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European Journal of Pharmacology 578 (2008) 57–64
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Ursodeoxycholic acid protects concanavalin A-induced mouse liver injury through inhibition of intrahepatic tumor necrosis factor-α and macrophage inflammatory protein-2 production

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Received 29 March 2007; received in revised form 9 August 2007; accepted 27 August 2007
Available online 4 September 2007

Abstract

Ursodeoxycholic acid (UDCA) is widely used for the therapy of liver dysfunction. In this study, we investigated the protective effect of UDCA in concanavalin A-induced mouse liver injury. The treatment with UDCA at oral doses of 50 and 150 mg/kg at 2 h before concanavalin A injection significantly reduced the elevated plasma levels of aminotransferases and the incidence of liver necrosis compared with concanavalin A-injected control group without affecting the concentrations of liver hydrophobic bile acids. UDCA significantly inhibited elevated levels of tumor necrosis factor-α (TNF-α), macrophage inflammatory protein-2 (MIP-2), and interleukin 6 (IL-6) in blood of concanavalin A-injected mice. To clarify the influence of UDCA on production of cytokines, we examined intrahepatic mRNA expressions and the protein levels of TNF-α, MIP-2, interferon-γ (IFN-γ), IL-4, IL-6, and IL-10 at 1 h after concanavalin A injection. The treatment with UDCA significantly decreased the intrahepatic levels of TNF- α and MIP-2, whereas this compound showed no clear effect on IFN-γ, IL-4, IL-6, or IL-10. Furthermore, UDCA significantly decreased myeloperoxidase activity as well as MIP-2 level in the liver and histological examination of liver tissue revealed that intrasinusoidal accumulation of neutrophils was decreased markedly by UDCA. In addition, UDCA significantly inhibited the production of TNF-α and MIP-2 when cultured with nonparenchymal and lymph node cells. In conclusion, these findings suggest that UDCA protects concanavalin A-induced liver injury in mice by inhibiting intrahepatic productions of TNF-α and MIP-2, and the infiltration of neutrophils into the liver.

Keywords: Concanavalin A; Liver bile acid; Liver nonparenchymal cell; Lymph node cell; Macrophage inflammatory protein-2; Neutrophil; Tumor necrosis factor-α; Ursodeoxycholic acid

1. Introduction

Ursodeoxycholic acid (UDCA) is widely used for the therapy of primary biliary cirrhosis and primary sclerosing cholangitis because orally administered UDCA significantly decreases the serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and γ-glutamyltransferase (Poupon et al., 1997; Pares et al., 2000; Corpechot et al., 2005; van de Meeberg et al., 1996; Mitchell et al., 2001). UDCA significantly reduces serum ALT level in chronic hepatitis C patients (Takano et al., 1994; Lirussi et al., 1999; Omata et al., in press) and maintaining ALT at a low level is an important therapy to prevent development of hepatocellular carcinoma and to increase survival rates (Tarao et al., 2004; Veldt et al., 2006). Moreover, UDCA exerts choleretic (Fickert et al., 2001), anti-apoptotic (Rodrigues et al., 1998), anti-oxidative (Serviddio et al., 2004; Mitsuyoshi et al., 1999), immunomodulating effects (Yoshikawa et al., 1992) and alternation of the bile acid

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doi:10.1016/j.ejphar.2007.08.031

composition (Combes et al., 1999; Lazzaridis et al., 2001). It has been suggested that UDCA reduces liver dysfunction by inducing the replacement of hydrophobic bile acids with hydrophilic bile acids (Takano et al., 1994; Combes et al., 1999).

Acute liver failure that is induced by intravenous injection of concanavalin A is a well-known mouse model for liver injury (Tiegs et al., 1992). Concanavalin A-induced liver injury in mice is characterized by inflammatory infiltration by neutrophils, macrophages, and T cells into the liver with a marked elevation of transaminases in the blood after concanavalin A injection. It has been shown that concanavalin A-induced liver injury depends on the production of inflammatory cytokines and chemokines, such as tumor necrosis factor-α (TNF-α), macrophage inflammatory protein 2 (MIP-2), interferon-γ (IFN-γ), interleukin 4 (IL-4), IL-6, and IL-10 (Tiegs et al., 1992; Imose et al., 2004; Nakamura et al., 2001; Sass et al., 2002).

In the present study, we demonstrated that administration of UDCA at 2 h before injection of concanavalin A significantly reduced levels of AST and ALT and the incidence of liver necrosis in concanavalin A-induced mouse liver injury. Our results suggest that the protective effects of UDCA on concanavalin A-induced mouse liver injury is mediated by inhibiting intrahepatic TNF-α and MIP-2 but not replacement of liver hydrophobic bile acids with hydrophilic bile acids including UDCA.

2. Materials and methods

2.1. Animals and reagents

Male BALB/c mice were purchased from CLEA Japan, Inc. (Tokyo, Japan) and used at 9 to 10 weeks of age. All animal experiments were approved by the Animal Ethical Committee of Mitsubishi Pharma Corporation and performed in accordance with guidelines of the Japanese Pharmacological Society. UDCA was synthesized with a purity of more than 99.0% in Mitsubishi Pharma Corporation and dissolved in 1 M NaOH followed by adjusting to pH 8.3 with HCl for oral administration. Concanavalin A was purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in physiological saline.

