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Glycyrrhizin prevents of lipopolysaccharide/ D-galactosamine-induced liver injury through down-regulation of matrix metalloproteinase-9 in mice

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Abstract

Glycyrrhizin, a biological active compound isolated from the liquorice root, has been used as a treatment for chronic hepatitis. We have examined the involvement of matrix metalloproteinase (MMP)-9 in the development of lipopolysaccharide (LPS) and D-galactosamine (GalN)-induced liver injury in mice. We also investigated the effect of glycyrrhizin on expression of MMP-9 in this model. Levels of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) increased after LPS/GalN treatment. Expression of MMP-9 mRNA and protein was markedly up-regulated in liver tissues 6–8 h after LPS/GalN treatment. Pretreatment with glycyrrhizin (50 mg kg^{-1}) and the MMP inhibitor (5 mg kg^{-1}) suppressed increases in serum levels of ALT and AST in mice treated with LPS/GalN. Furthermore, glycyrrhizin inhibited levels of both mRNA and protein for MMP-9. Immunohistochemical reaction for MMP-9 was observed in macrophages/monocytes infiltrated in the inflammatory area of liver injury. Glycyrrhizin reduced the infiltration of inflammatory cells and immunoreactive MMP-9 in liver injury. The results indicated that MMP-9 played a role in the development of LPS/GalN-induced mouse liver injury, and suggested that an inhibition by glycyrrhizin of the acute liver injury may have been due to a down-regulation of MMP-9.

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Introduction

Glycyrrhizin, a saponin isolated from liquorice roots (*Glycyrrhiza* spp), has been used as a folk medicine. Various pharmacological effects of glycyrrhizin are well known, such as anti-inflammatory (Ohuchi et al 1981; Okimasu et al 1983), antiviral (Cinatl et al 2003), anti-allergic (Shin et al 2007), hepatocyte-proliferative (Kimura et al 2001) and hepatoprotective activity (Nose et al 1994; Okamoto & Kanda 1999; Okamoto 2000). Intravenous administration of glycyrrhizin decreased serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in patients with chronic hepatitis. Especially in Japan, Stronger Neo-Minophagen C (SNMC) has been used as a remedy in patients with hepatitis C (Arase et al 1997; Ikeda et al 2006), and glycyrrhizin is the main ingredient of SNMC. The effect and safety of SNMC have also been confirmed in Europe (van Rossum et al 1999a b, 2001). However, the mechanisms by which glycyrrhizin inhibits liver injury have not been clearly identified.

Intravenous injection of a combination of small doses of lipopolysaccharide (LPS) and D-galactosamine (GalN) induces acute hepatitis, and is a well-established animal model of liver injury (Tiegs et al 1989; Leist et al 1995; Sass et al 2002). This model has been used widely to examine hepatic protection of compounds (Xiong et al 1999; Itokazu et al 2000). Upon stimulation by LPS, macrophages secrete pro-inflammatory cytokines including interleukin (IL)-1, IL-6, IL-10, IL-12 and tumour necrosis factor (TNF)-α (Sass et al 2002). Among them, TNF-α is a key mediator of hepatic apoptosis and necrosis in LPS/GalN-induced liver failure (Leist et al 1995; Morikawa et al 1996; Endo et al 1999; Nowak et al 2000).

Matrix metalloproteinase (MMP), a family of zinc- and calcium-dependent proteinases, participates in degradation of extracellular matrix (Visse & Nagase 2003). Various cell types such as neutrophils (Pugin et al 1999), macrophages and hepatic stellate cells have

been demonstrated to produce MMPs (Knittel et al 1999) in liver injury. Recently, increases in MMP-2 and -9 activity in the liver have been reported to be involved in animal hepatitis models (Wielockx et al 2001; Ito et al 2005, 2006). In this study, we have examined the involvement of MMP-9 in LPS/GalN-induced mouse liver injury and the effect of glycyrrhizin on expression of MMP-9 in this model.

Materials and Methods

Experimental animals

This study was performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society and the Ethical Committee of Animal Experiments of Tsurumi University School of Dental Medicine.

Six-week-old male BALB/c mice (20–23 g; Japan SLC, Hamamatsu, Japan) were kept in an environmentally controlled room (24 ± 1°C, 55 ± 10% humidity) and allowed free access to food and water.

