The Measurement of Platelet Aggregation and ATP-Release in Mice with Liver Damage Induced by Carbon Tetrachloride (CCl4) Using a Whole Blood Aggregometer

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Abstract—Time course change in platelet function on liver damaged mice was studied by a whole blood aggregometer. The liver damaged animals were produced by single and multiple injections (p.o.) of 20% CCl4 in olive oil to ddY mice (6 weeks). Platelet aggregation and ATP-release were induced with collagen (final conc. of 2 μg/ml), ADP (final conc. of 20 μM) and arachidonic acid (AA; final conc. of 100 μM). After a single injection of CCl4, platelet counts increased at 5 and 12 hr, and then they decreased from 24 to 120 hr. Multiple injections of CCl4 resulted in a significant increase in platelet counts. A single injection of CCl4 suppressed aggregation by collagen at 24 and 48 hr and diminished the rate of ATP-release from 12 to 48 hr. AA-induced platelet aggregation was depressed at 48 hr, and ATP-release was also diminished from 24 to 72 hr after a single injection. ADP-induced platelet aggregation was decreased 24 and 48 hr after a single injection and at 48 hr after multiple injections, while, the rate of ATP-release by ADP was significantly increased after single and multiple injections. These changes in platelet functions might be consistent with our original report on the alterations in coagulative and fibrinolytic activities with CCl4-induced liver injury.

An experimental model of liver disease is necessary for estimating the effect of therapeutically agents for liver disease. We have established a liver disease model in experimental animals using CCl4 and ethionine (1, 2). In our previous papers, we reported that CCl4 administration in mice induced severe liver damage with the depression of the reticuloendothelial system (RES) (1) and depression of the blood coagulative and fibrinolytic activities (3). It has been recognized that the activations of many coagulative factors and platelets were responsible for the initiation of the coagulative process. In addition, many papers indicated that platelet counts and the functions of these cells were changed in patients with liver diseases (4–6). Mild thrombocytopenia, even when platelet production is still active, was observed in many patients with fulminant hepatic failure and severe liver cirrhosis (7). In liver disease, multiple factors have a direct and/or indirect effect on hemostasis. Therefore, it is significant to examine platelet counts and its function in CCl4-induced liver injury in mice. Recently, the electrical impedance method for measuring platelet aggregation has been introduced. This method can monitor platelet aggregation in whole blood and measures the increase in impedance across electrodes placed in the blood samples as platelets accumulate on them (8). We used the impedance aggregometer, with which platelet function can be examined without damaging the cells, to study the platelet function in mice with CCl4-induced liver injury.

Materials and Methods

Animals and drug administration: Male ddY mice, 5 weeks of age at the start of the experiments, were fed with a stock diet (MF; Oriental Kobo Co., Ltd.) and tap water ad libitum under standard laboratory conditions. In the acute and chronic liver injury experiments,
the mice were divided into three groups: the saline control group, olive oil-treated group and CC14-treated group. CC14 and olive oil were purchased from Wako Pure Chemical Industries, Ltd. Each animal received 0.2 ml of 20% CC14 in olive oil or 0.9% NaCl solution. To induce chronic liver damage, 0.04 ml of CC14 per mouse was given to the animals 3 times a week (every other day) for 5 weeks. Platelet functions and blood examinations were determined at 5, 12, 24, 48, 72 and 120 hr after a single injection or at 48 hr after multiple injections of CC14.

Blood collection and preparation: Blood specimens were taken from the inferior vena cava with a plastic syringe and silicon-coated needle after pentobarbital anaesthesia (40 mg/kg nembutal, i.p.). Whole blood from mice was mixed with 3.2% sodium citrate solution at the ratio of nine to one.

Platelet aggregation and ATP-release in the impedance aggregometer: The determination of platelet aggregation and ATP-release was performed in siliconized glass cuvettes at 37°C using a whole blood aggregometer (model 540, Chrono-Log Corp. Havertown, PA, U.S.A.). A teflon-coated stirring bar was added and the electrodes placed in the cuvette. Aggregation and ATP-release activities in a sample were registered simultaneously and performed during 60–120 min after blood sampling. We used collagen, ADP and arachidonic acid (AA) as the platelet stimuli and used luciferase-luciferin reagent (LL) for the determination of ATP-release. Collagen, ADP and LL were obtained from Baxter Travenol. AA and ATP were purchased from Sigma.

