The inhibition of apoptosis by glycyrrhizin in hepatic injury induced by injection of lipopolysaccharide / d-galactosamine in mice*

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Summary. The inhibition of apoptosis by glycyrrhizin (GL) in hepatic injury induced by injection of lipopolysaccharide (LPS)/d-galactosamine (D-GalN) was examined in the present study. Morphological and biochemical analyses of LPS/D-GalN-induced mouse liver injury revealed that apoptosis occurred exclusively in injured hepatocytes of the centrilobular area. The degree of hepatic injury was associated with a substantial number of hepatocytes undergoing apoptosis. Transaminase levels were significantly increased at 6 to 8 h after the injection of LPS/D-GalN compared with controls. GL inhibited the elevation of serum transaminase levels when it was given to mice at 30 min before the administration of LPS/D-GalN. Morphological analyses using the TUNEL-method showed GL significantly reduced the number of TUNEL-labeled cells in acute hepatitis induced with LPS/D-GalN-treatment. Cells from the pericentral hepatic injury region were dissected out using a microdissection-method, and the DNA-ladder was clearly documented. Furthermore, results obtained through the TUNEL-method were confirmed with an oligonucleosome-bound DNA ELISA. From the current results, it seems reasonable to conclude that the protective role of GL in LPS/D-GalN-induced liver injury is performed through the inhibition of hepatic apoptosis.

Introduction

The well-defined model of hepatic injury induced by the injection of lipopolysaccharide (LPS)/d-galactosamine (D-GalN) has been widely used in studies of the mechanisms of human hepatitis. D-galactosamine is an aminosugar selectively metabolized by hepatocytes, which induces a depletion of the uridine triphosphate pool and thereby an inhibition of macromolecule (RNA, protein and glycogen) synthesis in the liver (Decker et al., 1974). The combination of LPS and d-GalN specifically causes hepatic failure in rodents (Galanos et al., 1979). Under stimulation by LPS, liver macrophages secrete various pro-inflammatory cytokines including the tumor necrosis factor (TNF)-α — which is a terminal mediator for apoptosis, subsequently leading to hepatic necrosis (Tiegs et al., 1989; Leist et al., 1995; Sass et al., 2002). The hepatic lesion in this model resembles that of human
hepatitis since the up-regulation of the TNF-α level and hepatic apoptosis have been reported as pathogenic symptoms in human hepatitis. Glycyrrhizin (GL), an aqueous extract of licorice root, has been used for the treatment of chronic hepatitis to reduce liver inflammation (Takahara et al., 1994; Ito et al., 1997; Yoshikawa et al., 1997), but its effects on acute hepatic injury are unclear. A recent report showed that Y-40138, a synthetic compound, inhibits liver injury evoked by LPS/D-GalN through the suppression of TNF-α and monocyte chemoattractant protein-1 and the augmentation of IL-10 (Fukuda et al., 2006). GL prevents anti-Fas antibody-induced mouse liver injury but has no effect on the upregulation of TNF-α mRNA expression in the liver (Okamoto, 2000). In a previous paper (Yoshida et al., 2007), we reported that levels in the serum of cytokines such as TNF-α, interleukin (IL)-6, IL-10, IL-12 and IL-18 as well as those of serum alanine aminotransferase (ALT) significantly increased after administration of LPS/D-GalN. GL had no effect on the production of TNF-α, IL-6, IL-10, or IL-12, whereas it significantly inhibited the increase in ALT levels and IL-18 production. Our previous results indicate that the inhibitory effect of GL differs from that of inhibitors for TNF-α production, such as Y-40138 (Fukuda et al., 2006) and bicyclol, a new synthetic anti-hepatitis drug (Wang et al., 2006).

IL-18 was originally identified as an interferon (IFN)-γ-inducing factor (Okamura et al., 1995) and has been suggested to be a potent inflammatory cytokine that regulates autoimmune and inflammatory diseases (Nakanishi et al., 2001; Dinarello et al., 2003; Gracie et al., 2003). Furthermore, IL-18 is produced by Kupffer cells (liver macrophages), B cells, and dendritic cells on LPS stimulation (Ghayur et al., 1997). There is evidence that IL-18 plays a critical role in the pathogenesis of acute hepatic injury (Okamura et al., 1995; Sakao et al., 1999). IL-18 stimulates gene expression and the synthesis of TNF-α, IL-1, the Fas ligand, and several chemokines (Dinarello, 1999; Nakanishi et al., 2001; Nakahira et al., 2002). IL-18 stimulation activates the DNA-binding activity of both transcription factors, NF-κB and the activator protein (AP)-1 (Shapiro et al., 1998; Kanakaraj et al., 1999; Nakahira et al., 2002). IL-18 deficient mice are resistant to LPS-induced liver injury (Sakao et al., 1999). In humans, IL-18 is involved in the pathogenesis of acute hepatic injury (Yumoto et al., 2002) and of chronic hepatitis C (Jia et al., 2002; Ludwiczek et al., 2002). Treatment with interferon and ribavirin induces a significant decrease in serum IL-18 concentration (Marin-Serrano et al., 2006). However, questions have remained as to whether or not IL-18 participates in the LPS/D-GalN-induced mouse liver injury.