2.2. Concanavalin A-induced liver injury in mice

In the liver injury studies, mice received an intravenous concanavalin A injection at 20 mg/kg body weight. UDCA was orally administered 2 h before concanavalin A injection and control mice received distilled water, instead of UDCA. Plasma samples or sera were collected from mice at indicated time points after concanavalin A injection to measure the levels of AST, ALT and cytokines. Plasma AST and ALT were determined using an automatic analyzer (Fuji Film Medical, Tokyo, Japan). For histological examinations, livers were fixed in 10% formalin, embedded in paraffin, and cut into 3-μm sections. The sections were stained with hematoxylin-eosin and examined under light microscopy to evaluate liver damage. The extent of liver damage was scored from 0 to 4 as follows: grade 0, normal histology; grade 1, minor necrosis covering <10%; grade 2, 10–25%; grade 3, 25–50%; grade 4, >50%.

2.3. Measurement of liver bile acid concentrations

The concentrations of UDCA, α-muricholic acid (α-MCA), β-muricholic acid (β-MCA), hyodeoxycholic acid (HDCA), cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA) and lithocholic acid (LCA) in the liver were measured by high-performance liquid chromatography (HPLC) according to the method described previously (Sakakura et al., 1998). The concentration of each bile acid including unconjugated, glycine-conjugated, and taurine-conjugated types was expressed as nmol/g liver.

2.4. Measurement of cytokine levels in the serum and liver

Serum samples from mice were obtained periodically after concanavalin A injection and the levels of TNF-α, MIP-2, and IL-6 were determined using enzyme-linked immunosorbent assay (ELISA) kits (TNF-α, Techne Corporation; MIP-2, R&D Systems; IL-6, Biosource International). The liver was homogenized in 5 ml ice-cold physiological saline and supernatants were obtained by centrifugation at 2000 ×*g* for 10 min. In some experiments, cytokine concentrations in the serum and liver homogenates were determined by cytometric bead array (CBA). Shortly, serum samples or liver homogenates (50 μl) were added to capture beads conjugated with anti-TNF-α, anti-MIP-2, anti-IL-6, anti-IFN-γ, anti-IL-4, and anti-IL-10 antibodies (BD Bioscience, San Diego). Then phycoerythrin detection reagent was added. After 2 h incubation, samples were analyzed with fluorescence-activated cell sorting and absolute values were obtained by comparisons with standards.

2.5. Cytokine messenger RNA quantification by real-time polymerase chain reaction (real-time PCR)

Total RNA was isolated from liver samples using SV Total RNA Isolation System (Promega, Madison, WI). Complementary DNA was synthesized with TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) using random hexamers and 0.5 μg of total RNA. Real-time PCR was performed with an ABI PRISM 7700 sequence detector (Applied Biosystems), using SYBR Green PCR master mix (Applied Biosystems). The initial step was at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of a denaturing step for 15 s at 95 °C and an annealing-extension step for 1 min at 60 °C. For normalization of data, determination of expression of a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was performed using predeveloped primers (TaqMan rodent GAPDH control reagents; Applied Biosystems). Data were analyzed using Sequence Detector software (Applied Biosystems). Relative quantification of mRNA levels was performed using the standard curve method and calculated as the ratio between each sample and GAPDH. Each experiment was performed in triplicate. The following primer pairs were used (5′ to 3′): TNF-α forward, AGC CGA TGG GTT GTA

CCT TGT CTA; TNF-α reverse, TGA GAT AGC AAA TCG GCT GAC GGT; MIP-2 forward, GTG AAC TGC GCT GTC AAT GC; MIP-2 reverse, CGC CCT TGA GAG TGG CTA TG; IL-6 forward, ATC CAG TTG CCT TCT TGG GAC TGA; IL-6 reverse, TAA GCC TCC GAC TTG TGA AGT GGT; IFN-γ forward, GAC AAT GAA CGC TAC ACA CTG CAT; IFN-γ reverse, GTG GCA GTA ACA GCC AGA AAC A; IL-4 forward, CGG AGA TGG ATG TGC CAA A; IL-4 reverse, AAG CCC TAC AGA CGA GCT CAC T; IL-10 forward, GAG ACT TGC TCT TGC ACT ACC AAA; IL-10 reverse, CAG TCA GTA AGA GCA GGC AGC AT.

2.6. Assay of hepatic myeloperoxidase activity

Hepatic myeloperoxidase (MPO) activity was measured by the method as described by Schierwagen et al. (1990). Briefly, liver samples obtained at 6 h after concanavalin A injection were homogenized and MPO activity was assayed by measuring absorbance at 492 nm using *o*-phenylenediamine as a substrate. Total MPO activity was quantified by a standard human sperm MPO (Elastin Products Co, Inc., Owensville, MS) and expressed as units/g of liver tissue.