Blood examination: Citrated whole blood was analyzed for platelet, white blood cell (WBC) and red blood cell (RBC) counts and the values of the hematocrit (Ht) and hemoglobin (Hb) using automatic equipment (Coulter Counter, S-plus size distributions, Coulter Electronics, Inc.).

Statistical significance was evaluated by Student's t-test.

Results

1. Typical patterns of platelet aggregation and ATP-release: Figure 1 shows the typical patterns of platelet aggregation and ATP-release after injections of three kinds of stimuli. Collagen at the concentrations of 1, 2 and 3 μg/ml dose-dependently induced platelet aggregation and at the same time, released ATP. ADP at 10, 20 and 30 μM caused the reversible platelet aggregation, but these amounts of ADP did not release ATP. AA at 50, 100 and 250 μM induced similar
degrees of platelet aggregation with the biphasic release of ATP. Among the three concentrations of AA, 100 μM AA more strongly induced platelet aggregation.

2. Collagen induced platelet aggregation and ATP-release: Figure 2 shows the changes of platelet aggregation and ATP-release activities by 2 μg/ml of collagen after single and multiple injections of CCl₄ in mice. Aggregation activity was determined at 7 min and the maximum value of ATP-release activity was measured after collagen addition. Collagen-induced aggregation was depressed significantly at 24 and 48 hr after a single injection of CCl₄ in whole blood. ATP-release activity was depressed at 12 and 48 hr after a single injection of CCl₄. The activity of aggregation and ATP-release after multiple injections of saline was a little higher than those after a single injection of saline. Therefore, no apparent changes were observed after multiple injections of CCl₄.

3. ADP induced platelet aggregation and ATP-release: Figure 3 shows the changes of platelet aggregation and ATP-release activities by 20 μM ADP after single and multiple injections of CCl₄. Aggregation and ATP-release activities were determined at each maximum value after ADP addition. ADP-induced aggregation was also depressed at 24 and 48 hr and increased at 120 hr after a single injection of CCl₄, while it was apparently depressed after multiple injections of CCl₄. In the controls that were injected with saline or olive oil, ADP-induced ATP-release was very low compared to the collagen induced one. ATP-release by ADP was apparently increased at 12, 24 and 48 hr after a single injection of CCl₄ and mildly increased at 48 hr after multiple injections.

4. AA induced platelet aggregation and ATP-release: Figure 4 showed the changes of platelet aggregation and ATP-release by 100 μM AA after single and multiple injections of CCl₄. Aggregation activity was determined at 7 min and ATP-release activity was measured.

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Fig. 2. Platelet aggregation and ATP-release induced by collagen (2 μg/ml). Control mice were given 0.2 ml of 0.9% NaCl solution (p.o.); olive oil-treated mice were injected with 0.2 ml of olive oil (p.o.); and CCl₄-treated mice were injected with 0.2 ml of 20% CCl₄ in olive oil (p.o.). The changes in platelet aggregation (lower panel) and ATP-release (upper panel) were shown with time course in μM and μM, respectively, after a single administration of CCl₄. "Multiple administration" means 15 injections (3 times a week) of each solution. Each value represents the mean±S.D. of 8 to 10 specimens. **:** significant difference from the saline-control value with P<0.01, P<0.05, respectively. ▲▲, ▲: significant difference from the olive oil-control value with P<0.01, P<0.05, respectively (Student's t-test).
at the maximum value after AA addition. AA-induced aggregation at 48 hr after a single injection of CCl₄ was depressed significantly. In contrast, the olive oil-treated group showed an increased activity of aggregation at 5 and 48 hr after a single injection. After a single injection of CCl₄, ATP-release was decreased significantly at 24 and 48 hr compared to the

Fig. 3. Platelet aggregation and ATP-release induced by ADP (20 μM). The changes in platelet aggregation (lower panel) and ATP-release (upper panel) are shown in Ω and μM, respectively. Each value represents the mean±S.D. of 8 to 11 specimens. **, *: significant difference from the saline-control value with P<0.01, P<0.05, respectively. LA, A: significant difference from the olive oil-control value with P<0.01, P<0.05, respectively (Student’s t-test). For details, see Fig. 2.