In the present investigation, we mainly examine the mouse hepatic injury 8 h after an injection of LPS/D-GalN, because levels in the serum of cytokines such as TNF-α, IL-6, IL-10, and IL-12 reached a maximum by 2 h after administration of LPS/D-GalN, and those of IL-18 and serum alanine aminotransferase (ALT) peaked at 8 h in the previous study (Yoshida et al., 2007). Findings show that GL might inhibit the LPS/D-GalN-induced liver injury through the prevention of hepatocyte apoptosis. Thus, our objective is to focus on a quantitative analysis of the concomitant administration of GL in comparison with LPS/D-GalN treatment alone and to evaluate the protective role of GL in acute hepatitis induced by an injection of LPS/D-GalN.

**Materials and Methods**

**Animals and materials**

Male mice of the Balb/c strain weighing 23–25 g and aged 6 weeks were purchased from Japan SLC (Hamamatsu). The animals were kept in an environmentally controlled room (24 ± 1°C, 55 ± 10% humidity) and their handling conformed to the guidelines for the care and use of experimental animals established by the Japanese Pharmacological Society and the Ethical Committee of Animal Experiments of Tsurumi University School of Dental Medicine. Lipopolysaccharide (LPS; Escherichia coli, O55:B5) and D-galactosamine (D-GalN) were purchased from Sigma (St. Louis, MO, USA). All drugs were dissolved with pyrogen-free saline. Glycyrrhizin (GL) was obtained from the Medical Chemistry Research Department of Minophagen Pharmaceutical CO., LTD. The Balb/c mice were intravenously injected with 25 ng LPS and 20 mg D-GalN per mouse. At 0.5 to 8 h after LPS/D-GalN treatment, mice were anesthetized with the diethyl ether to collect blood from a heart puncture. The intraperitoneal administration of GL (50 mg/kg body weight) was performed 30 min before LPS/D-GalN treatment, with control mice receiving saline. The effects of GL on liver damage were examined at 8 h after LPS/D-GalN treatment.

**Analysis of liver enzymes and released proteins**

Hepatocellular damage was evaluated by measuring serum alanine aminotransferase (ALT) and asparagine aminotransferase (AST) using a SPOT CHEM SP-4420 analyzer (ARKRAY, Kyoto). Serum TNF-α was measured by ELISA using commercially available kits (Biosource, CA, USA) following the manufacturer's
The tissue sections were deparaffinized and treated with 2% H2O2 for 5 min to block any endogenous peroxidase activity. The sections were then incubated with 0.3 U/ml terminal deoxynucleotidyl transferase (Invitrogen, Carlsbad, USA) and 0.04 nM biotinylated deoxyuridine triphosphate (Roche) in a terminal deoxynucleotidyl transferase buffer (Invitrogen, South San Francisco, USA) for 2 h at 37°C. After rinsing in distilled water, the sections were incubated in 2% bovine serum albumin (BSA: Sigma) in 0.1M phosphate-buffered saline (PBS) (pH 7.4) for 10 min and then incubated with peroxidase-conjugated streptavidin (DAKO, Copenhagen, Denmark) diluted 1:300 with PBS for 30 min. Peroxidase activity was visualized with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) in a 0.05M Tris-HCl buffer (pH 7.4) containing 0.01% CoCl2 and 0.01% H2O2 for 10 min. All steps were carried out at room temperature unless otherwise indicated.

**Light microscopy**

Some animals were perfused through their portal veins with 0.1M phosphate buffered (pH 7.4) 4% paraformaldehyde under deep narcosis. Small portions of the perfused-liver were immersed in the same fixative liquid for 4–6 h at 4°C and frozen in O.C.T. compound (Sakura Finetchnical, Tokyo) after rinsing with 15-30% sucrose in a 0.1M phosphate buffer (pH 7.4). Other portions of the specimens were dehydrated and embedded in paraffin wax. Serial cryostat or paraffin sections (6 μm in thickness) were cut and maintained at –20°C on slides coated with gelatin-chrome alum. Some sections were stained with hematoxylin and eosin (HE). Cells from pericentral regions of the hepatic injury were dissected out using the PALM MicroBeam system (P.A.L.M. Microlaser Technologies AG, Tokyo, Carl Zeiss Japan). This system allows for the non-contact capture of large cell areas, small cell cultures, and single cells. Basically, the whole procedure consists of two stages—laser microbeam microdissection followed by laser pressure catapulting. Caps briefly placed onto a section without laser activation were used as negative controls. Genomic DNA from laser microdissected liver tissues was purified by using the QIAamp DNA micro kit (QIAGEN, Hilden, Germany) according to the manufacture’s protocol with a few modifications. Apoptosis was measured using a PCR Kit for DNA Ladder Assay designed for the detection of nucleosomal ladders in apoptotic cells (Maxim Biotech, Inc., San Francisco, USA), according to the manufacture’s instructions with some modifications. Briefly, DNA was obtained from purified genomic DNA using proteinase-K. Dephosphorylated adaptors were ligated to 5’-phosphorylated blunt ends with T4DNA ligase to liver sample DNA. PCR products were electrophoresed on a 1.7% agarose gel. The bands were visualized using the Gel Doc EQ system (Bio-Rad, Hercules, CA, USA).