2.7. Co-culturing of liver nonparenchymal cells with lymph node cells

A single cell suspension of liver cells were prepared by the two-step collagenase perfusion of the liver from BALB/c mice according to the method described by Seglen (1973). Non-parenchymal cells isolated from the liver cells by the Percoll density gradient centrifugation (Smedsrod and Pertoft, 1985) were cultured at 3×10^5 cells in 48 wells for 2 h to remove non-adherent cells. Mesenteric lymph nodes removed from BALB/c mice were minced and passing through a stainless mesh (106 μm) to prepare a single cell suspension of lymph node cells. Adherent nonparenchymal cells were co-cultured with lymph node cells (10^6 cells) in the presence or absence of UDCA in RPMI 1640 medium (Sigma) containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2. After 1 h, these cells were stimulated with 2 μg/ml of concanavalin A in the presence or absence of UDCA for 24 h at 37 °C in 5% CO_2. The levels of TNF-α, MIP-2 and IL-6 in supernatants were determined using cytokine-specific ELISA kits (TNF-α, Techne Corporation; MIP-2, R&D Systems; IL-6, Biosource International).

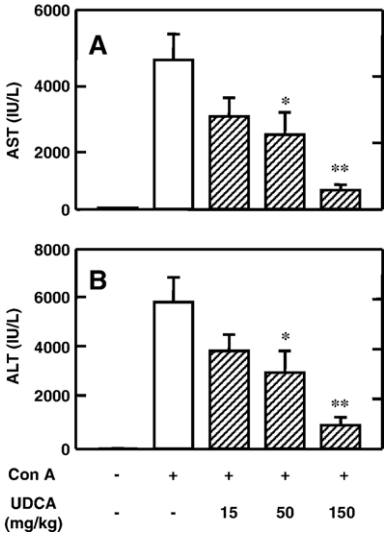

Fig. 1. Effect of UDCA on plasma AST and ALT in concanavalin A (Con A)-induced liver injury in mice. UDCA (15, 50 and 150 mg/kg) was administered orally 2 h before concanavalin A injection and plasma AST (A) and ALT (B) were measured at 24 h after concanavalin A injection. Results are expressed as the mean±S.E.M. of 12 mice. The statistical differences were calculated by Dunnett's multiple comparison test (*: $P<0.05$, **: $P<0.01$ vs. concanavalin A-injected control group).

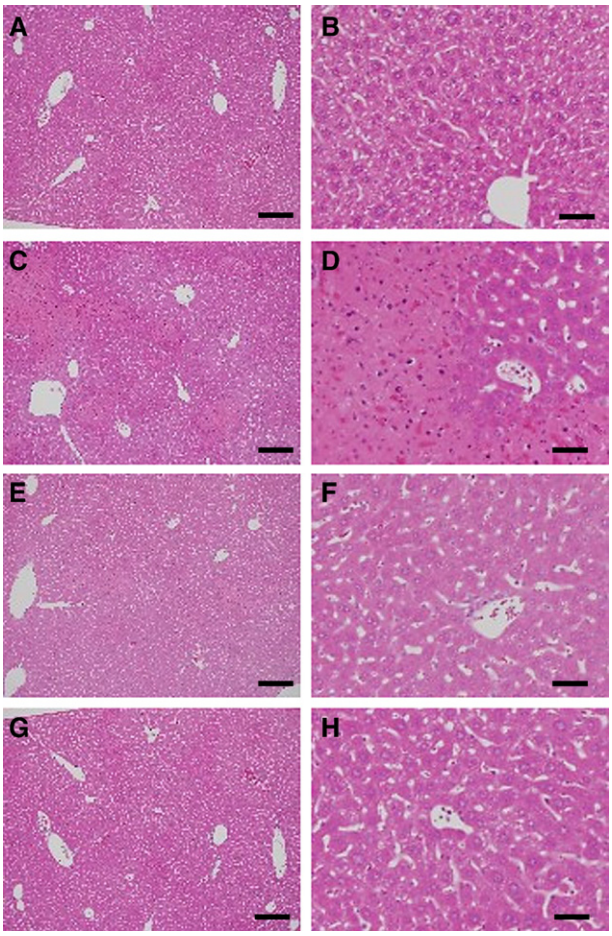

Fig. 2. Effect of UDCA on light micrographic changes of liver after concanavalin A injection. The liver samples were obtained 24 h after concanavalin A injection from untreated (A, B); concanavalin A-injected (C, D); concanavalin A injected with UDCA at 50 mg/kg pretreatment (E, F); and concanavalin A injected with UDCA at 150 mg/kg pretreatment (G, H) (hematoxylin-eosin staining). Bars indicate 200 μm (A, C, E, G) and 50 μm (B, D, F, H).

2.8. Statistical analyses

Results are expressed as the mean±S.E.M. The statistical analyses were performed using Dunnett's multiple comparison test for plasma transaminase levels and liver bile acid concentrations. For intrahepatic mRNA expression, cytokine concentrations and MPO activity, statistical significances were

determined using Student's *t*-test. Histological scores and the time course of serum cytokines were analyzed by Steel's method and 2-way ANOVA, respectively. Statistical significances *in vitro* study were determined using Dunnett's multiple comparison test (2-way layout). The difference was considered statistically significant when $P<0.05$.