Materials

LPS (*Escherichia coli*, O55:B5) and GalN were purchased from Sigma (St Louis, MO). All drugs were dissolved with pyrogen-free saline. MMP-2/-9 inhibitor, 2-[(4-biphenylsulfonyl) amino]-3-phenyl-propionic acid, was purchased from Calbiochem (San Diego, CA). Recombinant mouse MMP-9 and Mac-P-L-G-L-Dpa-A-R-NH2 fluorogenic peptide substrate I were obtained from R & D Systems (Minneapolis, MN, USA). Glycyrrhizin was prepared by the Medicinal Chemistry Research Department, Minophagen Pharmaceutical Co., Ltd. The purity of glycyrrhizin used in this study was approximately 76.5%. The pH of the solution including glycyrrhizin was adjusted to 7.0–7.5 using 1 M NaOH.

LPS/GalN-induced liver injury

Liver injury was induced by intravenously injecting a mixture of 25 ng LPS and 20 mg GalN per mouse. At 0.5–8 h after LPS/D-GalN treatment, mice were anaesthetized under diethyl ether to collect blood by heart puncture. Hepatocelluar damage was evaluated by measuring serum ALT and AST using a SPOT CHEM SP-4420 analyser (ARKRAY, Kyoto, Japan). Intraperitoneal administration of glycyrrhizin and the MMP-2/-9 inhibitor was performed 30 min before LPS/GalN treatment, respectively. Control mice received saline. Effects of test compounds on liver damage were examined 8 h after LPS/GalN treatment.

RNA isolation

Livers were removed from the animals and frozen immediately in liquid nitrogen. Total RNA was extracted from frozen livers by the acid-guanidine-phenol-chloroform method using ISOGEN (Nippon Gene, Toyama, Japan).

Reverse transcription polymerase chain reaction (RT-PCR) analysis

cDNAs were reverse transcribed from total RNA using an RNA-PCR kit (Takara Biomedical, Shiga, Japan) and served as a template for PCR. The PCR reaction of MMP-9 was performed using Ex Taq (TaKaRa, Japan). The synthetic MMP-2 forward primer (5′-ACC AGA ACA CCA TCG AGA CC -3′) and reverse primer (5′-CCA TCA GCG TTC CCA TAC TT -3′) were designed to amplify a 421-bp fragment. The synthetic MMP-9 forward primer (5′-GCG CCA CCA CAG CCA ACT ATG -3′) and reverse primer (5′-TGG ATG CCG TCT ATG TCG TCT TTA-3′) were designed to amplify a 379-bp fragment (Vaillant et al 2001). The PCR profile was as follows: denaturation at 94°C for 30 s, re-annealing at 60°C for 30 s, extension at 72°C for 30 s, total 30 or 32 cycles. Murine glyceraldehyde-3-phospate dehydrogenase (GAPDH) primer pair used the forward primer (5′-ACC ACA GTC CAT GCC ATC AC-3′) and the reverse primer (5′-CCA CCA CCC TGT TGC TGT AG-3′). This pair of primers amplified PCR products of 451 bp. PCR products were electrophoresed on 2% agarose gel and stained with SYBR gold (Molecular Probes, CA). These oligonucleotides were synthesized by Hokkaido System Science (Hokkaido, Japan). No PCR product was amplified without reverse transcription reaction. The fluorescence intensity of the band was measured with ATTO Lane and Spot analyser ver.6 (ATTO, Tokyo, Japan).

Measurement of proMMP-9 in the liver

Livers were removed from the animal, frozen in liquid nitrogen and stored at –80°C until assay. Liver tissues were homogenized in 50 mM Tris-HCl containing 1 mM monothioglycerol. The homogenized samples were centrifuged at 2000 *g* for 10 min. Protein concentrations were measured by DC protein assay reagents (Bio Rad Laboratories, CA, USA). The amount of proMMP-9 in liver was measured by a commercially available ELISA kit (R&D Systems, MN, USA).

