Chapter 9

Novel Mechanism Supporting Therapeutic Effects of Glycyrrhizin in Acute or Chronic Hepatitis

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Abstract

Glycyrrhizin (GL) isolated from the roots of licorice plant (*Glycyrrhiza glabra* L.) has been traditionally used for treating peptic ulcer, hepatitis, and pulmonary bronchitis. In addition to the protective effects of GL against liver injury or cancer proliferation by the membrane stabilization or via progesterone-receptor membrane component 1 (PGRMC1), the present chapter reports its new therapeutic mechanism through high-mobility group protein 1 (HMGB1) to which GL directly binds. In this study, we evaluated inflammation-promoting activity of HMGB1 and blockade of extracellular release of HMGB1 by GL in lipopolysaccharide (LPS)/D-galactosamine (GalN)-triggered mouse liver injury. In this experimental hepatitis model, apoptotic response of hepatocytes through the binding of HMGB1 protein to Glutathione transferase omega 1 (*Gsto1*), an apoptosis-associated gene, promoter region is caused, serum AST and ALT activities significantly increased, and GL-treatment prevented the apoptosis and inflammatory infiltrates induced with LPS/GalN-injection by disturbing the binding of HMGB1 protein to *Gsto1* promoter sequence. Analysis with chromatin immunoprecipitation (ChIP)-assay revealed inhibiting the binding of HMGB1 protein to *Gsto1* by the binding of GL to HMGB1.

Keywords: glycyrrhizin, liver injury, PAMPs, DAMPs, HMGB1

1. Introduction

Glycyrrhizin (GL), a triterpenoid glycoside isolated from the roots of licorice plant (*Glycyrrhiza glabra* L.), has been traditionally used for treating peptic ulcer, hepatitis, and pulmonary bronchitis. Various pharmacological effects of GL are well known, such as anti-inflammatory [1, 2], anti-allergic [3], and hepatoprotective activity [4–6]. In Japan, Stronger Neo-Minophagen C, the active ingredient of which is GL, has been used as a treatment for over 25 years for patients with chronic hepatitis. Intravenous administration of GL decreases serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in patients with chronic

hepatitis [7, 8]. The well-defined model of hepatic injury induced by the injection of lipopolysaccharide (LPS)/D-galactosamine (GalN) has been widely used in studies of the mechanisms of human hepatitis. GalN 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 [9]. Combination of LPS and GalN causes specifically hepatic failure in rodent [10]. Under the stimulation by LPS, liver macrophages secrete various pro-inflammatory cytokines including tumor necrosis factor (TNF)-α which is a terminal mediator for apoptosis, subsequently leading to hepatic necrosis [11–14]. The hepatic lesion in this model resembles that of human hepatitis since the up-regulation of TNF-α level and hepatic apoptosis have been reported as pathogenic symptoms in human hepatitis. GL, an aqueous extract of licorice root, has been used for the treatment of chronic hepatitis to reduce the liver inflammation [15–17], but its effects on acute hepatic injury have been unclear. A recent report showed that Y-40138, a synthetic compound, inhibits liver injury evoked by LPS/GalN through the suppression of TNF-α and monocyte chemoattractant protein-1 and the augmentation of IL-10 [18]. GL prevents anti-Fas antibody-induced mouse liver injury but has no effect on the upregulation of TNF-α mRNA expression in the liver [6]. In our previous study [19], we reported that levels of serum of cytokines such as TNF-α, interleukin (IL)-6, IL-10, IL-12, and IL-18 as well as those of serum ALT significantly increased after administration of LPS/GalN. GL had no effect on the production of TNF-α, IL-6, IL-10, and IL-12, whereas it significantly inhibited the increase in ALT levels and IL-18 production. We have so far indicated that the inhibitory effect of GL is different from that of inhibitor for TNF-α production, such as Y-40138 [18] and bicyclol, a new synthetic anti-hepatitis drug [20].