3. Results

3.1. Hepatoprotective effect of UDCA in concanavalin A-induced acute liver injury in mice

Acute hepatitis was induced in BALB/c mice by an intravenous concanavalin A injection. Plasma levels of AST and ALT are usually elevated significantly at 24 h after concanavalin A injection (Fig. 1). It has been reported that UDCA (600 mg/day) administered orally for 4 days in patients suffering chronic hepatitis C attained UDCA concentrations in their livers of 40.1±9.0 nmol/g (Setchell et al., 1997). When UDCA was administered at oral doses of 50 and 150 mg/kg to mice, liver UDCA concentrations were approximately 40 and 90 nmol/g, respectively. Therefore, we used 15–150 mg/kg UDCA to evaluate its hepatoprotective effect in this study. By treatment with UDCA orally at 50 and 150 mg/kg 2 h before concanavalin A injection, the levels of AST and ALT in plasma were significantly and dose-dependently lower as compared with those of vehicle-treated control group. As shown in Fig. 2, livers from concanavalin A-injected control mice exhibited lymphoid and neutrophilic inflammatory infiltrates, numerous apoptotic bodies, and widespread hepatocellular necrosis in the intermediate zone of the liver lobules. In contrast, the region of necrotic change was reduced by treatment with UDCA at 50 and 150 mg/kg. The histological scores in groups given UDCA at 50 and 150 mg/kg were significantly lower than that of control group (Table 1). UDCA at 150 mg/kg appeared to exert a striking hepatoprotective effect against concanavalin A-induced liver injury in mice.

3.2. UDCA did not affect the concentrations of liver hydrophobic bile acids in concanavalin A-induced liver injury

We examined the influence of UDCA on the concentrations of UDCA, α-MCA, β-MCA, HDCA, CA, CDCA, DCA, and LCA in the livers of concanavalin A-injected mice. The results are summarized in Table 2. The concentration of UDCA in the liver increased to 38.5±1.1 nmol/g (17.2±0.3% of total bile acids) in the 50 mg/kg group and 87.8±8.0 nmol/g (31.9±1.8% of total bile acids) in the 150 mg/kg group at 24 h after concanavalin A was injected. The concentrations of β-MCA and CA were decreased slightly, but significantly, by administration of UDCA. On the other hand, there were no clear changes in liver concentrations of either hydrophobic bile acids (CDCA, DCA and LCA) or total bile acids in groups treated with UDCA compared with untreated or a control group injected with concanavalin A. Similar results were obtained when concentrations of liver bile acids were determined at 4 h after concanavalin A was injected (data not shown). These results indicated that the hepatoprotective effect of UDCA in concanavalin A-induced hepatitis is not due to the replacement of hydrophobic bile acids with hydrophilic bile acids including UDCA.

Table 1
Effect of UDCA on histological extent of concanavalin A (Con A)-induced mouse liver injury

	Histological extent of liver injury (grade)				
	0	1	2	3	4
Con A (control)	0	0	1	10	1
Con A+UDCA 15 mg/kg	0	0	5	7	0
Con A+UDCA 50 mg/kg[a]	0	3	3	6	0
Con A+UDCA 150 mg/kg[b]	0	6	6	0	0

The extent of liver damage was scored from 0 to 4 as follows; grade 0, normal histology; grade 1, minor necrosis covering<10%; grade 2, 10–25%; grade 3; 25–50%, grade 4, >50%. The results are shown as the number of mice in each group and statistical differences were calculated by Steel's method ([a]: $P<0.05$, [b]: $P<0.01$ vs. concanavalin A-injected control group).

Table 2
Effect of UDCA on the concentrations of liver bile acids in concanavalin A (Con A)-induced liver injury in mice

	Concentrations of liver bile acids (nmol/g liver)			
	Untreated	Con A (Control)	Con A+UDCA 50 mg/kg	Con A+UDCA 150 mg/kg
UDCA	<1	2.4±2.4	38.5±1.1[b]	87.8±8.0[b]
α-MCA	16.1±2.6	13.1±1.1	11.1±1.0	18.7±2.1[a]
β-MCA	70.5±14.8	72.3±6.4	58.7±3.4	49.7±3.8[a]
HDCA	1.8±0.6	<1	<1	<1
CA	113.3±20.5	141.1±7.0	107.8±6.0[a]	105.5±11.0[a]
CDCA	<1	<1	<1	<1
DCA	10.3±1.3	10.0±0.7	6.5±0.7	12.4±1.5
LCA	<1	<1	<1	<1
Total	212.2±38.8	239.6±13.1	223.6±7.1	275.7±23.3

UDCA was administered orally to mice 2 h before concanavalin A injection. The concentrations of liver bile acids were measured by HPLC at 24 h after concanavalin A injection. The results were expressed as the mean±S.E.M. of 5 mice and statistical differences were calculated by Dunnett's multiple comparison test ([a]: $P<0.05$, [b]: $P<0.01$ vs. concanavalin A-injected control group). α-MCA:α-muricholic acid; β-MCA:β-muricholic acid; HDCA: hyodeoxycholic acid; CA: cholic acid; CDCA: chenodeoxycholic acid; DCA: deoxycholic acid; LCA: lithocholic acid.

