Studies on Alterations in Blood Coagulative and Fibrinolytic Activities after Single and Multiple Administrations of Carbon Tetrachloride in Mice

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Abstract—The time course of alterations in coagulative and fibrinolytic activities was studied in CCl₄-induced liver disease in mice. Liver disease was induced by administration of 20% CCl₄ in olive oil (p.o.). After single administration of CCl₄, significant prolongation of r and k values and decrease in the ma value of thromboelastogram and apparent prolongation of PT and PTT were seen at 24 hr. Fibrinogen content decreased from 12 to 72 hr after single administration, while a mild but significant decrease in fibrinogen was observed after multiple administrations. The activity of factor XIII increased from 5 to 12 hr and then decreased from 24 to 168 hr after single administration. The activity of the hepataplastin test and Antithrombin III decreased apparently after single and multiple administrations. The plasminogen content and the activity of α₂-plasmin inhibitor decreased severely after single and multiple administrations. These results indicate that the coagulative and fibrinolytic activities were decreased to the most lowest value at 24 or 48 hr after single administration of CCl₄, and the severe suppression of fibrinolysis and the mild decrease in coagulative activity were observed after multiple administrations of CCl₄. The reason for the different effects between single and multiple administrations on coagulative and fibrinolytic systems was discussed.

Oral administration of carbon tetrachloride (CCl₄) to animals leads to parenchymal cell injuries including centrilobular necrosis of the liver (1). In our previous studies (2), we reported that CCl₄ administration in mice induced severe liver damage with abnormal values in blood components and with the depression of reticuloendothelial system (RES). Many factors of the coagulative and fibrinolytic systems are synthesized by the liver. In fact, Shaw has confirmed the functional capacity of the isolated liver for synthesizing proteins and vitamin K-dependent coagulation factors (3); and patients with liver disease often exhibit multiple coagulation defects (4, 5). Therefore, CCl₄ treatment is considered to induce the decrease in the activity of many factors of the coagulative and fibrinolytic systems, because the metabolic activation of CCl₄ is associated with the formation in the endoplasmic reticulum of free radicals which interact with cell constituents (6). However, experimental studies on the changes in the coagulative and fibrinolytic activities have not yet been reported, especially using mice. The clotting time is dependent on numerous factors including temperature, water quality, pH, ionic strength, test system and anticoagulant used and the technique for specimen collection. These factors seemed to make it more difficult to investigate the activity of coagulation and fibrinolysis in mice. If the basic data on the activity of coagulation and fibrinolysis are obtained using mice with liver injury, it will be to our benefit when we examine the effect of various kinds of therapeutic agents for liver disease. Because mice are so small, easy to handle and very cheap, it is convenient for us to use them for
this experimental model. Therefore, it is important to know the time course of coagulative and fibrinolytic activities in acute and chronic liver injury induced by \( \text{CCl}_4 \) administrations. The purpose of this paper is to examine the alterations in blood coagulative and fibrinolytic activities in mice after single and multiple administrations of \( \text{CCl}_4 \).

**Materials and Methods**

**Animal and drug administration**

Male ddY mice, 5 weeks of age at the start of experiment, were fed with a stock diet (MF, Oriental Kobo Co. Ltd.) and tap water ad libitum under standard laboratory conditions. The mice were divided into three groups: saline control group, olive oil treated group and \( \text{CCl}_4 \) treated group, in acute and chronic liver injury experiments, respectively. \( \text{CCl}_4 \) and olive oil were purchased from Wako pure chemical industries, Ltd. Each animal received 0.2 ml of 20% \( \text{CCl}_4 \) in olive oil (\( \text{CCl}_4 \): 0.04 ml) by stomach tubes to induce acute liver damage. The control group received 0.2 ml of olive oil or 0.9% NaCl solution per mouse. To induce chronic liver damage, 0.04 ml \( \text{CCl}_4 \) per mouse was given to animals 3 times a week (every other day) for 5 weeks, that is, the total number of 0.04 ml \( \text{CCl}_4 \) injections were 15. We examined all items of the experiment at 5, 12, 24, 48, 72 and 120 hr after single administration and 48 hr after multiple administrations.

