The Role of Kupffer Cells in Carbon Tetrachloride Intoxication in Mice

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Carbon tetrachloride (CCl_4)-induced acute hepatitis is assumed to involve two phases. The initial phase, initiated within 2 h after CCl_4 administration, involves the generation of reactive oxygen species. The second phase is assumed to start about 8 h subsequent to CCl_4 administration and involves the oxidant-induced activation of Kupffer cells, which release various pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6). We investigated the role of Kupffer cells during CCl_4 intoxication using Nucling-knockout mice (the KO group), in which the number of Kupffer cells is largely reduced. Plasma alanine transaminase and aspartate transaminase levels demonstrated that the liver necrosis during the second phase was significantly alleviated in the KO group compared with that in the wild-type mice (the WT group). Plasma TNF-α concentrations in the WT group significantly increased 24 h after CCl_4 intoxication, whereas those in the KO group did not significantly increase. Plasma IL-6 levels also significantly increased in the WT group 24 h after CCl_4 administration, but those in the KO group did not increase at any time point. These results indicated that excess reactions of Kupffer cells, once primed by oxidants, were involved in the exacerbation of oxidative stress and liver damage during the second phase of CCl_4 intoxication.

Key words carbon tetrachloride; Kupffer; nucling; interleukin; tumor necrosis factor; oxidative stress

Carbon tetrachloride (CCl_4) is a typical hepatotoxin causing acute centrizonal necrosis, and its chronic application causes liver fibrosis.[1)] Many studies have demonstrated that lipid peroxidation of liver cell endoplasmic reticulum initiated by the trichloromethyl radical generated by the reaction between CCl_4 and cytochrome P450 is the initial event that occurs as early as 2 h after CCl_4 intoxication in rats.[1,2)] Thereafter, the resulting severe oxidative stress persisted for at least 36 h[3)] despite the concentration of CCl_4 in the liver having decreased rapidly from the maximal level attained at 1.5 h.[4)]

CCl_4 causes many intracellular events including activation of mitogen activated protein kinase (MAPK),[5,6)] sphingomyelinase,[3)] nuclear factor-κB (NF-κB),[7)] and cyclooxygenase-2 (COX-2).[8)] Humoral factors may be involved in these inflammatory events and, therefore, CCl_4-induced hepatic injury is assumed to involve two phases.[9)] The initial phase, initiated within 2 h after CCl_4 administration, is the generation of reactive radicals, which destroy endoplasmic reticulum[1,2)] and activate MAPK[6)] and sphingomyelinase.[3)]

The second phase, assumed to start about 8 h subsequent to CCl_4 administration, is oxidant-induced activation of Kupffer cells which releases various pro-inflammatory mediators such as tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), interleukin-1 (IL-1), and IL-6.[10)] These cytokines activate NF-κB and COX-2, leading to necrosis and apoptosis[11)] of liver cells *via* caspase-3 activation.[12)] Along with extensive cell death maximally occurring 24–48 h after CCl_4 administration, processes for survival and regeneration of the liver cells occur simultaneously.[13,14)] Furthermore, these cytokines also contribute to the regeneration process.[15)]

Although the involvement of Kupffer cells in the liver cell death caused by CCl_4 has been evaluated utilizing gadolinium chloride ($GdCl_3$),[9)] assumed to be a specific inhibitor of Kupffer cells, recent studies have indicated that $GdCl_3$ exerts hepatoprotective effects other than depleting Kupffer cells.[16–18)] In this study, we investigated the role of Kupffer cells during the second phase of CCl_4 intoxication using Nucling-knockout (KO) mice.[19)] Nucling is a stress-inducible protein associated with apoptosomes[20)] and it also regulates the expression of galectin-3 *via* the suppression of nuclear factor (NF)-κB signaling.[19)] As the most prominent character of Nucling-knockout mice, it was found that the population of Kupffer cells in the liver was largely decreased to approximately 3.1%, while that in the wild-type was 13.6%.[21)]

MATERIALS AND METHODS

Animals This study was approved by the Animal Care Committee of Nara Women's University. The animals were housed in a room at 24±2°C, with a 12-h light/dark cycle. The animals were fed commercial laboratory chow (MF, Oriental Yeast Co., Osaka, Japan) and water *ad libitum*. After a 12-h fast, 8-week-old KO or wild-type (WT: C57BL/6J strain) male mice were administered 100 μL of a mixture of CCl_4 and mineral oil (1 mL/kg body weight as CCl_4) through an intragastric tube. Control mice received mineral oil (100 μL) alone, and assessments were made 24 h after administration. The control groups were designated as the "vehicle" groups.

Analytical Methods Mice were anesthetized with sodium 5-ethyl-5-(1-methylbutyl) barbiturate, and blood samples were collected by left-ventricular puncture using a syringe containing sodium heparin as an anticoagulant. Livers were excised after perfusion with phosphate buffered saline (pH 7.4) containing 2 mM of ethylenediaminetetraacetic acid (EDTA). The blood was centrifuged to separate the plasma.