MMP-9 enzymatic activity in-vitro

Recombinant mouse MMP-9 (20 ng) was activated by incubation of 1 mM APMA (p-aminophenylmercuric acetate) at 37°C for 2 h. Activated MMP-9 was incubated with 10 μM substrate (Mac-P-L-G-L-Dpa-A-R-NH2 fluorogenic peptide substrate I) in 100 μL TCNB (50 mM Tris-HCl, pH 7.5, 10 mM CCl_2, 150 mM NaCl, 0.05% Brij 35) at room temperature for 60 min. Test compounds were pre-incubated with enzyme for 5 min. Enzymatic activity was measured by a fluorescence plate reader with excitation at 320 nm and emission at 405 nm.

Immunohistochemistry for MMP-9

Under ether narcosis, the mouse livers were perfused through a portal vein with 0.1 M phosphate-buffered saline (PBS; pH 7.4) followed by 0.1 M phosphate-buffered (pH 7.4) 4% paraformaldehyde. Serial cryostat sections were cut at 6-μm thickness and maintained at –20°C on slides coated with gelatin

until used for immunohistochemistry. Anti-MMP-9 polyclonal antibody (M-17; Santa Cruz Biotechnology, CA, USA) was used for detection of MMP-9. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in 100% methanol. Liver sections were immersed in 20% normal rabbit serum in PBS for 30 min to block non-specific binding. Primary antibody was added at an appropriate dilution (1:500) in 1% bovine serum albumin (BSA; Sigma, MO, USA) in PBS with 0.03% Triton X-100 (Wako, Osaka, Japan) and incubated overnight at room temperature. The sections were then incubated in biotinylated bovine anti-goat Ig (1:400; Santa Cruz Biotechnology) diluted in 1% BSA in PBS for 30 min at room temperature. This was followed by a further 30-min incubation at room temperature with peroxidase-conjugated streptavidin (Dako, Copenhagen, Denmark) diluted 1:300. Sections were washed in PBS between each step. The immunoreaction was visualized using 0.025% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) and 0.01% hydrogen peroxide in 0.05 M Tris-HCl buffer (pH 7.3). The sections were counterstained with haematoxylin before mounting. No specific immunoreaction was detected in control sections without primary antibody.

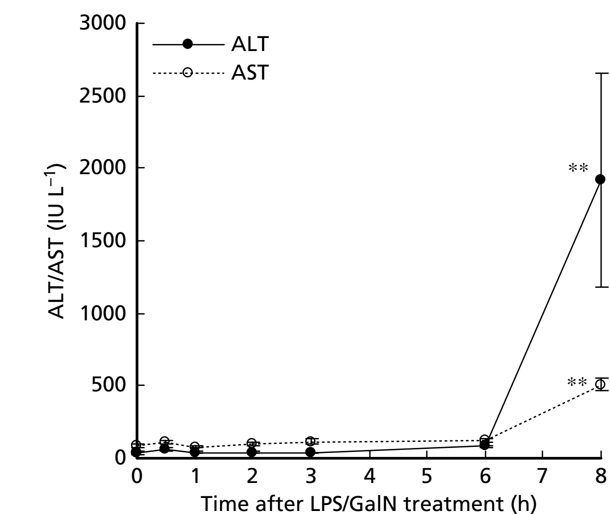

Figure 1 Serum levels of ALT and AST at various times after LPS/GalN treatment. Each point represents the mean ± s.e.m. of five to six animals. $^{**}P < 0.01$ compared with the value for untreated mice (0 h).

Statistical analysis

Data are shown as the mean ± standard error of mean (s.e.m.). The Grubbs and Smirnov methods were used to exclude outliers, and statistical significance of differences between the LPS/GalN-treated group and test group was evaluated by the Tukey-Kramer multiple comparison test after one-way analysis of variance. Differences were considered to be significant for *P* values less than 0.05.

Results

Changes in serum ALT and AST activity in LPS/GalN-induced liver injury

Serum ALT and AST activity did not change markedly for up to 6 h after intravenous treatment with LPS/GalN. However, significant increases in ALT and AST levels were observed at 8 h (Figure 1). Thereafter, increased ALT and AST levels were reduced with time after treatment (data not shown).

Expression of MMP-9 mRNA and proMMP-9 in the liver of mice treated with LPS/GalN

Presence of mRNA for MMP-9 was confirmed in control liver. MMP-9 mRNA was up-regulated following treatment of mice with LPS/GalN. Significant increases in levels of mRNA for MMP-9 were observed 6 and 8 h after LPS/GalN treatment (Figure 2A). However, levels of MMP-2 mRNA were not increased in the lives of mice treated with LPS/GalN (data not shown). Thus, it seemed unlikely that MMP-2 played an important role in the development of liver injury evoked by LPS/GalN in mice.