**Blood collection and preparation**

Blood specimens were taken from the vena cava inferior with a plastic syringe and silicon-coated needle after pentobarbital anaesthesia (nembutal, 40 mg/kg, i.p.).

Citrated plasma was prepared as follows: whole blood from mice was mixed with 3.2% sodium citrate solution at the ratio of nine to one, and then it was centrifuged at 3,000 r.p.m. for 15 min at 4°C. The supernatant was used for the specimen as platelet-poor plasma. We used whole blood to determine thromboelastogram (TEG), venous blood mixed by 3.2% of sodium citrate for the heparplastintest (HPT) and platelet-poor plasma for other factors of coagulative and fibrinolytic activities.

**Assay of coagulative activity**

1. **Thromboelastogram (TEG):** The total coagulative process was depicted by thromboelastography (Clot-tracer TE-30, Erma Co., Ltd.). From the thromboelastogram obtained using 0.35 ml of a whole blood sample, \( r, k \) and \( ma \) values were measured. \( R \) stands for the reaction time from the beginning of blood collection to the time the amplitude reached 1 mm. \( k \) stands for clot formation time from the \( r \) value to the time the amplitude reached 20 mm. The \( ma \) stands for the maximum amplitude (Fig. 1).

2. **Prothrombin time (PT) and partial thromboplastin time (PTT):** PT was measured using Simplastin (Warnar-Lambert Co. Ltd.) in a modified one-stage method. Simplastin is a general diagnostics, and it contains a tissue thromboplastin reagent (rabbit brain and lung). \( \text{CaCl}_2 \) and NaCl. PTT was measured using Platelin (Warnar-Lambert Co. Ltd.) by the method of Langdell, et al. (7). Platelin contains plasma thromboplastin reagent (rabbit brain phospholipid) and NaCl. Measurement of PT and PTT was conducted using clot digitim TE-20 (Erma Co., Ltd.).

3. **Fibrinogen level:** The fibrinogen level was measured by the method of Tomikawa (8). Citrated plasma was mixed with \( \text{CaCl}_2 \) and tranexamic acid (Sigma Chemical Co.), and the mixture was incubated at 37°C. After removal of non-clottable proteins from the diluted plasma clot by centrifugation, 1% monochloracetic acid was added to the plasma.
fibrin precipitate. After washing of the fibrin precipitate with H₂O, the protein content of the fibrin precipitate was determined by the method of Lowry et al. (9).

4. Factor XIII: The conventional method using the latron-FL kit, F. XIII. The activities of factor XIII were determined as the dansylcadaverine complex fluorescence by a fluorescence spectrofluorometer (Hitachi 650–10M Fluorescence spectrophotometer).

5. Antithrombin III (AT III): AT III activity was determined by the method using synthetic chromogenic substrates (Tosyl-Gly-Pro-Arg-pNA: chromolate AT III, latron Laboratories, Inc.).

6. Hepaplastintest (HPT): HPT was achieved by utilizing a specially prepared rabbit brain thromboplastin (Hepaplastintest: Eisai Co., Ltd.) with venous blood mixed with 3.2% sodium citrate (10).

Assay of fibrinolytic activity

1. Plasminogen (PLG): PLG analysis was conducted by the chromogenic method using S-2251 as a substrate (Kabi Diagnostica, Daich chemical pharmacy Co., Ltd.). We used urokinase (Uronase, Mochida pharmaceutical Co., Ltd.) as PLG activator because the PLG of rat and mouse was activated by urokinase but not by streptokinase (11).

2. α₂-Plasmin inhibitor (α₂-PI): Activity of α₂-PI was determined by a method using a synthetic chromogenic substrate, S-2251. It is the same substrate used for PLG determination (Kabi Diagnostica, Daich Chemical Pharmacy Co., Ltd.).

Statistical significance was evaluated using Student’s t-test.