Blood was centrifuged at 8400×g for 5 min at 4°C to separate the plasma. The activities of plasma aspartate aminotransferase (AST: EC 2.6.1.1) and alanine aminotransferase (ALT:

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EC 2.6.1.2) were determined using diagnostic kits (GOT and GPT-UV Test Wako, Wako Pure Chemicals Co., Osaka, Japan) and expressed as Karmen units.

Plasma TNF-α and IL-6 levels were determined using ELISA kits (Thermo Scientific, Rockford, IL, U.S.A.).

Statistical analysis for multiple comparisons was performed using one-way analysis of variance followed by Tukey–Kramer *post hoc* test and values of $p<0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Change in Plasma Levels of ALT and AST as an Index of Liver Necrosis Twelve hours after CCl_4 administration, the plasma AST levels in the WT group significantly increased compared with those in the vehicle groups given only mineral oil, whereas those in the KO group did not significantly increase (Table 1). The plasma AST level in the WT group increased further 24 h after CCl_4 administration, and was significantly higher than that at 12 h, whereas that in KO mice tended to increase but the increase was not statistically significant compared with that in the vehicle groups. Furthermore, plasma ALT activity increased 24 h after CCl_4 administration in the WT and KO groups, but the level in the WT group was significantly higher than that in the KO group.

These results demonstrated that liver necrosis during the second phase of CCl_4 intoxication was significantly alleviated in the livers of the KO group compared with that of the WT group, indicating the involvement of Kupffer cells in the progression of liver inflammation. Moreover, it is possible that the decrease in liver necrosis by CCl_4 in KO mice was not only due to the decrease of Kupffer cells but also due to the deficiency of Nucling, resulting in a resistance to apoptosis,[20] because CCl_4 causes both apoptosis and necrosis in the liver, and plasma ALT and AST levels at 24 h represent both necrosis and secondary necrosis followed by apoptosis.[12]

The Concentration of Plasma TNF-α Plasma concentrations of TNF-α significantly increased in the WT group 24 h after CCl_4 intoxication, whereas those in the KO group did not significantly increase (Fig. 1). The concentration of serum TNF-α increased in mice after CCl_4 administration[22,23] and treatment with a soluble TNF-α receptor to bind TNF-α decreased the degree of liver injury by CCl_4.[24] These studies indicated the central role of TNF-α in hepatitis caused by CCl_4. In addition TNF-α promoted inflammation and liver cell death by activating NF-κB, MAPK, and apoptosis signal-regulated kinase 1 (ASK1).[25–27] Therefore, our present results indicated that TNF-α, released from the Kupffer cells by CCl_4,[28] was reduced in KO mice; thus, contributing to loss of an inflammatory response in the livers of KO mice. It is also established that reactive oxygen species are involved in TNF-α-induced cell death,[29] explaining why severe oxidative stress persisted more than 24 h after CCl_4 intoxication[3] when CCl_4 had almost totally disappeared from the liver.[4]

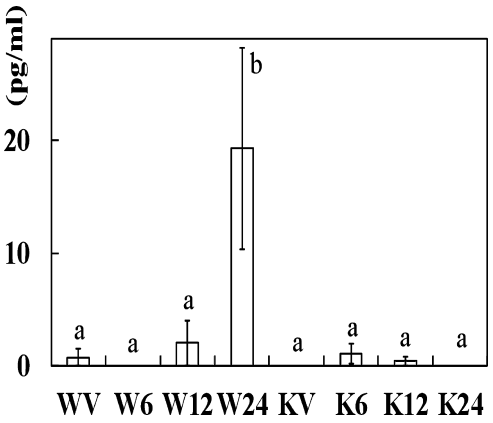

Fig. 1. Plasma TNF-α Concentrations in the WT and KO Groups

CCl_4 (1 mL/kg body weight as a mixture of CCl_4 and mineral oil) was orally administered to mice and ELISA analyses were conducted 6, 12, and 24 h later. The vehicle groups received mineral oil. WV, W6, W12, and W24 represent the vehicle group of the WT mice, and the WT groups, 6, 12, and 24 h after CCl_4 administration, respectively. KV, K6, K12, and K24 represent the vehicle group of the KO mice, and the KO groups, 6, 12, and 24 h after CCl_4 administration, respectively. Values are represented as mean±S.E. for 4 mice. Different superscript letters indicate significant differences at $p<0.05$ (Tukey–Kramer *post hoc* test).