High-mobility group proteins (HMGBs) possess a unique DNA-binding domain that is subject to transcriptional regulation [21]. One of these proteins, HMGB1 (amphoterin), can be secreted into the extracellular milieu as a late-acting mediator of LPS-induced or sepsis-induced lethality in mice [22]. Although HMGB1 is a non-histone nuclear protein, it is passively released from necrotic cells [23] or actively secreted from stress-received cells such as monocytes/macrophages as an inflammatory cytokine in response to endotoxin, tumor necrosis factor (TNF)-α, or interleukin (IL)-1β [22, 24–26]. HMGB1, which was released into the intravascular area, has great potential as a local inflammatory activator through intensifying the release of cytokines and chemokines from stimulated cells [27] and interact with endothelial cells by up-regulating surface receptors and causing the secretion of soluble pro-inflammatory mediators [28]. Extracellular HMGB1 works properly as a damage-associated molecular patterns (DAMPs) molecule and increases powers of pro-inflammatory signaling paths by activating pattern recognition receptors (PRRs) including toll-like receptor 4 (TLR4) and/or the receptor for advanced glycation endproducts (RAGE) [25, 29]. Increasing evidence suggests that HMGB1 may also operate so as to assist the progress of the recognition of other immune co-activators such as LPS, DNA, and IL-1 by being excessively desirous of the binding to these molecules [30–32]. However, the mechanisms by which GL inhibits inflammation induced with pathogen-associated molecular patterns (PAMPs) such as LPS or endogenous DAMPs such as HMGB1 have not been clearly revealed.

Previous results have suggested that glycyrrhizin (GL) and glycyrrhetinic acid (GA) exert their protective effects by the membrane stabilization which results in inhibiting the prolongation of oxidative stress [33]. Furthermore, progesterone-receptor membrane component 1 (PGRMC1) was proposed as a new target protein for GL. PGRMC1 is a haem-containing protein that inter-

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acts with epidermal growth factor (FGFR) and cytochrome P450 to regulate cancer proliferation and chemoresistance [34]. GL is thought to reduce cancer proliferation via PGRMC1. On the other hand, a research work utilizing nuclear magnetic resonance (NMR) and fluorescence methods revealed the supplementary mechanism by which GL directly binds to HMGB1 and suppresses the HMGB1 chemoattractant and mitogenic activities [35]. Recent studies, furthermore, have reported that GL reduces inflammatory infiltrates by inhibiting the cellular proliferation and migration, and formation of blood vessels induced by HMGB1 [36]. In this chapter, we evaluated the underlying new mechanism supporting various pharmacological effects of GL on the basis of upcoming data of our experiment on hepatitis induced by an injection of LPS/GalN.

2. Results and discussion

In the present study, we explored inflammation-promoting activity of HMGB1 and blockade of extracellular release of HMGB1 by GL in LPS/GalN-triggered mouse liver injury. Male BALB/c mice were intravenously injected with LPS/GalN. At 1–10 h after LPS/GalN treatment, mice were anesthetized to collect blood by heart puncture, and serum transaminase and HMGB1 were evaluated. Intraperitoneal administration of GL was performed 30 min before treatment. Effects of GL on liver damage were examined 8 h after stimulation with endotoxin. The injection of LPS/GalN significantly increases serum AST and ALT activities as compared with controls. The enhancement of AST and ALT levels is significantly suppressed by an intraperitoneal administration of GL (**Figure 1**; [19, 37, 38]). Administration of LPS/GalN precipitate tissue injury associated with time-dependent alteration in HMGB1 serum levels. Immunohistochemistry with antibodies to HMGB1 reveals a distinct nuclear expression in the hepatocytes of control mice. Immunoreactivity to HMGB1 begins to be suppressed in the nuclei 6 h after administration of LPS/GalN. At 8 h nuclear immunoreactive products are remarkably reduced and extracellular HMGB1 expression is found exclusively in the pericentral foci. Double-immunofluorescence staining for HMGB1/F4/80 or HMGB1/CD11c demonstrates that some cell populations of $F4/80^{+}$ and $CD11c^{+}$ cells located in the inflammatory foci are immunolabeled simultaneously with HMGB1 8 h after stimulation with LPS/GalN [39]. The GL-treatment significantly reduces the serum levels of ALT, AST, and HMGB1 besides the strong inhibition of inflammatory tissue damage, and cytoplasmic and extracellular immunoreactive-response to both the HMGB1 and acetylated-lysine. The acetylation of HMGB1 is physiologically involved in regulating HMGB1 DNA binding properties along with the subcellular location. The lysine residues of HMGB1 between 27 and 43 represent functional nuclear localizing signals. An administration of GL brings about a significant decrease in the number of apoptotic hepatocytes labeled with TUNEL-method. On the basis of these results, we have identified an apoptosis-associated gene, Glutathione transferase omega 1 (*Gsto1*), using microarray analysis and real-time PCR (**Figure 2**). In addition, the chromatin immunoprecipitation (ChIP)-assay have revealed the binding of HMGB1 protein to *Gsto1* promoter sequence and the remarkable decrease in the volume of bound HMGB1 protein by administration of GL (**Figure 3**). Our findings claim that GL treatment might prevent the apoptosis and inflammatory infiltrate caused with LPS/GalN-treatment by disturbing the binding of HMGB1 protein to *Gsto1* promoter sequence. We provide *in vivo* evidence showing that HMGB1 is involved in the apoptosis of hepatocytes caused by LPS/GalN-treatment and administration of GL significantly improves hepatic injury,