Results

TEG: Figure 2 shows the typical TEG figures with time course after single injection of CCl₄. Manifest changes in figures were not seen at 5 and 12 hr, but abnormal patterns were observed with severe inhibition of coagulation or with abnormal enhancement of fibrinolysis at 24 hr. TEG figures 48, 72 and 120 hr after CCl₄ injection showed gradual recovering tendencies, and at 168 hr, they were almost the same as that of the control. On the other hand, multiple treatments of CCl₄ produced different TEG figures, making it impossible to pinpoint one typical figure of TEG as a result of multiple treatments of CCl₄. Figure 3 shows the r, k and ma values of TEG after single and multiple treatments of CCl₄. Here we see that the r value of the control was about 8 min and that it increased about 1.7 times that of the olive oil and/or control values at 24 hr after single administration of CCl₄. However, the increasing tendency of the r value continued up to 48 hr after CCl₄ treatment. The k value of the control was about 2.5 min and increased dramatically 24 hr after single treatment of CCl₄. At 168 hr after single injection of CCl₄, the k value recovered to the normal range. The ma value of the control was about 68 mm, and that of CCl₄ 24 hr after single administration decreased to half in comparison to that of control. It gradually recovered to the normal range and showed the same value compared to that before injection of CCl₄. On the other hand, no significant effects were observed after multiple treatments of CCl₄ because of the variability of r, k and ma values.

PT and PTT: Figure 4 shows the time course of PT and PTT following CCl₄ administration. After single treatment of olive oil, PT shortened at 5, 72 and 120 hr. At 5 hr after single administration of CCl₄, PT was short; however, at 12 and 24 hr, CCl₄ injection caused a significant elongation compared to saline and olive oil treated mice. On the other hand, PTT shortened significantly at 12 hr after olive oil treatment. It become longer for
the CCl₄ treated group compared to that of the olive oil treated group at 12 hr. At 24 hr, CCl₄ caused a prolongation of PTT, whereas olive oil caused a shortening. At 72 hr, olive oil and CCl₄ treatment caused significant shortening. After multiple treatments, olive oil caused the shortening of PT, while CCl₄ caused the prolongation of PT compared to olive oil. No change in PTT was observed.

**Fibrinogen:** Figure 5 shows the time course of fibrinogen content after single and multiple administrations of CCl₄. Single administration of CCl₄ caused a significant decrease in fibrinogen content from 12 to 72 hr, and this tendency was especially strong at 24, 48 and 72 hr. On the other hand, multiple administrations of CCl₄ induced a mild decrease.

**Factor XIII:** As shown in Fig. 6, factor XIII was activated 5 hr and 12 hr after single administration of CCl₄. However, single treatment of CCl₄ induced a marked decrease in the activity of factor XIII to about 20% that of the saline control at 24, 48 and 72 hr. and this depressed tendency continued up to 168 hr. At 5 and 120 hr after single treatment of olive oil, the activity of factor XIII was high compared to that of the saline control. After multiple administrations of olive oil, a significant increase in the activity of factor XIII was observed. Therefore, multiple administrations of CCl₄ was seen to decrease the activity of factor XIII compared to that of the olive oil control.

**AT III:** As shown in Fig. 7, the activity of AT III decreased from 12 to 72 hr after single administration of CCl₄. They were
Fig. 4. Effect of single and multiple administrations of CCl₄ on PT and PTT. PT (lower panel) and PTT (upper panel) are shown in sec by using Simplastin and Platelin (Warnar-Lambert Co., Ltd.). ***: significant difference from the saline control value with P<0.01, P<0.05. #: significant difference from the olive oil control value with P<0.01, P<0.05 (Student's t-test). For details, see Fig. 3.

Fig. 5. Effect of single and multiple administrations of CCl₄ on fibrinogen content. Fibrinogen contents are shown in mg/dl. **:** significant difference from the saline control value with P<0.01, P<0.05. #$: significant difference from the olive oil control value with P<0.01, P<0.05 (Student's t-test). For details, see Fig. 3.
Fig. 6. Effect of single and multiple administrations of CCl₄ on the activity of factor XIII. The activities of factor XIII are shown as Dansylcadaverine complex fluorescence determined by fluorescence spectrofluorometer. **, *: significant difference from the saline control value with P<0.01, P<0.05. #, #: significant difference from the olive oil control value with P<0.01, P<0.05 (Student’s t-test). For details, see Fig. 3.