**TUNEL staining**

The nuclear DNA fragmentation of apoptotic cells was labeled in situ by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling (TUNEL) method (Sasaki et al., 2001). The tissue sections were deparaffinized and treated with 20 mg/ml proteinase K (Roche Diagnostics GmbH, Mannheim, Germany) in a 0.1M Tris-HCl buffer (pH 7.4) for 15 min. After rinsing with distilled water, the sections were treated in 2% H2O2 for 5 min to block any endogenous peroxidase activity. The sections were then washed with distilled water and incubated with 0.3 U/ml terminal deoxynucleotidyl transferase (Invitrogen, Carlsbad, USA) and 0.04 nM biotinylated deoxyuridine triphosphate (Roche) in a terminal deoxynucleotidyl transferase buffer (Invitrogen) for 2 h at 37°C. After rinsing in distilled water, the sections were incubated in 2% bovine serum albumin (BSA: Sigma) in 0.1M phosphate-buffered saline (PBS) (pH 7.4) for 10 min and then incubated with peroxidase-conjugated streptavidin (DAKO, Copenhagen, Denmark) diluted 1:300 with PBS for 30 min. Peroxidase activity was visualized with 0.05% 3,3’-diaminobenzidine tetrahydrochloride (DAB; Sigma) in a 0.05M Tris-HCl buffer (pH 7.4) containing 0.01% CoCl2 and 0.01% H2O2 for 10 min. All steps were carried out at room temperature unless otherwise indicated.

**Microdissection and DNA extraction**

Cells from pericentral regions of the hepatic injury were dissected out using the PALM MicroBeam system (P.A.L.M. Microlaser Technologies AG, Tokyo, Carl Zeiss Japan). This system allows for the non-contact capture of large cell areas, small cell cultures, and single cells. Basiclly, the whole procedure consists of two stages—laser microbeam microdissection followed by laser pressure catapulting. Caps briefly placed onto a section without laser activation were used as negative controls. Genomic DNA from laser microdissected liver tissues was purified by using the QIAamp DNA micro kit (QIAGEN, Hilden, Germany) according to the manufacture’s protocol with a few modifications. Apoptosis was measured using a PCR Kit for DNA Ladder Assay designed for the detection of nucleosomal ladders in apoptotic cells (Maxim Biotech, Inc., San Francisco, USA), according to the manufacture’s instructions with some modifications. Briefly, DNA was obtained from purified genomic DNA using proteinase-K. Dephosphorylated adaptors were ligated to 5’-phosphorylated blunt ends with T4DNA ligase to liver sample DNA. PCR products were electrophoresed on a 1.7% agarose gel. The bands were visualized using the Gel Doc EQ system (Bio-Rad, Hercules, CA, USA).

**Quantitative analysis of DNA fragmentation**

The livers collected after the measurement of serum levels were washed with PBS and maintained at –80°C until a quantitative analysis of DNA fragmentation. A cell death detection ELISA kit (Roche Diagnostics GmbH) was used according to the manufacture’s protocol adapted for the use of tissue fragments. Briefly, small pieces (1mg) from hepatic tissues were lysed using the provided lysis buffer, incubated for 30 min at room temperature, and—with a 10 min centrifugation at 1,500 rpm—20 μl of the supernatant as a sample was submitted to the ELISA test. Absorbance was measured at 405 nm using Multiskan (Thermo ELECTRON, Vantaa, Finland). DNA fragmentation was expressed as a relative percentage in comparison with that of control animals.

**Caspase-1, -3 and -8 activity assay**

At 0, 0.5, 1, 2, 3, 6, and 8 h after the injection of LPS/β-GalN, mice were sacrificed for the removal of liver tissue to examine caspase-1, -3 and -8 activities. The effect of GL on caspase activities was examined at 8 h after the administration of LPS/β-GalN. Mouse livers were homogenized in a lysis buffer containing 25 mM HEPES-KOH (pH 7.5), 5 mM MgCl2, 1 mM EGTA, and 1 mM PMSF. The homogenates were centrifuged at 15,000 rpm for 20 min at 4°C. The supernate was assayed
for caspase-1, -3 and -8 activities. The extracted lysates were incubated with acetyl-YVAD-pNA for caspase-1, acetyl-DEVD-pNA for caspase-3, and acetyl-IETD-pNA for caspase-8. After 90 min incubation at 37°C, caspase-1, -3 and -8 activity-dependent cleavages of pNA were monitored at 405 nm by a microtitre plate reader.