in parallel with suppression of exaggerated apoptotic cell death and enhanced expression of regeneration mediator. Several recent investigations including our research [40] have reported that GL may protect against liver injury by reducing the expression of HMGB1, a mediator of inflammation [41, 42]. The induction of liver injury in mice by LPS/GalN represents a promising animal model for elucidating the mechanism of clinical dysfunction and for evaluating the efficacy of hepatoprotectives. The liver injury induced by LPS has been reported to be abrogated by treatment with anti-TNF-α mAb or p55 TNF receptors in mice [43–45]. TNF-α can induces 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 [46, 47]. In our study [19], the serum levels of TNF-α were markedly increased 0.5–1 h after LPS/GalN-treatment. Treatment with anti-TNF-α antibody reduced the elevated ALT level by LPS/GalN. Thus, it appears that TNF-α plays an important role in the pathogenesis of this model. There is evidence for the cytoprotection by IL-6 of liver injury induced by LPS [48]. In addition, both endogenous and exogenous IL-10 protect against LPS/GalN-induced liver injury [49]. Others have reported that at a 2-h timepoint, IL-12 is increased in plasma of mice treated with LPS/GalN [13]. We have confirmed that the serum levels of IL-6, IL-10, and IL-12 can reach a maximum by 2 h after LPS/GalN treatment [19]. GL had no effect on the production of TNF-α, IL-6, IL-10, and IL-12 in the same model mice, whereas it significantly inhibited increase in ALT levels.

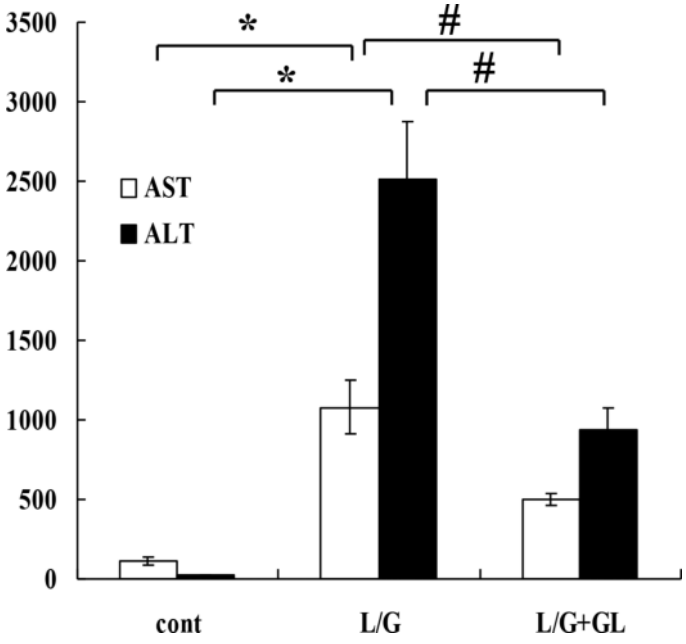

Figure 1. The effect of GL on serum AST and ALT levels at 8 h after administration of LPS/D-GalN. Increased serum AST and ALT levels are significantly inhibited by combined treatment with LPS/D-GalN + GL (L/G + GL) compared with mice treated with L/G. *Significant difference compared with 0 h or control ($P < 0.05$); #Significant difference between L/G and L/G + GL ($P < 0.05$); each value represents the mean ± SEM of six mice (Cited from Ref. [38]).

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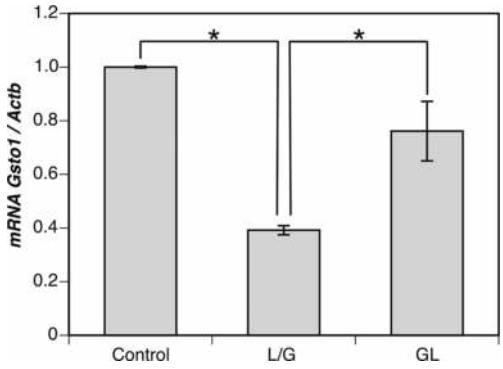

Figure 2. mRNA expression of *Gsto1* gene involved in apoptosis by LPS/GalN-treatment. After an injection of LPS/GalN, the expression level of *Gsto1* mRNAs is significantly decreased. The expression level of *Gsto1* mRNA is significantly recovered by administration of GL. *Significant difference between two groups (Control versus LPS/GalN or LPS/GalN versus GL + LPS/GalN) approved ($P < 0.05$). L/G: LPS/GalN, GL: GL + LPS/GalN (Cited from Ref. [40]).