Fig. 7. Effect of single and multiple administrations of CCl₄ on the activity of antithrombin III. The activities of antithrombin III are shown as a ratio compared to that of normal human plasma. **: significant difference from the saline control value with P<0.01. #: significant difference from the olive oil control value with P<0.01 (Student’s t-test). For details, see Fig. 3.
about 45–67% compared to the saline control between 12 and 48 hr. After multiple administrations of CCl₄, the activity of AT III was about 80% that of the saline and olive oil controls.

HPT: Figure 8 shows that HPT activity decreased significantly from 12 to 48 hr after single administration of CCl₄. At 24 and 48 hr, they were about 30–36% that of the saline control. Twelve hr after a single

![Graph showing changes in HPT activity](image)

**Fig. 8.** Effect of single and multiple administrations of CCl₄ on the activity of hepaplastintest. The activities of hepaplastintest are shown as the percentage of activity percent corresponding with coagulation time. ****: significant difference from the saline control value with P<0.01. **:** significant difference from the olive oil control value with P<0.01 (Student's t-test). For details, see Fig. 3.

![Graph showing changes in plasminogen content](image)

**Fig. 9.** Effect of single and multiple administrations of CCl₄ on plasminogen content. Plasminogen contents are shown in ratio compared to that of normal human plasma. **,**: significant difference from the saline control value with P<0.01, P<0.05. **:** significant difference from the olive oil control value with P<0.01 (Student's t-test). For details, see Fig. 3.
administration of olive oil, a significant increase in HPT activity was observed. After multiple administrations of CCl₄, it was about 60% that of both controls.

PLG: Figure 9 shows that an apparent change in PLG content was observed starting 12 hr after single administration of CCl₄. The content of PLG was about 53% that of the saline control at 12 hr and decreased drastically thereafter to reach the bottom value at 48 hr. Here, the content was about 5% that of the saline control. It recovered to normal level at 120 hr. On the other hand, the increase in PLG content was observed 5 hr after single administration of olive oil. After multiple administrations of CCl₄, the content of PLG was about 30% that of the saline control.

α₂-PI: As shown in the Fig. 10, α₂-PI activity decreased slightly but evidently 12 hr after single administration of CCl₄. A drastic decrease in α₂-PI activity was observed at 24, 48 and 72 hr. Each activity was 8, 25 and 38% that of the saline control, respectively. Twenty-four hr after single administration of olive oil, a slight but evident decrease in α₂-PI activity was observed. After multiple administrations of CCl₄, α₂-PI activity was about 45% that of the saline control.

Discussion

There have been many clinical reports on abnormal situations in the coagulative and fibrinolytic systems in patients with various types of liver disease (4, 5). However, there have been only a very few reports on the changes in these systems using experimental animals other than rabbits (12) and rats (13, 14). In order to know the degree of acute and chronic liver damage, the time course of coagulative and fibrinolytic activities following single and multiple administrations of CCl₄ was examined using mice. Our results with mice show that after a single administration of CCl₄, whole blood coagulative and fibrinolytic factors begin to be suppressed at 12 hr, and this becomes drastic at 24 and 48 hr. Especially, the concentration of fibrinogen, activity of factor XIII, PLG level and α₂-PI activity continued to show very low values up to 72 hr. After multiple administrations of CCl₄, coagulative factors showed a mild but evident depression to about 60–85% that of the saline control. On the other hand, fibrinolytic factors, PLG and
α2-PI showed a more severe decrease to about 47–55% that of the saline control.

