid, including  $\text{TXA}_2$ , as well as the formation of PMP in the platelet membrane.

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