**Immunohistochemistry**

For immunohistochemical identification of the mouse IL-18 (MBL) and F4/80 (macrophages; Serotec, Kidlington, UK), the streptavidin-biotin-peroxidase complex (SAB) method was applied (Sato et al., 1998). Specific cellular populations were stained with rat monoclonal antibodies (mAbs) that recognize IL-18 (Okamura et al., 1995) or a cytoplasmic antigen (F4/80) in monocyte-derived macrophages and Kupffer cells (Austyn et al., 1981; Naito et al., 1991). Sections to be stained with mAbs were blocked with 20% normal goat serum in phosphate-buffered saline (PBS) for 30 min. Primary antibodies were then used at an appropriate dilution of mAb (1:500) in PBS with 1% BSA (Sigma) and 0.03% Triton X-100 (Wako, Osaka) overnight at room temperature. Sections were washed in PBS without Triton X-100 (3 × 20 min). The sections were incubated (30 min, room temperature) in biotinylated goat anti-rat Ig (1:10,000; Caltag Lab, Burlingame, CA, USA), diluted in 1% BSA in PBS, and then incubated (30 min, room temperature) with peroxidase-conjugated streptavidin (Dako), diluted 1:300. Endogenous peroxidase was blocked with hydrogen peroxide (0.3%) in 100% methanol. The immunoreaction was visualized using 0.025% DAB (Sigma) and 0.01% hydrogen peroxide in a 0.05M Tris-HCl buffer (pH 7.3) for 10 min. After being counterstained with hematoxylin, slides were dehydrated in a graded ethanol series and mounted in Permount®. The specificity of the immunoreactive staining was controlled by the incubation of adjacent sections with normal rat serum matched for protein concentration, in place of the primary antibodies.

**Quantification of F4/80-immunoreactive macrophages and TUNEL-positive cells**

We counted TUNEL-positive cells and F4/80-immunoreactive macrophages on clearly stained cryostat sections at the light-microscopic level. Hepatic lobules were divided into two areas: the pericentral zone located within 120 μm from the walls of central veins, and the periportal zone, which encompassed an area 120 μm from the outer edge of Glisson’s sheath. Four to five mouse livers were examined, and the number of labeled cells was determined in one to six fields per liver. The volume of the area was measured by an Image Processor and Analyzer (TRI/2 D-MES; RATOC, Tokyo), and cellular density was expressed as the cell number per square millimeter.

**Statistical Analysis**

The analysis was performed with a commercially available statistical package (Dr. SPSS II; SPSS Japan Inc., Tokyo). Data were shown as the means ± standard error.
Fig. 2. Light microscopic photographs of the liver tissues of controls (cont; a, b), LPS/D-GalN-treatment (L/G; c, d), and combined treatment with LPS/D-GalN+GL (L/G+GL; e, f), stained with hematoxylin and eosin. Inflammatory changes are scarcely found in the periportal area of all livers examined (b, d, f). In the liver section of an L/G-treated mouse (c), the injured structural change is observed in the centrilobular area. A higher magnification of the black rectangle in Figure c reveals intensely hematoxylin-stained features of chromatin condensation (arrow). No damaged hepatocytes are identified in the control (a, b). Such inflammatory foci in the pericentral area are remarkably inhibited by the intraperitoneal administration of GL (e). CV: central vein, PV: portal vein. Bars = 100 μm (a–f), 20 μm (inset)
Fig. 3. TUNEL-staining for the liver tissue of controls (cont; a, b), for LPS/D-GalN-treatment (L/G; c, d), and for combined treatment with LPS/D-GalN+GL (L/G+GL; e, f). The nuclei of cells labeled with the TUNEL-method yield a brown color. TUNEL-positive cells are more frequently distributed in pericentral (c, e) than periportal areas (d, f) at 8 h after treatment. CV: central vein, PV: portal vein. Bars = 100 μm (a–f)
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of mean (SEM) on the serum ALT, AST and cytokine levels, and caspase activities. Statistical significance of difference between LPS/D-GalN-treated group and test group was evaluated by Student's $t$-test or Turkey's multiple comparison test. On the quantification of F4/80-immunoreactive macrophages and TUNEL-positive cells, data were expressed as the means ± SEM, and Dunnett's $t$-test or Turkey's multiple comparison test was used to evaluate statistical significance in distributional density between respective areas. A level of $P < 0.05$ was considered to be statistically significant.