Production of proMMP-9 slightly increased up to 3 h but significantly up-regulated 6 and 8 h after LPS/GalN treatment (Figure 2B). This suggested that LPS/GalN induced the production of proMMP-9 in the liver. However, serum levels of MMP-9 were under the detection limit (data not shown).

Effect of MMP inhibitor and glycyrrhizin on LPS/GalN-induced liver injury

We examined whether MMP-9 activity was involved in the increased ALT and AST levels using the MMP-2/-9 inhibitor in this model. Pretreatment with the MMP inhibitor dose-dependently decreased ALT and AST levels in the serum at 8 h after LPS/GalN treatment (Table 1). ALT and AST values in control mice were 78.8 ± 7.0 and 15.8 ± 2.9 IU L^{-1}, respectively, whereas those in LPS/GalN-treated mice were 11547.0 ± 2138.0 and 8903.3 ± 1293.1 IU L^{-1}, respectively. The MMP inhibitor at 5 mg kg^{-1} significantly suppressed the increased levels in ALT and AST by more than 90%. To investigate the effect of glycyrrhizin on liver injury induced by LPS/Ga1N, glycyrrhizin (50 mg kg^{-1}, i.p.) was administered to mice 30 min before LPS/GalN treatment. Glycyrrhizin significantly inhibited increased ALT and AST levels (Figure 3). The ED50 value of glycyrrhizin for the LPS/GalN-induced liver injury was 14.3 mg kg^{-1} (Yoshida et al 2007).

Effect of glycyrrhizin on expression of mRNA and protein for MMP-9 in the liver of mice treated with LPS/GalN

We examined whether glycyrrhizin (50 mg kg^{-1}, i.p.) had an effect on the expression of mRNA for MMP-9 in the liver injury. At 8 h after LPS/GalN treatment, expression of MMP-9 mRNA was markedly induced. Pretreatment with glycyrrhizin resulted in a significant inhibition of mRNA level for MMP-9 in LPS/GalN-treated liver tissue (Figure 4A). Furthermore, glycyrrhizin significantly decreased the level of proMMP-9 in

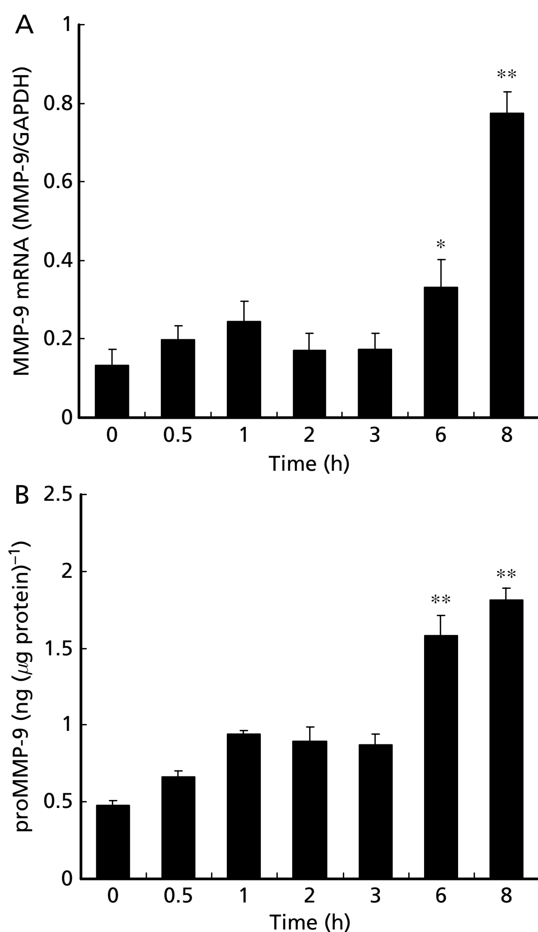

Figure 2 Time-course of (A) MMP-9 mRNA expression and (B) proMMP-9 production (A) in the liver of mice treated with LPS/GalN. Total RNA was isolated from liver at various times after treatment with LPS/GalN. The intensity of the MMP-9 bands is shown relative to that of the GAPDH band (mean ± s.e.m.; n = 5). Livers were removed from the animal at various times after treatment with LPS/GalN. Each bar represents mean ± s.e.m. of five to six animals . $*P < 0.05$, $**P < 0.01$ compared with the value for the untreated mice (0 h).