NF-κB, which was activated by TNF-α in turn induced TNF-α mRNA transcription[30] and selective inactivation of NF-κB in the liver using an NF-κB decoy suppressed CCl_4-induced liver injury with a concomitant decrease in concentration of serum TNF-α.[23] These studies indicated that excess reactions of activated Kupffer cells increased proinflammatory cytokines such as TNF-α, which induced an exacerbation cycle resulting in extensive cell death. This exacerbation cycle includes TNF-α upregulation, which is also associated with COX-2 induction,[31,32] which successively causes a marked induction of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6.[33] Then, it is reasonable that inhibiting only COX-2, a component in this exacerbation cycle, ameliorated hepatitis in the COX-2 transgenic mice[33] as well as in CCl_4-administered rats.[34] Therefore it is conceivable that the reduced number of Kupffer cells and resulting decrease in TNF-α release intercepted the exacerbation cycle in KO mice.

On the other hand, NF-κB activation protected hepatocytes from TNF-induced cell death, and crosstalk between TNF-α-induced NF-κB and c-Jun activating kinase (JNK) pathways was important to determine the biological outcome of TNF stimulation.[35] Since Nucling is an NF-κB suppressor,[36] NF-κB was activated spontaneously in KO mice.[21] Furthermore, a deficiency of Nucling conferred resistance to cellular apoptotic

Table 1. Plasma AST and ALT Levels (Karmen Units) of the WT and KO Groups

	Vehicle		6 h		12 h		24 h	
	WT	KO	WT	KO	WT	KO	WT	KO
AST	98.2±4.3^{a}	76.4±11^{a}	369±71^{ad}	202±33^{ad}	993±386^{b}	369±35^{ad}	3675±221^{c}	449±145^{ad}
ALT	24.3±2.3^{a}	25.2±2.2^{a}	157±110^{a}	39.7±8.9^{a}	524±302^{ac}	246±29^{ac}	3255±478^{b}	700±69^{c}

After a 12-h of fast, 8-week-old Nucling deficient (KO) or wild-type (WT) male mice were orally administered 100 μL of a mixture of CCl_4 and mineral oil (1 mL/kg body weight as CCl_4). Assessments were made 6, 12, and 24 h after CCl_4 administration. Control mice received mineral oil (100 μL) alone and assessments were made 24 h after administration. The control groups were designated as the "vehicle" groups. Values are represented as mean±S.E. for 4 mice. Different superscript letters indicate significant differences at $p<0.05$ (Tukey–Kramer *post hoc* test).

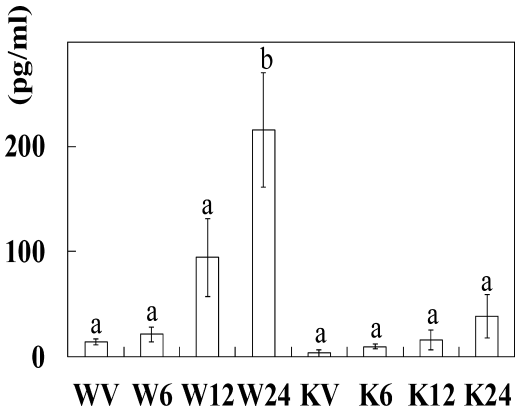

Fig. 2. Plasma IL-6 Concentrations in the WT and KO Groups

CCl_4 (1 mL/kg body weight as a mixture of CCl_4 and mineral oil) was orally administered to mice and ELISA analyses were conducted after 6, 12, and 24 h. The vehicle groups received mineral oil. WV, W6, W12, and W24 represent the vehicle group of the WT mice, and the WT groups 6, 12, and 24 h after CCl_4 administration, respectively. KV, K6, K12, and K24 represent the vehicle group of the KO mice, and the KO groups 6, 12, and 24 h after CCl_4 administration, respectively. Values are represented as mean±S.E. for 4 mice. Different superscript letters indicate significant differences at $p<0.05$ (Tukey–Kramer *post hoc* test).

stress.[20] Moreover, this resistance to cell death and NF-κB activation, which has anti-oxidative functions[37] may have contributed to inhibition of liver cell necrosis caused by CCl_4.

Plasma Concentration of IL-6 Plasma IL-6 levels significantly increased in the WT group 24 h after CCl_4 administration but those in the KO group did not increase at all time points (Fig. 2). This may be due to the decrease of Kupffer cells in the KO mice and the resulting lower IL-6 levels in the KO group may cause less damage to the liver compared with that in the WT group.

CONCLUSION

The role of Kupffer cells during the second phase of acute CCl_4 intoxication was evaluated using Nucling-knockout mice (the KO group), in which the number of Kupffer cells is largely reduced. Plasma ALT and AST levels demonstrated that the liver necrosis was significantly alleviated in the KO group compared with that in the WT group after CCl_4 intoxication. The attenuation of liver damage in the KO mice may have been due to lower plasma levels of inflammatory cytokines such as TNF-α and IL-6, which are released from Kupffer cells compared with that in WT mice. These results indicated that excess reactions of Kupffer cells, previously activated by oxidants, were involved in the exacerbation of oxidative stress and liver damage during the second phase of CCl_4 intoxication.

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