Results

LPS/D-GalN-induced liver injury

In the present study, we injected LPS/D-GalN to produce an inflammatory infiltration and hepatocellular damage. Apoptosis-like changes of liver cells appeared to provide visible and morphologic processes of acute injury in the liver. In comparison with a single LPS/D-GalN-injection, the current results led us to assume a protective role for glycyrrhizin (GL) in acute hepatic injury. The intravenous administration of LPS/D-GalN to the mice transformed their liver environments into cytokine-mediated inflammatory sites. The injection of LPS/D-GalN significantly increased serum AST and ALT activities compared with saline-injected control mice 6 to 8 h after treatment (Fig. 1a). The enhancement of AST and ALT levels was significantly suppressed by an intraperitoneal administration of GL (50mg/kg, i.p.) ($P < 0.05$; Fig. 1b). A light-microscopic analysis of hepatic sections stained with HE showed that pericentral injury occurred 8 h after the injection of LPS/D-GalN, with inflammatory mononuclear cells concentrated in this area (Fig. 2c) but periportal injury considerably weakened (Fig. 2d). Furthermore, many apoptosis-like features of injured hepatocytes with condensed chromatin were observed in such inflammatory foci (inset in Fig. 2c, d), whereas hepatocytes with a condensation of chromatin were not apparently recognized in controls (Fig. 2a, b). Inflammatory changes such as the infiltration of neutrophils and macrophages appeared to be remarkably reduced by the intraperitoneal administration of GL (compare Fig. 2c with 2e). There was little difference between the periportal areas of LPS/D-GalN- and LPS/D-GalN+GL-treatment (Fig. 2d, f). These results indicate that histological findings almost parallel the changes in AST and ALT levels in the livers of mice treated with LPS/D-GalN or LPS/D-GalN+GL.

Fig. 4. Intralobular distribution of TUNEL-labeled cells in controls (cont; a), LPS/D-GalN-treatment (L/G; b) and in combined treatment with LPS/D-GalN+GL (L/G+GL; c). Dots indicate TUNEL-stained cells. They are probably not identified in the control mice (a). TUNEL-positive cells are more frequently found in the centrilobular zone than in the peripheral one at 8 h after LPS/D-GalN treatment (b). Positive cells appear to be diffusely distributed throughout the lobule after the administration of GL (c).
Apoptotic cells in liver injury induced by LPS/D-GalN-treatment

Apoptotic liver cells that showed DNA fragmentation were evaluated and their distributional patterns were examined using the TUNEL-method. As expected, few TUNEL-positive cells were observed in the liver of saline-injected control mice (Figs. 3a, b, 4a, 5). However, quantities of TUNEL-positive cells with sharply delineated masses or crescents of condensed chromatin were identified in the inflammatory areas at 8 h post-injection of LPS/D-GalN (Fig. 3c, d). The distribution of intensely hematoxylin-stained substances found in the pericentral inflammatory area was almost identical to that of the structures labeled with TUNEL-staining in the adjacent section. The number of TUNEL-positive cells was significantly increased in both pericentral and periportal areas of the LPS/D-GalN-treated liver compared with controls (Fig. 3a–d, 5). The intraperitoneal administration of GL significantly suppressed the number of TUNEL-labeled cells in pericentral areas compared with mice treated with LPS/D-GalN but not in periportal regions (Fig. 5). TUNEL-positive cells in pericentral areas were significantly higher in distributional density compared with the periportal areas in the livers of mice treated with LPS/D-GalN or LPS/D-GalN+GL, respectively (Fig. 3c–f, 5). Thus, intralobular TUNEL+ cells were more frequently found in pericentral rather than peripheral zones in both treated livers (Fig. 4b, c). These results indicate that the increase in TUNEL-labeled cells in the pericentral areas of mice treated with LPS/D-GalN is significantly inhibited by an injection of GL.

Furthermore, the DNA ladder and DNA fragmentation were measured as markers of the apoptosis of liver cells. Cells from a hepatic pericentral injury caused by the injection of LPS/D-GalN were dissected out using the PALM MicroBeam system, and a representative ladder pattern of liver oligonucleosomal DNA was demonstrated on agarose gel electrophoresis (Fig. 6). Thus, intensely hematoxylin-stained granule-like structures and TUNEL+...
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products were confirmed as apoptotic cells or bodies. This DNA ladder was effectively suppressed by an administration of GL (Fig. 6). The expression of cytosolic oligonucleosome-bound DNA within the hepatic injured area was significantly enhanced 8 h following LPS/d-GalN administration compared with that for saline-injected control mice (Table 1). A quantitative determination of DNA fragmentation revealed that the DNA fragmentation of liver cells induced by the injection of LPS/d-GalN was significantly suppressed by an administration of GL (Table 1).

**Caspase-like activity in LPS/d-GalN-induced hepatic injury**

The time-course changes showed that caspase-3 and -8 activities began to significantly increase from 2 to 3 h after LPS/d-GalN-treatment compared with the controls, whereas caspase-1 activity was not significantly increased (Fig. 7). Caspase activities peaked at 8 h after the injection of LPS/d-GalN. We examined the effect of GL on caspase-3 and -8 activities at 8 h after LPS/d-GalN-treatment. Increased caspase-3 and -8 activities enhanced by the injection of LPS/d-GalN were not significantly inhibited by administration of GL (Fig. 8a, b). These results show that GL has no direct, inhibitory effect on either caspase activity.