3.3. UDCA decreased the serum levels of TNF-α, MIP-2, and IL-6 in concanavalin A-induced liver injury in mice

We next examined the influence of orally administered UDCA on the time course of the serum levels of TNF-α, MIP-2, and IL-6 in mice injected with concanavalin A, because it is well-known that the blood levels of these inflammatory cytokines are elevated rapidly and markedly from the time concanavalin A is injected (Nakamura et al., 2001; Sass et al., 2002). We used UDCA at 150 mg/kg, because the most suppressive effect of ALT level was shown at this dose. The serum level of TNF-α in the control group elevated rapidly to a maximum of approximately 150 pg/ml at 1 h after concanavalin A was injected (Fig. 3A). In the group treated with UDCA the serum TNF-α was markedly lower at 1 h and remained 20% lower than that of control group until 2 h after concanavalin A

was injected. Furthermore, the serum levels of MIP-2 in the control group were also markedly elevated to a maximal level of approximately 2200 pg/ml at 2 to 3 h after concanavalin A injection (Fig. 3B). In the UDCA group, serum levels of MIP-2 were significantly lower initially and remained low until 8 h after concanavalin A injection. The serum IL-6 in the control group increased at 3 h and reached a maximum level (approximately 8000 pg/ml) at 4 to 5 h and thereafter decreased gradually (Fig. 3C). The serum IL-6 in groups treated with UDCA was limited to half the level in the concanavalin A-injected control group throughout the assay period. Furthermore, we confirmed that there was no clear change in the plasma levels of IL-2, IL-4, IL-12 and IL-10 by treatment with UDCA during 8 h after concanavalin A injection (data not shown). Our results demonstrated that the elevation of TNF-α, MIP-2, and IL-6 in the blood is significantly prevented by UDCA in concanavalin A-induced mouse liver injury.

3.4. UDCA inhibited intrahepatic TNF-α and MIP-2 production and neutrophil infiltration into the liver in concanavalin A-induced liver injury

To clarify, more precisely, the influence of UDCA on the production of cytokines in concanavalin A-induced mouse liver injury, we examined intrahepatic mRNA expressions and the protein levels of TNF-α, MIP-2, IFN-γ, IL-4, IL-6, and IL-10 at 1 h after concanavalin A was injected. As shown in Fig. 4, the intrahepatic mRNA expressions of TNF-α and MIP-2 in the group treated with UDCA were reduced significantly compared

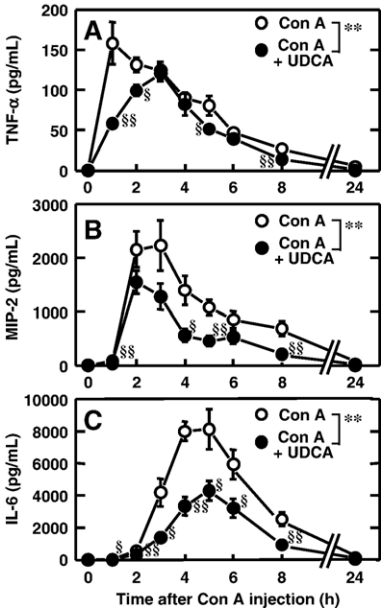

Fig. 3. Effect of UDCA on serum TNF-α, MIP-2, and IL-6 in concanavalin A (Con A)-induced liver injury in mice. UDCA (150 mg/kg) was administered orally 2 h before concanavalin A injection and serum levels of TNF-α (A), MIP-2 (B), and IL-6 (C) were determined by ELISA. Results are expressed as the mean±S.E.M. of 6 mice. The statistical differences were calculated by 2-way ANOVA (**: $P<0.01$ vs. concanavalin A-injected control group). The statistical differences were calculated by Student's *t*-test (§:$P<0.05$, §§:$P<0.01$ vs concanavalin A-injected control group).

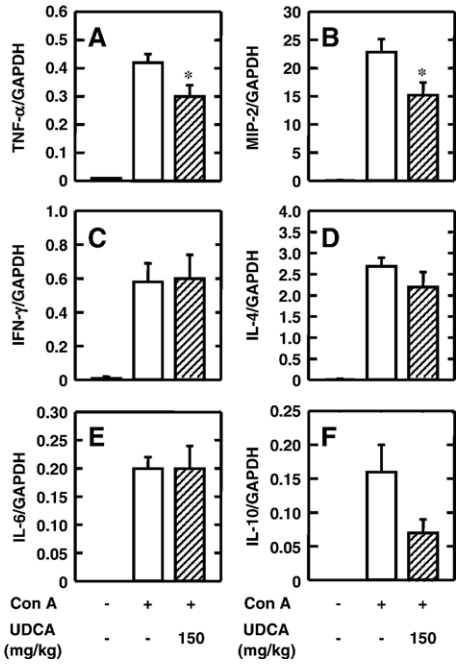

Fig. 4. Effect of UDCA on intrahepatic cytokine mRNA expressions in concanavalin A (Con A)-induced liver injury in mice. UDCA (150 mg/kg) was administered orally 2 h before concanavalin A injection and intrahepatic mRNA expressions of TNF-α (A), MIP-2 (B), IFN-γ (C), IL-4 (D), IL-6 (E), and IL-10 (F) were determined at 1 h after concanavalin A injection by real time RT-PCR. Relative quantification of mRNA levels was calculated as the ratio between each sample and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results are expressed as the mean±S.E.M. of 6 mice. The statistical differences were calculated by Student's *t*-test (*: $P<0.05$ vs. concanavalin A-injected control group).

with concanavalin A-injected control group. On the other hand, there was no clear effect on the intrahepatic mRNA expressions of IFN-γ and IL-6; while levels of IL-4 and IL-10 were lower, but not significantly, compared with control. Fig. 5 shows the levels of cytokines in liver homogenates at 1 h after concanavalin A was injected. Consistent with the intrahepatic mRNA expression patterns of cytokines, the intrahepatic levels of TNF-α and MIP-2 decreased significantly in the group that was treated with UDCA. Intrahepatic IFN-γ, IL-4, and IL-6 in the UDCA-treated group were lower, but not significantly; whereas the level of IL-10 in the liver tended to increase compared with untreated or concanavalin A-injected groups. These results indicated that UDCA inhibits intrahepatic TNF-α and MIP-2 production in the early phase of concanavalin A-induced liver injury.