Table 1 Effect of MMP-2/-9 dual inhibitor on LPS/GalN-induced liver injury

Dose (mg kg^{-1})	Inhibition (%)	
	ALT	AST
0.5	35.4 ± 24.7	52.6 ± 11.4
1	53.9 ± 16.4	46.1 ± 20.6
5	92.0 ± 1.5**	92.6 ± 1.7**

Test compound was administered 30min before LPS/GalN treatment. Serum ALT and AST were examined 8h after LPS/GalN treatment. Data are expressed as mean ± s.e.m. of five to six animals. $**P$ 0.01 compared with the value for LPS/GalN-treated mice.

the liver injury (Figure 4B). However, glycyrrhizin had no direct effect on the enzymatic activity of recombinant mouse MMP-9, even at a high concentration of 10^{-3} M (data not shown).

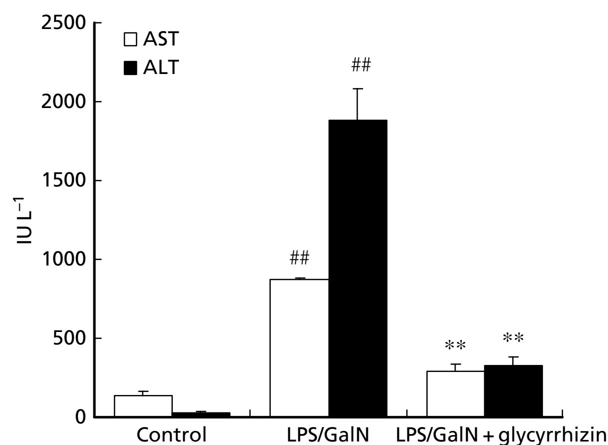

Figure 3 Effect of glycyrrhizin on LPS/GalN-induced liver injury. Glycyrrhizin (50 mg kg^{-1}, i.p.) was administered 30 min before LPS/GalN treatment. ALT and AST activity were measured at 8 h after LPS/GalN treatment. Each bar represents mean ± s.e.m. of five mice. $^{\#\#}P < 0.01$ compared with the value for control mice. $**P < 0.01$ compared with the value for LPS/GalN-treated mice.

Histopathological effect of glycyrrhizin

Immunoreactive MMP-9 was predominantly detected in the pericentral inflammatory area containing many macrophages and neutrophils at 8 h after LPS/GalN treatment (Figure 5B), whereas no reaction was observed in the liver of non-treated mouse (Figure 5B). The localization of the MMP-9-immunostaining was consistent with that of macrophages immunolabelled with F4/80, a specific marker of macrophages (data not shown). The MMP-9-immunoreaction was remarkably reduced in the LPS/GalN-injured liver when administration of glycyrrhizin (50 mg kg^{-1}, i.p.) was performed 30 min before LPS/GalN treatment (Figure 5C).

Discussion

We have confirmed that intravenous injection of LPS/GalN can cause acute hepatitis in mice. This animal model takes advantage of the ability of the transcriptional inhibitor, GalN, to potentiate the toxic effect of LPS producing typical apoptosis of hepatocytes followed by fulminant hepatitis (Leist et al 1995). Liver macrophages (Kupffer cells) stimulated by LPS secrete various pro-inflammatory cytokines including TNF-α (Sass et al 2002). TNF-α can induce apoptosis of hepatocytes at an early stage in LPS/GalN-induced liver injury, and neutrophil transmigration can represent a critical step leading to necrosis of hepatocytes at a later stage (Tiegs 1994; Chosay et al 1997). In fact, glycyrrhizin inhibits LPS/GalN-induced liver injury but has no effect on increases in serum TNF-α level in mice (Yoshida et al 2007). However, glycyrrhizin can inhibit apoptosis in the injured hepatocytes of the centrilobular area, though it failed to prevent caspases 3 and 8 in LPS/GalN-induced liver injury (Ikeda et al unpublished data).