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**Table 1. Effect of GL on hepatic DNA fragmentation after LPS/d-GalN-treatment.**

| Treatment   | OD_{405}   | % of control |
|-------------|------------|--------------|
| cont        | 0.015 ± 0.011 | 100 ± 72    |
| L/G         | 1.414 ± 0.312 | 9639 ± 2127  |
| L/G+GL      | 0.069 ± 0.034 | 427 ± 243*   |

GL was intraperitoneally administered 30 min before LPS/d-GalN-treatment. Liver samples were taken at 8 h after treatment. Hepatic DNA fragmentation is expressed as the relative ratio (%) in comparison with control. Each value represents the mean ± SEM of 5-6 mice. *P < 0.05 compared with the value of LPS/d-GalN-treatment.

**Table 2. Effect of GL on serum cytokine release after LPS/d-GalN-treatment.**

| Treatment | TNF-α (pg/ml) | IL-18 (pg/ml) |
|-----------|---------------|---------------|
| cont      | n.d           | 100.3 ± 10.7  |
| L/G       | 415.0 ± 114.4* | 737.6 ± 157.9* |
| L/G+GL    | 464.9 ± 45.1  | 276.2 ± 63.9* |

GL was intraperitoneally administered 30 min before LPS/d-GalN-treatment. Serum TNF-α and IL-18 after treatment were measured at 1 h and 8 h after treatment, respectively. Each value represents the mean ± SEM of 5-6 mice. *P < 0.05 compared with the value of control. *P < 0.05 compared with the value of LPS/d-GalN-treatment.

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**Fig. 7.** Time-course changes of caspase-1, -3, and -8 activities after an injection of LPS/d-GalN. Caspase-3 and -8 activities begin to significantly increase from 2 or 3 h after an injection of LPS/d-GalN, while caspase-1 activity does not. Each point represents the mean ± SEM of 6 mice. *Significant difference compared with each caspase activity at 0 h (P < 0.05).
Fig. 8. Effect of GL on caspase-3 and -8 activities at 8 h after treatment of LPS/d-GalN (L/G). Caspase-3 (a) and -8 (b) activities are significantly increased after L/G-treatment (L/G) compared with the controls (cont) but the intraperitoneal administration of GL (L/G+GL) does not inhibit increases in caspase-3 and -8 activities. Each value represents the mean ± SEM of 4-6 mice. *A level of $P < 0.05$ is considered to be statistically significant.

Fig. 9. Immunostaining for IL-18 in the mouse liver of controls (cont; a), with LPS/d-GalN-treatment (L/G; b) and with a combined treatment with LPS/d-GalN+GL (L/G+GL; c). Immunoreactive products of IL-18 are intensely found in the cytoplasm of macrophages or neutrophils distributed in the injured pericentral area of the mice treated with LPS/d-GalN (b), whereas they are not recognized in the controls (a). The increased expression of IL-18 is markedly suppressed by an intraperitoneal administration of GL (c). CV: central vein. Bars = 100 μm (a–c)
Fig. 10. Immunostaining with anti-F4/80 antibody in the liver tissue of controls (cont; a, b), LPS/D-GalN-treatment (L/G; c, d), and LPS/D-GalN+GL-treatment (L/G+GL; e, f). F4/80-immunoreactive cells are more frequently observed in periportal area (b, d, f) than pericentral ones (a, c, e) in all the livers examined. In the mice treated with LPS/D-GalN (c) or LPS/D-GalN+GL (e), immunolabeled cells are concentrated in the injured region of pericentral areas. Positive cells are irregular in shape, with several long cytoplasmic processes except for cells distributed in the inflamed areas. CV: central vein, PV: portal vein. Bars = 100 μm (a–f)
LPS/d-GalN-induced cytokine release

The concomitant administration of LPS/d-GalN induced the release of cytokines containing TNF-α and IL-18 into serum (Table 2). In a previous paper (Yoshida et al., 2007), we showed that levels in the serum of cytokines such as TNF-α, IL-6, IL-10 and IL-12 reached a maximum by 2 h after treatment, while levels of IL-18 were maximal at 8 h, as was ALT activity. Furthermore, GL had no effect on the production of TNF-α, IL-6, IL-10, or IL-12, whereas it significantly inhibited IL-18 production (Yoshida et al., 2007). Thus, in the present experiment, levels of TNF-α and IL-18 in serum were measured at 1 h and 8 h after treatment, respectively. The inhibitory effect of GL on the cytokine release of TNF-α and IL-18 by LPS/d-GalN was examined as shown in Table 2. GL (50 mg/kg, i.p.) had little effect on the circulating maximal levels of TNF-α, but the compound significantly inhibited the release of IL-18. Immunohistochemistry for IL-18 revealed that immunoreaction was labeled in the cytoplasm of neutrophils and macrophages distributed densely in the hepatic pericentral area of LPS/d-GalN-treated mice (Fig. 9b) but was rare in the hepatic pericentral of GL-treated or control animals (Fig. 9a, c). These findings indicate that the administration of GL reduces the immunoreactive response to IL-18 in livers of mice treated with LPS/d-GalN.