PT is sensitive to deficiencies in the extrinsic coagulation system, that is, factor II, V, VII and X. The normal PT range for mice at our laboratory lies between 7.5 and 8.6 sec. PTT is sensitive to deficiencies in the intrinsic system, that is, factors II, V, VIII, IX, X, XI and XII. The normal PTT range for mice lies between 45 and 47 sec. In our results, olive oil treatment caused a shortening of PT and PTT, and a drastic prolongation of PT (31.4 sec) and PTT (66.1 sec) was observed 24 hr after single administration of CCl4, whereas no significant prolongation at 48 hr was observed because of the wide variety of all the values. In this experimental liver disease by CCl4, the intrinsic coagulation system was abnormal at just only 24 hr after single administration, and no change was seen after multiple ones. On the other hand, the abnormality of the extrinsic coagulation system continued from 12 to 72 hr after single administration. A mild elongation was observed in comparison to olive oil after multiple ones. Therefore, CCl4 seemed to induce the deficiencies of extrinsic coagulation factors rather more strongly than those of intrinsic factors. In other animal experiments using CCl4, in this case rabbits, the prolongation of PT and PTT was also observed with the decrease in fibrinogen content 24 hr after single administration (12).

TEG is a very convenient method to find out the entire mobilization of coagulative and fibrinolytic activity. At 24 hr after single administration of CCl4, abnormal patterns of TEG figures, inhibition of coagulation and enhancement of fibrinolysis were observed. Concerning r, k and ma values after single administration of CCl4, a severe elongation of the r value at 24 hr and the long-term elongation of k value were observed. The r value contributed to thromboplastin formation time and the k value was equivalent to thrombin formation time. Therefore, single administration of CCl4 was considered to cause the strong inhibition of thrombin formation. The ma value indicated the strength of the clot, that is, the platelet numbers and function and fibrinogen content could be judged from it. Our results showed the ma value decreasing drastically at 24 hr and then recovering to the normal range at 120 hr after single administration of CCl4. These results showed that changes of the ma value was related to fibrinogen content after single administration of CCl4. While, no change in the ma value after multiple administrations of CCl4 was observed, although fibrinogen content showed mild but significant decrease. Weston et al. (15) has previously reported that platelets reduced in numbers and were smaller than those of healthy controls in 34 patients with fulminant hepatic failure. Therefore, it is important to examine platelet numbers and function after single and multiple injections, and this is now under study in our laboratory.

Fibrinogen is synthesized largely or exclusively in the liver, as demonstrated in the isolated perfused rat liver (3) and in hepatic slices (16). The site of synthesis has been localized to the parenchymal cells (17). In experimental liver disease using rabbits (12) and rats (13, 14), an apparent decrease in fibrinogen content was observed 24 hr after single injection of CCl4, as in our data using mice. The turnover rate of fibrinogen is known to be 23–41 hr in rats (18), compared to 60–90 hr in humans, but it is not known in mice. Our results showed a decrease in fibrinogen content up to 72 hr after single administration of CCl4 (0.04 ml/body). If the turnover rate of fibrinogen in mice is the same as the one in rats, the continued decrease in fibrinogen up to 72 hr is considered a consistent result.

Factor XIII is important in the cross linking of fibrin monomers. It is also said that it is a fibrin stabilizing factor (FSF). Factor XIII decreases in the case of disseminated intravascular coagulation syndrome (DIC). The decrease in factor XIII is possibly due to (a) consumption to crosslink fibrin monomer, (b) absorption on the fibrin clot, and (c) destruction by the serine proteases (19). The concentration of factor XIII is decreased in the plasma of some patients with hepatic disease (21–22). Whether the deficiency of factor XIII in hepatic disease is due to failure of synthesis is not known. Our results showed that the activity of factor
XIII was enhanced at 5 and 12 hr after single administration of CCl₄. However, it is difficult to distinguish the effect between CCl₄ and olive oil, because the apparent increase in factor XIII activity was observed 5 hr after single administration of olive oil. At 24 hr, this activity decreased drastically, and its long-term decrease continued up to 168 hr after single administration of CCl₄. One of the reasons for this decrease is the consumption to crosslink fibrin monomer, since the fibrinogen content decreased apparently up to 72 hr and recovered to the normal range at 120 hr after single administration of CCl₄. Conceivably, in this case with the decrease in factor XIII, formed fibrin clots are more susceptible to fibrinolysis than is normal fibrin.