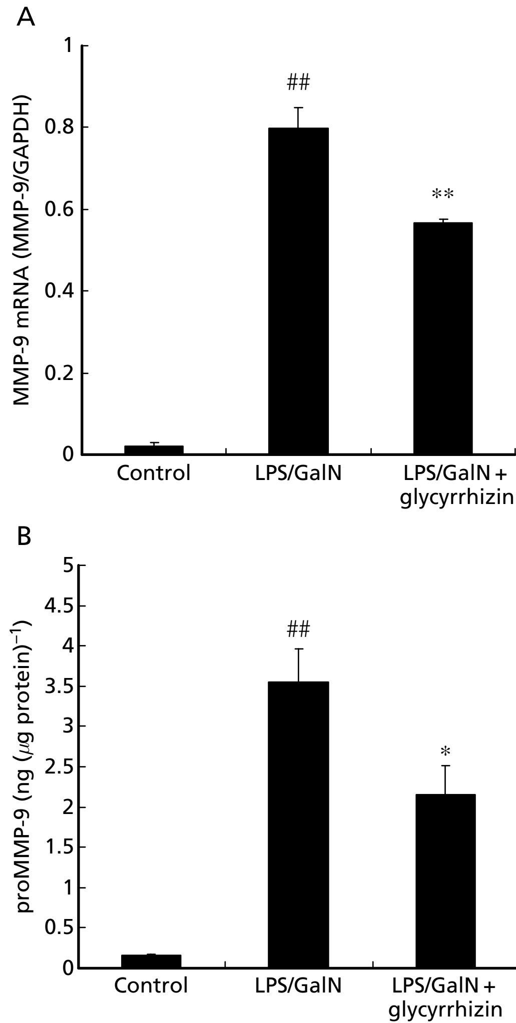

Figure 4 Effect of glycyrrhizin on (A) MMP-9 mRNA expression and (B) proMMP-9 production in the liver of mice treated with LPS/GalN. Glycyrrhizin (50 mg kg^{-1}, i.p.) was given 30 min before LPS/GalN treatment. Total RNA was isolated from livers 8 h after LPS/GalN treatment. The intensity of MMP-9 bands is shown relative to that of the GAPDH band (mean ± s.e.m.; n = 5). Levels of proMMP-9 were determined in liver 8 h after LPS/GalN-treatment. Each par represents mean ± s.e.m. of five mice. ##$P < 0.01$ compared with the value for control mice. *$P < 0.05$, **$P < 0.01$ compared with the value for LPS/GalN-treated mice.

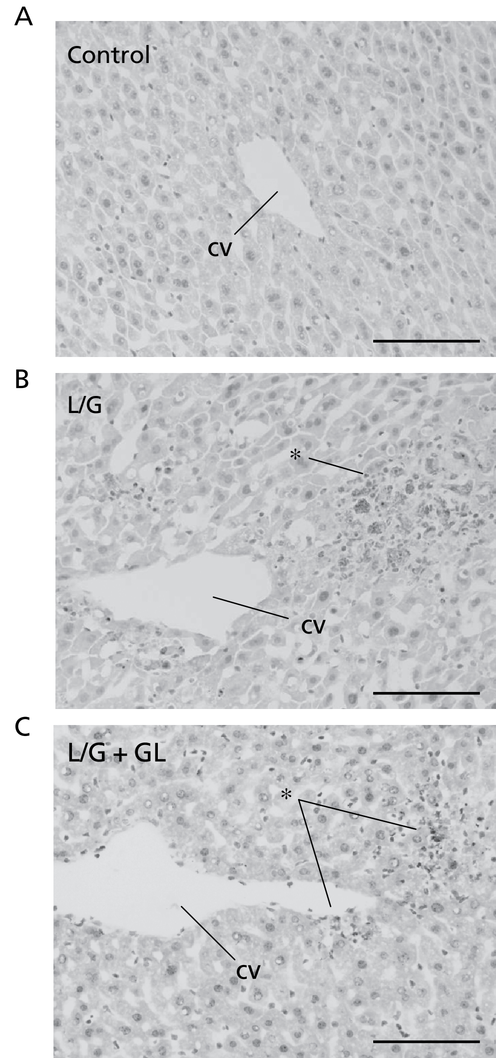

Figure 5 Immunohistochemical staining for MMP-9 expression in liver injury induced by LPS/GalN. MMP-9 immunoreactive cells were localized in the pericentral inflammatory area 8 h after LPS/GalN treatment (* in B), whereas any MMP-9 immunoreactivity was not detected in the control liver (A). Glycyrrhizin (50 mg kg^{-1}, i.p.) reduced an inflammatory area and suppressed immunoexpression (* in C) for MMP-9 in LPS/GalN-injured liver. L/G, LPS/GalN; GL, glycyrrhizin; CV, central vein. Bars = 100 μm for A–C.