F4/80-immunoreactive macrophages

Kupffer cells as well as monocytes/macrophages located in hepatic sinusoids of control animals showed a specific F4/80-positive immunoreactivity (Fig. 10a, b). F4/80+ macrophages were densely distributed in the pericentral damaged areas of mice 8 h after the administration of LPS/d-GalN (Fig. 10c). Immunolabeled cells were irregular in shape with several long cytoplasmic processes—except for cells situated in the inflammatory areas (Fig. 10). F4/80-immunoreactive cells were more frequently found in periportal than pericentral areas in all the livers of control animals (Fig. 10a, b), LPS/d-GalN-treated (Fig. 10c, d), and LPS/d-GalN+GL-treated mice (Fig. 10e, f). Thus, intralobular F4/80+ cells were more densely distributed in peripheral than pericentral zones in the treated and untreated livers (Fig. 11a–c). A quantitative analysis for F4/80-immunohistochemistry revealed that immunostained cells were significantly higher in their distributional density in periportal than pericentral areas in all the livers—regardless of treatment, when macrophages identified from frozen sections which were labeled by F4/80 mAb were quantified (Fig. 12). The
number of F4/80-positive cells was significantly increased in both the pericentral and periportal areas at 8 h after the administration of LPS/d-GalN compared with the controls \((P < 0.05)\) (Fig. 12). Combined treatment with LPS/d-GalN+GL showed a moderate decrease in the number of F4/80-positive cells compared with LPS/d-GalN-treatment, though this was not significant (Fig. 12).

Discussion

Transaminase levels were significantly increased at 6 to 8 h after an injection of LPS/d-GalN, but a combined treatment with glycyrrhizin (GL) significantly suppressed the elevation of serum transaminase levels. The administration of treated doses did not induce any changes in histological appearance or serum levels of liver enzymes in this study. We have performed the analysis of apoptotic cells using the TUNEL assay, which is based on the direct, specific labeling of DNA breaks in the nuclei. This method enables the visualization of the apoptotic process and identification of apoptotic cells among injured hepatocytes at the level of a single cell \textit{in situ} (Gavrieli et al., 1992; Ansari et al., 1993). The number of TUNEL-positive apoptotic cells was significantly increased in both hepatic pericentral and periportal areas of the LPS/d-GalN-treated animals compared with the controls. TUNEL-positive cells were more remarkably increased in the pericentral area than the periportal one. The intraperitoneal administration of GL significantly suppressed an increase in the number of apoptotic cells occurring in pericentral areas compared with mice treated with LPS/d-GalN, but not in periportal areas. These findings indicate that the administration of GL might inhibit the enhancement of apoptotic cells exclusively in the pericentral areas of mice treated with LPS/d-GalN. The combined treatment with LPS/d-GalN+GL did not significantly suppress an increase in the number of F4/80-positive macrophages in pericentral and periportal areas, whereas treatment with LPS/d-GalN significantly increased the number of macrophages in either area compared with the controls. This might reflect regional differences among liver cells within a protective role of GL. However, further experiments will be required to examine the effect of GL on the liver of mice depleted of monocytes/macrophages.

GL inhibited the increase in AST and ALT levels when it was given to mice at 30 min before the administration of LPS/d-GalN in the present study. Furthermore, GL can reduce an increase in ALT levels at an administration of 10 min or 60 min but not 3 h, even after LPS/d-GalN treatment (Yoshida et al., 2007). In contrast, Hase et al. (1999) reported that pretreatment with GL did not inhibit hepatic DNA fragmentation, although this compound significantly protected against serum ALT elevation. In our experiment, a representative ladder pattern of liver oligonucleosomal DNA was confirmed on agarose gel electrophoresis by dissecting cells from the hepatic injury area using the PALM MicroBeam system. The expression of this DNA ladder was effectively suppressed by an administration of GL. The amount of cytosolic oligonucleosome-bound DNA within the hepatic damaged area was significantly enhanced at 8 h following LPS/d-GalN treatment compared with saline-injected control mice. The quantitative determination of DNA fragmentation clearly demonstrated that the DNA fragmentation of liver cells was significantly increased in quantity by the injection of LPS/d-GalN, which was significantly inhibited by an administration of GL. In the experiment by Hase et al. (1999), GL was orally administered twice at 18 and 2 h before intoxication. It has been reported that an oral administration of GL did