Fig. 4. Platelet aggregation and ATP-release induced by arachidonic acid (100 μM). The changes in platelet aggregation (lower panel) and ATP-release (upper panel) are shown in Ω and μM, respectively. Each value represents the mean±S.D. of 7 to 10 specimens. **, *: significant difference from the saline-control value with P<0.01, P<0.05, respectively. ▲▲, ▲: significant difference from the olive oil-control value with P<0.01, P<0.05, respectively (Student’s t-test). For details, see Fig. 2.
Table 1. Time course of the counts of various blood cells and hemoglobin and hematocrit values after a single injection of CCl₄

|        | Saline (×10⁹/µl) | 5 hr     | 12 hr    | 24 hr    | 48 hr    | 72 hr    | 120 hr   |
|--------|------------------|----------|----------|----------|----------|----------|----------|
| PLA    | 9.82± 1.04       | 10.86± 0.78 | 7.87± 2.24* | 10.93± 0.97 | 8.45± 1.55 | 8.94± 1.05 |
| CCl₄   |                 | 11.20± 1.25* | 11.67± 0.85* | 7.34± 1.48** | 6.24± 1.95*** | 7.17± 1.29** | 7.90± 0.56** |
| WBC    | 4.03± 0.69       | 4.64± 2.17 | 1.84± 0.36** | 4.79± 1.61* | 4.22± 0.54 | 4.35± 0.26 |
| CCl₄   |                 | 3.87± 1.10 | 3.96± 0.59 | 2.15± 0.99** | 3.82± 0.78 | 3.39± 0.40# | 4.18± 0.68 |
| RBC    | 8.88± 0.48       | 8.57± 0.48 | 9.00± 0.41 | 8.71± 0.27 | 8.66± 0.32 | 8.70± 0.23 | 8.98± 0.60 |
| CCl₄   | 8.71± 0.19       | 8.98± 0.31 | 9.24± 0.36 | 9.34± 0.37## | 8.94± 0.11 | 9.31± 0.28## |
| Hb     | 15.0± 0.4        | 14.4± 0.7 | 15.8± 0.5 | 14.5± 0.6# | 14.2± 0.5# | 15.3± 0.2 | 15.7± 0.5 # |
| CCl₄   | 14.4± 0.7        | 15.6± 0.5 | 15.7± 0.6# | 14.9± 0.5# | 15.6± 0.2 | 15.6± 0.5 |
| Ht (%) | 41.3± 1.3        | 40.0± 1.3 | 42.7± 1.7 | 40.0± 1.9## | 40.0± 1.8## | 41.1± 1.8## | 42.0± 0.7 |

Each value represents the mean±S.D. of 7 specimens. PLA=platelet, WBC=white blood cell, RBC=red blood cell, Hb=hemoglobin, Ht=hematocrit. **, *: significant difference from the saline-control value with P<0.01, P<0.05, respectively. ##, #: significant difference from the olive oil-control value with P<0.01, P<0.05, respectively (Student's t-test). For details, see Fig. 2.
saline or olive oil-control, and also at 72 hr as compared to the olive oil-one. On the other hand, multiple injections of olive oil and CCl₄ produced no changes in the activity of AA induced aggregation and ATP-release.

5. Blood examinations: Table 1 shows the time course of the change in the counts of blood cells after a single injection of CCl₄. Platelet counts increased at 5 and 12 hr and then decreased from 24 to 120 hr after a single injection of CCl₄. WBC counts decreased at 24 hr after olive oil and CCl₄ injections, and it increased at 48 hr after olive oil injection. At 72 hr, WBC counts of CCl₄-injected mice decreased compared to those of olive oil-injected mice. RBC counts showed a moderate increase at 24, 48 and 120 hr after a single injection of CCl₄. The Hb value of CCl₄-injected mice increased moderately compared to the saline or olive oil-control, while the Ht value of the CCl₄-injected mice increased significantly compared to the saline or olive oil-control. Table 2 shows the effects of multiple injections of CCl₄ and olive oil. Platelet counts significantly increased by multiple injections of CCl₄ as compared to the counts in the saline and olive oil-controls, but RBC counts showed no changes. The Ht value of CCl₄-injected mice increased moderately compared to the olive oil-control, but the Hb value of mice injected with olive oil or CCl₄ decreased significantly compared to the saline-control.