To elaborate on these results, we analyzed the correlation between intrahepatic MIP-2 production and neutrophil infiltration into the liver in response to UDCA in this model. Mice treated with UDCA at 150 mg/kg experienced a significant reduction of intrahepatic expression of MIP-2 mRNA and MIP-2 in the liver and serum at 4 h after concanavalin A was injected (Fig. 6A, B, and C). Moreover, MPO activity in the liver and accumulation of neutrophils into the hepatic sinusoids was decreased by UDCA at 6 h after concanavalin A injection (Figs. 6D and 7). Liver MPO activity was approximately 20 U/g liver 1 h after concanavalin A injection, increased gradually, and

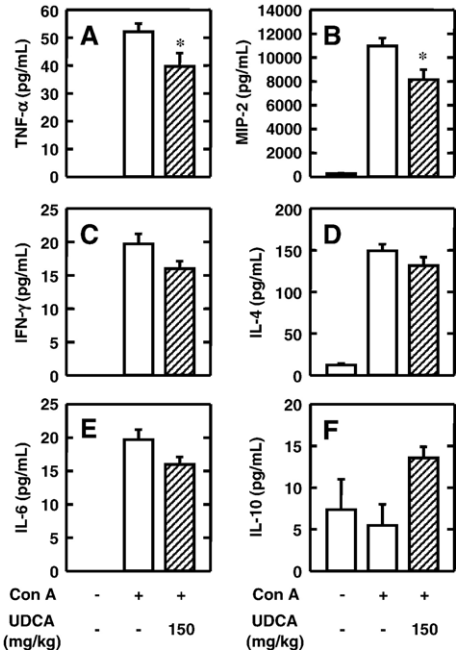

Fig. 5. Effect of UDCA on intrahepatic cytokine levels in concanavalin A (Con A)-induced liver injury in mice. UDCA (150 mg/kg) was administered orally 2 h before concanavalin A injection and intrahepatic levels of TNF-α (A), MIP-2 (B), IFN-γ (C), IL-4 (D), IL-6 (E), and IL-10 (F) were determined at 1 h after concanavalin A injection by CBA. Results are expressed as the mean±S.E.M. of 6 mice. The statistical differences were calculated by Student's *t*-test (*: $P<0.05$ vs. concanavalin A-injected control group).

reached to a maximal level (approximately 60 U/g liver) at 6 h. Thereafter liver MPO activity decreased gradually and returned to approximately 20 U/g liver 24 h after concanavalin A injection (data not shown). As our data suggests, infiltration of neutrophils into the liver is maximal at 6 h after concanavalin A; therefore, we evaluated the effect of UDCA at this time point. These findings suggested that UDCA prevented concanavalin A-induced liver injury in mice by inhibiting intrahepatic production of TNF-α and MIP-2 and the infiltration of neutrophils into the liver.

3.5. Effect of UDCA on TNF-α, MIP-2 and IL-6 productions in co-culturing with nonparenchymal cells and lymph node cells

As shown in Table 3, the significant amounts of TNF-α, MIP-2 and IL-6 were produced by concanavalin A stimulation when nonparenchymal cells were co-cultured with lymph node cells for 24 h. On the other hand, nonparenchymal cells or lymph node cells alone produced no detectable level or only sight level of these cytokines by concanavalin A stimulation. Because the liver UDCA concentration was 87.5±8.2 nmol/g (87.5±8.2 μM) when mice were given UDCA at 150 mg/kg (Table 2), we used 50 to 200 μM UDCA to evaluate its effect *in vitro*. UDCA at concentrations of 100 to 200 μM significantly suppressed the release of TNF-α and IL-6. In addition, UDCA at concentrations of 50 μM or higher significantly suppressed the MIP-2 production. Similar results were obtained when we examined the effect of UDCA on the production of these cytokines at 3 h after concanavalin A stimulation (data not shown).

4. Discussion

The oral administration of UDCA significantly decreases the serum levels of aminotransferases in primary biliary cirrhoses (Poupon et al., 1997; Pares et al., 2000; Corpechot et al., 2005), primary sclerosing cholangitis (van de Meeberg et al., 1996; Mitchell et al., 2001), and in chronic hepatitis C patients (Takano et al., 1994; Lirussi et al., 1999). The hepatoprotective effect of UDCA is considered to be due mainly to the replacement of hydrophobic bile acids with hydrophilic bile acids (Takano et al., 1994; Combes et al., 1999; Omata et al., in press); however UDCA exerts choleretic (Fickert et al., 2001), anti-apoptotic (Rodrigues et al., 1998), anti-oxidative (Serviddio et al., 2004; Mitsuyoshi et al., 1999), and immunomodulating effects (Yoshikawa et al., 1992). Because the progression of chronic liver disease may be, at least in part, involved in a repetition of acute liver injury, in this study we utilized concanavalin A-induced mouse acute liver injury as one of models that reflect the part of chronic liver diseases in human.