\[\text{Fig. 12. Statistical analysis of the number of macrophages immunostained with the anti-F4/80 antibody. The number of F4/80-immunolabeled cells is significantly increased in both the pericentral and periportal areas of the mouse liver at 8 h after LPS/d-GalN-treatment (L/G) compared with the controls (cont). The increase in the number of positive macrophages is not significantly suppressed by the intraperitoneal administration of GL (L/G+GL). F4/80-positive cells are more frequently distributed in periportal rather than pericentral areas of all the livers examined. *A level of } P < 0.05 \text{ is considered to be statistically significant. Data are expressed as means ± SEM.} \]
not inhibit serum ALT elevation because of the poor absorption of GL from the intestinal tract (Yamamura et al., 1995). The discrepancy between the present results and theirs may be due to variances in the manner of administration.

In the present study, the serum levels of TNF-α were markedly increased 1 h after LPS/d-GalN treatment. Exogenous TNF-α dose-dependently enhanced IL-18 production as well as ALT activity (data not shown), suggesting that TNF-α is involved in the IL-18 production of an LPS/d-GalN-induced liver injury. Thus, it appears that TNF-α induced by LPS/d-GalN plays an important role in the pathogenesis of this model. A recent report showed that Y-40138, a synthetic compound, inhibits liver injury evoked by LPS/d-GalN through the suppression of TNF-α and monocyte chemoattractant protein-1 and the augmentation of IL-10 (Fukuda et al., 2006). According to Okamoto (2000), GL prevents anti-Fas antibody-induced mouse liver injury but has no effect on the upregulation of TNF-α mRNA expression in the liver. His findings are consistent with the present results where treatment with GL markedly reduced serum ALT- and AST-activity and inflammatory liver damage in the LPS/d-GalN-injected mice but had little effect on maximal levels of TNF-α.

DNA degradation in apoptotic cells is under the regulation of caspase-activated DNase (CAD) within the dying cell and DNase II within the lysosomes of phagocytes (Danial et al., 2004). Select members (caspase-1, -11) of a family of protease dependent on a cysteine nucleophile are involved in the specific processing of pro-inflammatory cytokines, including IL-1 and IL-18. Other effectors, such as caspase-3 and -7, are the executioners of apoptosis as processing of their substrates leads to morphological changes associated with apoptosis, including DNA degradation, chromatin condensation, and membrane blebbing (Danial et al., 2004). Importantly, activation of caspase-3 was shown to cause an apoptotic nuclear morphology, which could be blocked by a peptide inhibitor of caspase (Nicholson et al., 1995). Treatment with GL inhibits anti-Fas antibody-induced hepatitis and the induced activation of caspase-3-like protease in vivo (Okamoto, 2000). Caspase-3 is a cysteine protease that is involved in apoptosis (Nagata, 1997), and the activation of caspase-3-like protease is critically involved in the development of hepatitis induced by anti-Fas antibodies (Rodriguez et al., 1996). However, no direct effect of GL on caspase-3-like activity has been identified (Okamoto, 2000). In the present study, the injection of LPS/d-GalN significantly increased caspase-3 and -8 activities compared with the controls, but both enhanced caspase activities were not significantly inhibited by the administration of GL. Thus, GL seems to have no direct effect on either caspase activity. However, apoptotic changes in liver cells induced with LPS/d-GalN treatment were apparently inhibited by intraperitoneal administration of GL. The inability of caspase inhibitors to completely protect cells and their organelles from damage following intrinsic death signals suggests that caspase-independent death might occur (Danial et al., 2004). Apoptosis is triggered by two major apoptosis-initiating pathways which are designated as inner/intrinsic/mitochondria and endoplasmic reticulum-mediated, and outer/extrinsic/receptor-mediated (Klener et al., 2006). In mammalian cells, caspase-independent apoptotic DNA degradation has been attributed to two mitochondrial proteins—endonuclease G (endG) and apoptosis-inducing factor (AIF)—that translocate to the nucleus upon release (Sisin et al., 1999; Li et al., 2001). AIF induces nuclear condensation and large-scale DNA fragmentation and is required for apoptosis during embryoid body cavitation (Jozza et al., 2001). Precisely how AIF and endG affect DNA degradation remains to be fully understood.

The most important findings of the present study are that GL suppresses the apoptosis of liver cells induced by LPS in d-GalN-sensitized mice. GL inhibited the increase in serum levels of IL-18 in this model. However, this compound did not significantly suppress the infiltration of macrophages into the injured liver. Thus, GL-induced reduction in the serum levels and immunoreactive products of IL-18 does not appear to be due to an inhibition of cell infiltration in the liver. In conclusion, in mice treated with LPS/d-GalN, GL is assumed to caspase-independently inhibit the apoptosis of liver cells through the prevention of an IL-18-mediated inflammatory response. However, further investigations will be required to clarify the mechanism by which GL inhibits apoptotic changes in liver cells in an LPS/d-GalN-induced liver injury.

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