Discussion

The platelet functions in experimental animal models of liver damage were studied by using the electrical impedance method in whole blood. Generally, platelet aggregation has been studied by the turbidometric method described by Born (9) using platelet-rich plasma (PRP). One of the limitations of photometric aggregometry is the centrifugation required to separate platelets from other blood cells, which may modify platelet behavior. Conveniently, whole blood aggregometry can be used to screen the functions of platelets and detect platelet dysfunctions. In addition, platelet aggregation testing in whole blood proved to be easy and time sparing (10). We have used this whole blood technique to examine platelet function in CCl₄-induced liver injury.

Collagen (1, 2, 3 μg/ml) induced strong dose-related responses in control whole blood. The platelet aggregation occurs after a lag phase accompanied with ATP-release. At 24 hr after a single injection of CCl₄, collagen induced aggregation and ATP-release were depressed with the decrease in platelet counts. With the whole blood technique, we can observe that the aggregation started slightly earlier than the start of ATP-release. On the other hand, ADP (10, 20, 30 μM) only induced reversible aggregation without ATP-release from the mouse platelets. The cause of this is platelet deaggregation and the platelets become refractory towards to ADP. The development of platelet refractoriness to ADP may be related to the function of the platelet contractile proteins, and this may be at least partly responsible for platelet deaggregation (11). After a single injection of CCl₄, ADP-induced ATP-release was increased from 12 to 48 hr, although platelet aggregation was depressed at 24 and 48 hr and increased at 120 hr. These results indicate
that platelet aggregation and ATP-release do not always occur in parallel. These two phenomena are different processes in activated platelets (12). Intracellular Ca\(^{2+}\) plays an essential role in exocytosis in platelets (12). An increase in free cytoplasmic Ca\(^{2+}\) might be induced by ADP in CCl\(_4\)-induced liver injury, and it may serve to activate ATP-release. Further studies will be needed on the mechanism of the decrease in platelet activities in CCl\(_4\)-induced liver injury. On the other hand, fibrinogen is required for ADP-induced aggregation of washed human platelets and enhances the ADP-induced aggregation of rabbit platelets. Mustard et al. (13) showed that fibrinogen may be involved in transient linkages between platelets in ADP-induced aggregation. Our previous report showed that fibrinogen content decreased significantly after a single injection of CCl\(_4\) (3). We think that the decrease in fibrinogen content after CCl\(_4\) injection is one of the reasons for the depression of platelet aggregation. The increase in ATP-release after a single injection of CCl\(_4\) means that many factors from the platelets are released. Therefore, our results with ADP in this liver injury suggested that the some factors from platelets interacted with the endothelial cells of blood vessels. These cells can produce factors that regulate smooth muscle cell proliferation (14) and then may be associated with the development of arteriosclerotic lesions (15).

AA showed similar degrees of aggregation and ATP-release at three different concentrations (50, 100, 250 \(\mu\)M). As to the ATP-release by AA, we can find a sharp increase as soon as the stimulus was added, and this was followed by the second increase. The first increase in ATP-release was dependent on the added amounts of AA (data not shown). AA-induced aggregation was suppressed at 48 hr after a single injection of CCl\(_4\). AA is converted in the platelets to short-lived prostaglandin endoperoxides (PGG\(_2\) and PGH\(_2\)), thromboxane (TX)A\(_2\) and more stable prostaglandins (16, 17). Both the prostaglandin endoperoxides and TXA\(_2\) can induce platelets to undergo shape change, to stick to each other and to release their granule contents. Among these AA metabolites, TXA\(_2\) is a strong stimulus of platelet aggregation, but Hashimoto et al. have reported that the mechanisms of the actions of more than 30 \(\mu\)M AA on platelets is independent of TXA\(_2\). The mechanism of AA itself which induces platelet responses is not clear (18).