In this study, expression of mRNA and protein for MMP-9 in mouse liver tissue was induced by LPS/GalN treatment. There is evidence for the involvement of MMPs in animal hepatitis models (Wielockx et al 2001). Others have suggested that MMP-2 and -9 participate in the paracetamol-induced hepatotoxicity mediated by sinusoidal endothelial cell injury, which results in impairment of microcirculation (Ito et al 2005, 2006). It is known that MMPs cleave the extracellular matrix, leading to disintergration of the tissue integrity and infiltration of neutrophils and macrophages (Wielockx et al 2001). Levels of mRNA and protein for MMP-9 in liver tissue, as well as serum levels of ALT and AST, began to increase 6 h after LPS/GalN treatment, suggesting that MMP-9 was involved in the development of LPS/GalN-induced liver injury. This was supported by our finding that the MMP-2 and -9 dual inhibitor inhibited increases in serum levels of ALT and AST.

Wielockx et al (2001) reported that a major source of MMP-9 was the infiltrating cells, including neutrophils and macrophages, in TNF-α/GalN-induced hepatitis. This report was consistent with our immunohistochemical evidence showing that hepatic macrophages/monocytes and infiltrated cells including neutrophils may have predominantly produced MMP-9 in the LPS/GalN-induced liver injury. Production of MMPs is regulated by growth factor and cytokine in liver (Knittel et al 1999). MMPs and their specific inhibitors, tissue inhibitor of matrix metalloproteinases (TIMPs), play essential roles in liver injury (Pagenstecher et al 2000). Although levels

of TIMP-1 mRNA, but not TIMP-2 mRNA, were induced in the liver of mice treated with LPS/GalN, glycyrrhizin had no effect on the expression of TIMP-1 (data not shown). Glycyrrhizin has been reported to prevent anti-Fas antibody- and concanavalin A-induced mouse liver injury without affecting cytokine expression (Okamoto & Kanda 1999; Okamoto 2000). Furthermore, it has been proved that glycyrrhizin protects the liver from various kinds of injury, such as ischaemia-reperfusion and hepatotoxic chemicals (Shibayama 1989; Nagai et al 1991). Glycyrrhizin also prevented lysis of hepatocyte membranes induced by anti-liver cell membrane antibody through the inhibition of elevated phospholipase A_2 activity (Shiki et al 1992). These reports indicate that glycyrrhizin is a hepatoprotective agent.

Others have reported that IL-18 is involved in the pathogenesis of acute liver injury in mice (Sakano et al 1999) and man (Yumoto et al 2002). In fact, IL-18 can stimulate gene expression and the synthesis of TNF-α, IL-1, Fas ligand and several chemokines (Nakanishi et al 2001). Glycyrrhizin modulates mouse liver injury evoked by LPS/GalN through the inhibition of increased serum IL-18 (Yoshida et al 2007). Thus, it seems that glycyrrhizin has various mechanisms of liver protection against acute liver injury including chemical compound- and drug-induced hepatitis.

In this study, glycyrrhizin inhibited expression of mRNA encoding MMP-9 in the LPS/GalN-induced liver injury. Moreover, glycyrrhizin reduced inflammatory cells staining for MMP-9 in the liver injury. Therefore, it was conceivable that glycyrrhizin inhibited the production of MMP-9 through the prevention of the signalling pathway leading to the expression of mRNA for MMP-9 in the process of liver injury. This study also suggested that an inhibitory effect of glycyrrhizin on the liver injury may have been due to the prevention of MMP-9 production in the development of acute hepatitis.

Conclusion

The results indicated that the production of MMP-9 played a role in the development of LPS/GalN-induced mouse liver injury, and suggested that a hepatoprotective effect of glycyrrhizin may have been due to a down-regulation of MMP-9 in infiltrated cells of the liver.

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