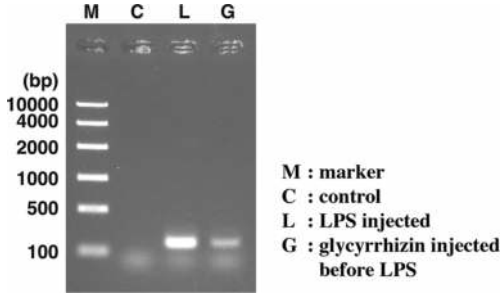

Figure 3. Analysis of HMGB1-binding to *Gsto1* with chromatin immunoprecipitation (ChIP)-assay. Hmgb1 protein intensely binds to *Gsto1* promoter sequence in LPS/GalN-induced liver injury and an administration of GL remarkably inhibits the binding of Hmgb1 to *Gsto1* promoter sequence. M: marker, C: control, L: LPS/GalN, G: GL + LPS/GalN (Cited from Ref. [40]).

HMGB1 is a multifunctional protein: the earliest studies reported it as a nonhistone DNA-binding nuclear protein. HMGB1 binds to DNA in a sequence-independent manner and changes the structure of DNA so as to assist the progress of transcription, replication, and repair [50, 51]. These functions are essential for survival, as HMGB1-deficient mice die of hypoglycemia within 24 h after birth [52]. Recent researches have identified HMGB1 as a new inflammatory factor and a late mediator of endotoxin lethality in mice [53]. Extracellularly released HMGB1 mobilizes a great number of various physiological reactions in different cell types [54]. HMGB1 may be released both through active secretion from various cells, including activated monocytes/macrophages [22], neutrophils [55], and endothelial cells [56], and passively from necrotic or damaged cells [23]. HMGB1 is released as a danger signal from damaged cells [24]. Even when cellular integrity is maintained, hepatocyte HMGB1 expression increases markedly after noxious stimuli [13, 24]. In our experiment, immunohistochemistry revealed

HMGB1 overexpression was found predominantly in the inflammatory foci located close to the central veins, that is, in areas most susceptible to LPS/GalN-treatment.

3. Conclusion

Apoptotic response of hepatocytes through the binding of HMGB1 protein to *Gsto1* promoter region is caused in this experimental hepatitis model and GL-treatment prevents the apoptosis and inflammatory infiltrates caused with LPS/GalN-injection by disturbing the binding of HMGB1 protein to *Gsto1* promoter sequence. The present findings claim a new mechanism supporting therapeutic effects of GL in hepatitis (**Figure 4**).

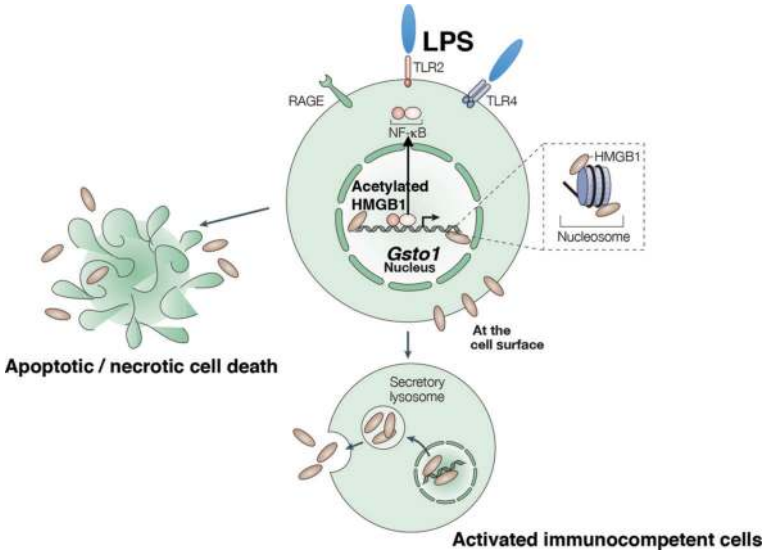

Figure 4. Action point of acetylated HMGB1. An administration of GL suppresses apoptotic cell death through inhibiting the binding of Hmgb1 to *Gsto1* (Scheme modified from Nature Reviews Immunology 2005:5:334).

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