Blood examinations indicated that platelet counts increased at 5 and 12 hr and then decreased from 24 to 120 hr after a single injection of CCl\(_4\). On the other hand, multiple injections increased the increase of platelet counts compared to the saline or olive oil control, although the decrease of platelet counts was reported in patients with liver disease (4–6). The most plausible reason for the increase in platelet counts in CCl\(_4\)-induced liver injury may be the stimulation of megakaryocyte-platelet lineage (19). Moreover, the transference of platelets to peripheral blood vessels may happen, since it is necessary to inhibit the abnormal bleeding due to liver injury. In CCl\(_4\)-induced liver injury, we had already reported that the coagulative activities were strongly depressed at 24 and 48 hr (3). Therefore, platelets in the circulation must be consumed to regulate the hemostasis (20). After a single injection of CCl\(_4\), WBC counts decreased at 24 hr; on the other hand, RBC counts increased moderately from 24 to 120 hr. In this experiment, with the impedance method using whole blood, we can consider the interaction between platelets and other blood cells. The increase of RBC counts and the decrease of platelet counts occurred simultaneously. Jackson et al. (21) reported that the degree of thrombocytopenia depended upon the degree of polycythemia induced. Machi et al. (22) demonstrated a growth inhibition of platelet aggregates by addition of washed RBC to PRP with a high resolution ultrasound technique. In our experiments, one of the causes of the decrease in platelet aggregation may be due to the increase in RBC counts after a single injection of CCl\(_4\). It is not clear whether the decrease of WBC counts involve the suppression of platelet activity at 24 hr after a single injection of CCl\(_4\).

Platelet aggregation velocity was directly proportional to platelet volume or the number of large platelets, but correlated poorly with platelet mean or mode (23). In this experi-
ment, the blood was enriched with large platelets at 24 hr after a single injection of olive oil or CCl₄, and this tendency persisted to 120 hr in CCl₄-treated mice. On the other hand, small platelets and large platelets were mixed after multiple injections of CCl₄ (data not shown). The existence of large platelets might be consistent with the mild decrease of platelet functions in this chronic liver injury.

Platelet aggregation had been shown to contribute to the clearance of colloidal carbon and RE-depressing substance depressed platelet aggregation induced by ADP or collagen (24). Van Aken et al. had already reported that the clearance of carbon particles in rabbits was accompanied by aggregation of blood platelets. The disappearance of carbon particles is accelerated by platelet transfusion. These observations suggest that platelet aggregation is essential for transport of carbon to the RES (25). It is possible that the decrease in the carbon clearance rate in chronic liver injury by CCl₄ (1) was due to the depression of platelet aggregation by ADP.

Our results with mice demonstrated that the platelet counts and functions except for ADP-induced ATP-release significantly decreased at 24 and 48 hr after a single injection of CCl₄. We had already reported the decrease in the activities of the heparplastin test and antithrombin III, which were undoubtedly considered to reflect the damage of the liver cells (3). In addition, our previous experiment showed the decrease in the main value of the thromboelastogram, a value which reflects the changes of platelet counts and function, from 24 to 72 hr after a single injection of CCl₄ (3). These results indicate that there is an increased risk of bleeding in this acute liver injury model produced by a single injection of CCl₄. After multiple injections of CCl₄, an increase in platelet counts was found and no apparent dysfunction of platelets could be observed.

In summarizing our experimental conditions using mice, a single injection of CCl₄ induced acute liver injury with a strong decrease in the coagulative and fibrinolytic activities (1, 3). On the other hand, multiple injections of CCl₄ induced chronic liver injury with severe histological changes and the significant suppression of RES phagocytosis (1), but the decrease in coagulative activity was mild (3). The decrease in coagulative factors was attributable to the decrease in the synthesizing activity of the parenchymal cells because of liver damage by CCl₄. However, a high dosage of CCl₄ after a single treatment does not show high toxicity for the cells because of the decrease in CCl₄ formation in rats (26). Therefore, the hyperproduction of coagulative factors might be induced and a complex counterbalance between the coagulative and fibrinolytic systems was shown in chronic liver injury by CCl₄ (3). The results of the present investigation on the platelet counts, volume and functions might be consistent with our original report (3) on the alterations in coagulative and fibrinolytic activities in CCl₄-induced liver injury in mice.

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