In the present study, we demonstrated in mice, for the first time, that treatment with UDCA before concanavalin A injection dose-dependently inhibited development of elevated plasma AST and ALT and decreased the incidence of liver necrosis in concanavalin A-induced mouse liver injury (Fig. 1). The UDCA treatment decreased the liver concentrations of β-MCA and CA; however UDCA did not affect the liver concentrations of either hydrophobic bile acids (CDCA, DCA and LCA) or total bile acids. Consequently, we concluded that the hepatoprotective effect of UDCA was not due to the replacement of hydrophobic

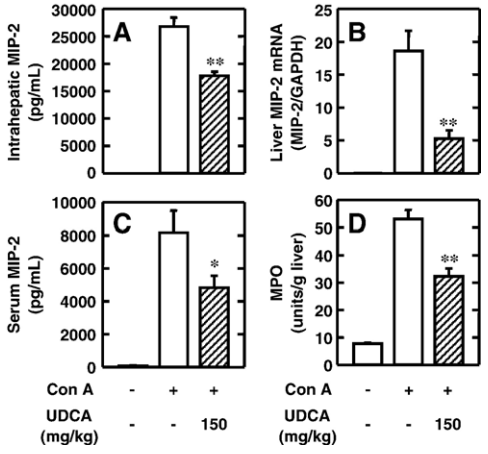

Fig. 6. Effect of UDCA on intrahepatic MIP-2 production, serum MIP-2 levels, and MPO activity in the liver in concanavalin A (Con A)-induced liver injury in mice. UDCA (150 mg/kg) was administered orally 2 h before concanavalin A injection. Intrahepatic MIP-2 level (A), intrahepatic MIP-2 mRNA expression (B), and serum MIP-2 were determined 4 h after concanavalin A injection. MPO activity in the liver was determined 6 h after concanavalin A injection. Relative quantification of MIP-2 mRNA level was calculated as the ratio between each sample and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Results are expressed as the mean±S.E.M. of 6 mice. The statistical differences were calculated by Student's *t*-test (*: $P<0.05$, **: $P<0.01$ vs. concanavalin A-injected control group).

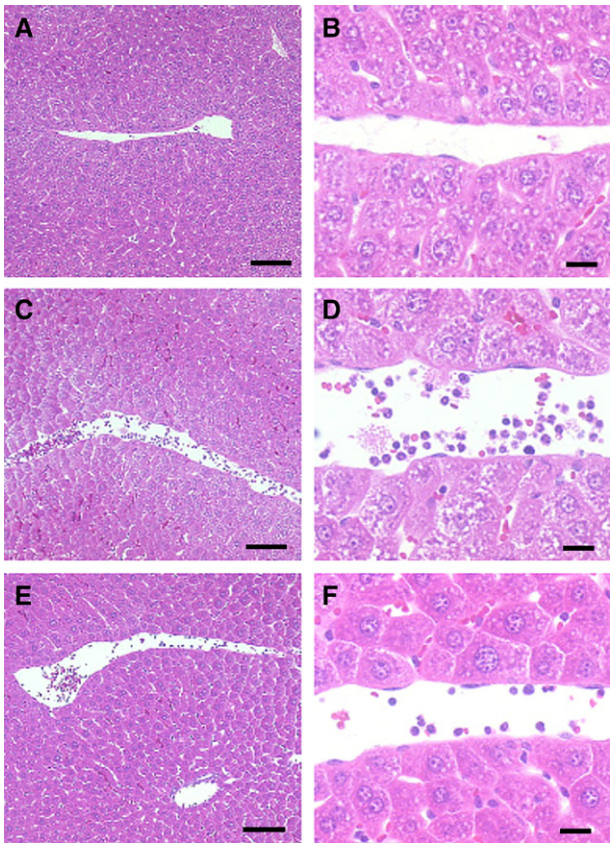

Fig. 7. Effect of UDCA on light micrographic changes of liver at 6 h after concanavalin A injection. The liver samples were obtained 6 h after concanavalin A injection from untreated (A, B); concanavalin A-injected (C, D); and concanavalin A injected with UDCA at 150 mg/kg pretreatment (E, F) (hematoxylin-eosin staining). Bars indicate 100 μm (A, C, E) and 20 μm (B, D, F).

bile acids with hydrophilic bile acid including UDCA in the liver of concanavalin A-injected mice.