It was recognized that two proteins, α₂-macroglobulin and AT III, were responsible for the progressive inactivation of thrombin. Also, the activity of AT III is undoubtedly considered to reflect the damage of the liver cells where AT III is synthesized. Clinically, the concentration of AT III diminishes in patients with chronic hepatic disease. From our results, CCl₄ induced liver injury caused an apparent decrease in AT III activity after single and multiple administrations of CCl₄. In addition, HPT, which determines the true activity of coagulation factors (II, VII and X), was decreased after single and multiple administrations of CCl₄. This means that the depression of the productive ability of these factors in liver cells was actual.

Lysis of fibrin is brought about by a proteolytic enzyme, plasmin. PLG is converted to plasmin enzymatically by kinase or activators found in plasma (plasma activator), in urine (urokinase), in the tissues (tissue activator), and in body secretions. In humans, monkeys and dogs, streptokinase activates plasma PLG; while in rabbits and rats, urokinases activates it (23). In our experiments, longer incubation time was necessary for activating plasma PLG in mice than in rats. Natural inhibitors of fibrinolysis inhibit the activation of PLG already formed (24). Since the affinity of α₂-PI for plasmin is far greater than that of other inhibitors, this inhibitor is considered the most important in the regulation of fibrinolysis. From our results, a severe decrease in PLG content and α₂-PI activity was observed up to 72 hr after single administration and 48 hr after multiple ones of CCl₄. Especially, multiple administrations of CCl₄ seemed to cause a more drastic inhibition of the fibrinolytic system than the coagulative system. These results are considered as one of the causes of a more mild decrease in the coagulative system after multiple administrations of CCl₄ than after single administration.

The reticuloendothelial system (RES) is important in removing clotting products from circulating blood. It is well-known that RE cells play an important role in the removal of intravascular fibrin (25). RES blockade by thorotrast or trypan blue increases the sensitivity of rabbits to the generalized Shwartzman reaction (26). In a previous study (2), a significant decrease in RES function with a more mild change of biochemical parameters was observed after multiple injections of CCl₄ than after single injection. In this paper, a mild but significant decrease in fibrinogen content, AT III and HPT activity was reported after multiple administrations of CCl₄. 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, high dosage of CCl₄ after a single treatment does not show high toxicity for cells because of the decrease in CCl₃ formation in rats (27), and Busuttil et al. (28) reported that biochemical manifestation of hepatic injury during the development of cirrhosis was prominent only in cases of acute injury from intermittent doses in rats. Our results in the coagulative system also seemed to show a more apparent change in liver injury by a single treatment of CCl₄ than that by multiple treatments. Sugai et al. (29) postulated that hyperproduction of coagulative factors might be induced by the regeneration of liver cells after inflammation or by the inflammatory stimulation on hepatic parenchymal cells. In the previous paper, we showed severe steatosis and necrotic inflammation in chronic liver injury after multiple administrations of CCl₄ (2). Therefore, we can also postulate that these multiple administrations of CCl₄ induce the
hyperproduction of coagulative factors. On the other hand, the activities of the fibrinolytic system, PLG and α2-PI, were suppressed severely after multiple treatments of CCl4. This severe decrease in PLG and α2-PI might be due to the consumptive decrease by activation of fibrinolysis except the decrease in synthesizing activity of the liver cells, because in multiple administrations of CCl4 during 5 weeks, necrotic hepatocytes are considered to activate clotting factors within the circulating plasma.

In these experimental conditions using mice, the decrease in coagulative and fibrinolytic factors seemed to reflect accurately liver cell injury induced by single administration of CCl4, while multiple administrations of CCl4 might induce the hyper-production of coagulative factors and the complex counterbalance during the coagulative and fibrinolytic systems. However, it is important to know the situation of both systems in chronic liver injury using experimental animals. Therefore, the present experimental model using mice is useful for studying the effect of therapeutic agents for liver disease.

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