Concanavalin A-induced liver injury is a well-established experimental mouse model (Tiegs et al., 1992), that is characterized by markedly increased serum levels of transaminases and simultaneous infiltration of neutrophils, macrophages, and T cells into the liver. The hepatic natural killer T cells play essential roles in concanavalin A-induced liver injury by releasing a variety of cytokines, including IL-4, IFN-γ, and TNF-α (Nicoletti et al., 2000). Furthermore, many other cytokines are involved in concanavalin A-induced liver injury: for example, IL-6 and IL-10 show hepatoprotective effects, whereas IL-12 and IL-18 aggravate hepatitis induced by concanavalin A (Sass et al., 2002; Nicoletti et al., 2000; Gantner et al., 1997; Louis et al., 1997; Fiorrucci et al., 2001). Among these cytokines, TNF-α and IFN-γ are considered to play critical roles in the development of massive hepatocellular apoptosis and necrosis, whereas IL-10 prevents liver injury through inhibition of TNF-α and IFN-γ production (Gantner et al., 1997; Louis et al., 1997). TNF-α plays a particularly important role in the early stage of concanavalin A-induced liver injury in mice, because the pretreatment with anti-TNF-α antibody markedly prevents injury, whereas treatment with anti-TNF-α antibody after concanavalin A injection shows no clear effect (Nakamura et al., 2001; Fukuda et al., 2005).

One of the main functions of TNF-α was revealed to be the up-regulation of the neutrophil-attracting CXC-chemokine, MIP-2, in concanavalin A-induced mouse liver injury. The elevated MIP-2 levels in plasma after concanavalin A injection were significantly reduced, compared to controls, by anti-TNF-α antibody (Nakamura et al., 2001). MIP-2 has been reported to be an important mediator involved in concanavalin A-induced liver injury. Pretreatment with anti-MIP-2 antibody markedly inhibited elevated ALT levels, hepatic necrosis, and infiltration of neutrophils into the liver (Nakamura et al., 2001). From these results, it is presumed that production of TNF-α induced by injection of concanavalin A stimulates MIP-2 induction and that the released MIP-2 plays a pivotal role in the progression of liver injury mediated through the recruitment of neutrophils into the liver.

In the present study, we demonstrated that pretreatment with UDCA significantly inhibited the elevation of TNF-α and MIP-2 not only in the blood but also in the livers of mice injected with concanavalin A. On the other hand, UDCA showed no clear effect on the intrahepatic levels of IFN-γ, IL-4, IL-6, or IL-10. Serum IL-6 was markedly reduced by UDCA treatment; however we believe that serum IL-6 reflects the degree of inflammation, including liver injury in the whole body, because UDCA did not reduce intrahepatic IL-6. In addition, histological examination revealed that intrasinusoidal accumulation of neutrophils was decreased markedly by UDCA. Thus, our findings suggested that UDCA prevents concanavalin A-induced liver injury through inhibiting production of intrahepatic TNF-α and MIP-2 and infiltration of neutrophils into the liver. These actions may contribute to the hepatoprotective effect of UDCA in chronic liver disease. The molecular mechanism by which production of intrahepatic TNF-α and MIP-2 is inhibited by UDCA remains unclear. However, our data indicate that UDCA directly inhibits release of TNF-α and MIP-2 from liver nonparenchymal cells co-cultured with lymph node cells (Table 3). Furthermore, the hepatoprotective effect of UDCA may be mediated partly by a glucocorticoid receptor (Tanaka et al., 1996; Miura et al., 2001).

Table 3
Effect of UDCA on concanavalin A (Con A)-induced TNF-α, MIP-2 and IL-6 release in a co-culture system of liver nonparenchymal cells (NPCs)/ lymph node cells (LNCs)

	Cells	UDCA (μM)	TNF-α (pg/ml)	MIP-2 (pg/ml)	IL-6 (pg/ml)
–	NPCs	0	11.9±1.0	138.2±10.7	78.6±7.3
Con A	NPCs	0	19.6±9.2	111.8±11.0	83.7±10.1
–	LNCs	0	4.2±1.6	0.0±0.0	1.3±0.6
Con A	LNCs	0	71.0±4.2[a]	15.6±2.5[a]	6.2±0.4[a]
–	NPCs+LNCs	0	15.8±1.5	315.1±6.3	52.6±5.2
Con A	NPCs+LNCs	0	1154.1±42.4[a]	2829.6±146.8[a]	863.1±48.1[a]
Con A	NPCs+LNCs	50	1089.8±59.1	2605.0±168.8[b]	800.5±35.1
Con A	NPCs+LNCs	100	882.2±43.7[b]	2331.6±137.4[b]	632.7±19.0[b]
Con A	NPCs+LNCs	200	810.2±29.8[b]	2111.3±132.1[b]	571.1±23.1[b]

Values are expressed as means±S.E.M., $n=8$. Nonparenchymal cells (3×10^5/well), lymph node cells (10^6/well) and nonparenchymal cells (3×10^5/well) / lymph node cells (10^6/well) co-cultures were incubated with or without concanavalin A (2 μg/ml) at 37 °C for 24 h. UDCA was pretreated 1 h before concanavalin A addition. [a]:$P<0.01$ difference from corresponding values in concanavalin A (−) (paired t-test), [b]:$P<0.01$ difference from corresponding values in UDCA 0 μM (Dunnett's multiple comparison test (2-way layout)).

In pursuit of these directions, we are performing further studies to clarify the molecular mechanisms underlying the hepatoprotective effect of UDCA.

Acknowledgements

The authors would like to thank Mr. Akihiro Fujii for the helpful advice and discussion and Ms. Naomi Takeba, Ms. Yoshie Anabuki and Ms. Yasuko Ogawa for the excellent technical assistance.

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