4. Discussion

In the present study, the results of surface plasmon resonance experiments clearly showed that glycyrrhizin binds to HMGB1, but not to sRAGE, with moderate affinity (Kd value of 4.03 μM). The binding of HMGB1 with sRAGE was inhibited by the addition of glycyrrhizin, with the IC50 value being similar to the Kd value of glycyrrhizin for HMGB1. Thus, glycyrrhizin binding to HMGB1 inhibited HMGB1 binding to sRAGE, leading to the diminution of RAGE signaling. In fact, x-ray crystallographic analysis revealed that GL interacts with the A- and B-box of HMGB1 molecules (Mollica et al., 2007). Although the amino acid sequence in the B-box has been suggested to be involved in RAGE recognition (Andersson and Tracey, 2011), modification of the A- and B-box by GL may strongly interfere with RAGE binding.

Glycyrrhizin is a major constituent of licorice root that has often been used in traditional medicine in Japan and China (Arase et al., 1997; Kumada, 2002). Injection of glycyrrhizin is available for the treatment of hepatitis and allergic inflammation in Japan. Plural action mechanisms have been suggested for the anti-inflammatory activity of glycyrrhizin, including inhibition of NF-κB activation (Cherng et al., 2006; Takei et al., 2008), suppression of inflammatory cytokine production (Michaelis et al., 2011) and inhibition of the migration of inflammatory cells (Andersson and Tracey,

2011). These effects are interrelated, and some of them may be ascribed to the inhibition of HMGB1 activity and HMGB1 translocation/secretion (Kim et al., 2012; Hwang et al., 2006). The in vivo kinetics of glycyrrhizin indicate that the therapeutic concentration of glycyrrhizin is about 0.4-1.0 μM in humans (Michaelis et al., 2011; van et al., 1999), which is close to the Kd value of glycyrrhizin binding to HMGB1 determined in the present study. Therefore, a routine dose of glycyrrhizin (40 mg/injection) may yield sufficient plasma levels of glycyrrhizin to interfere with HMGB1-RAGE binding.

We observed a marked translocation of HMGB1 in pyramidal neurons in both the cerebral cortex and CA1 hippocampus under percussion injury (Fig. 1C). The reduced HMGB1 levels detected by western blotting in the injured cerebral cortex (Fig. 1D,E) strongly suggested that the rate of translocation and release of HMGB1 from neuronal nuclei into extracellular space exceeded substantially that of synthesis in the injury core. As in the case of anti-HMGB1 monoclonal antibody treatment (Okuma et al., 2012), glycyrrhizin significantly inhibited the translocation of HMGB1 in neurons (Fig. 1C). This strongly suggested the existence of a cycle in which HMGB1 is released, and then induces its own translocation, leading to further HMGB1 release, that was inhibited by glycyrrhizin. Since glycyrrhizin inhibited HMGB1 and sRAGE binding, it was

speculated that inhibition of RAGE signaling may in turn lead to the suppression of HMGB1 translocation by GL. Thus, the direct binding of GL to HMGB1 and subsequent inhibition of HMGB1 translocation support the notion that GL may be an inhibitor of HMGB1 secretion (Kim et al., 2012; Gong et al., 2012; Ohnishi et al., 2011).

Glycyrrhizin therapy is considered to be rather safe due to its long clinical experience of use. Pseudo-aldosteronism is the sole major side effect of glycyrrhizin and is generally limited to cases requiring long-term treatment. In the present study, two administrations of glycyrrhizin at 5 min and at 6 h after injury achieved a greater than 90% inhibition of the expressions of TNF-α, IL-1β, and IL-6. These anti-inflammatory effects of glycyrrhizin and inhibition of HMGB1 mobilization suggested by immunohistochemical study as well as reduction of plasma levels of HMGB1 probably contributed to the protection of the blood-brain barrier (BBB), since in vitro experiments showed that HMGB1 induced a breakdown of the BBB directly (Zhang et al., 2011). Thus, glycyrrhizin inhibited BBB breakdown, expression of inflammation-related cytokines and elevation of plasma levels of HMGB1, probably through its binding to HMGB1, which would have downregulated the RAGE signaling pathway. In fact, the beneficial effects of glycyrrhizin disappeared in RAGE-knockout

mice in the present study.

The beneficial effects of glycyrrhizin on sensorimotor and cognitive functions continued for up to at least 7 days after injury. The numbers of reactive astrocytes in the lesion site were reduced in glycyrrhizin-treated rats. Therefore, it is speculated that the acute effects of glycyrrhizin on BBB disruption as well as expression of inflammation-related molecules were followed by a reduced activation of astrocytes. One of the major complications of traumatic brain injury is post-traumatic epilepsy. Since the epileptic foci probably exhibits the sustained activation of astrocytes and microglia (Pascual et al., 2012; Zurolo et al., 2011; Mattson et al., 2001), the reduced activation of astrocytes and microglia may lead to a reduction in the severity of epileptic status. Because of the absence of severe side effects, glycyrrhizin may be applicable for the treatment of traumatic brain injury and prevention of post-traumatic epilepsy.

5. Conclusion

It was demonstrated that GL exerts beneficial effects on acute traumatic brain injury as well as subacute neural functions through binding to HMGB1 and interference with its RAGE ligation. Glycyrrhizin has long been used in the clinic setting and is a safe drug. Therefore, a clinical study evaluating the efficacy of GL on TBI should be

considered.

Conflict of interest

The authors declare no conflict of interest.

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Figure legends

Fig. 1.

(A) GL binding to HMGB1 in vitro. Recombinant human HMGB1 (40 μg/ml) was immobilized on a CM5 BIAcore chip and different concentrations of GL were flowed at time zero for 120 sec. Surface plasmon resonance (BIAcore) showed a rapid increase in RU indicating binding of GL to the immobilized HMGB1. The Kd for GL binding to HMGB1 was determined to be 4.0 μM. Similarly, recombinant human sRAGE (53 μg/ml) was immobilized on a CM5 BIAcore chip and the binding of GL to was determined. The GL binding to sRAGE was minimal. (B) The effect of GL on HMGB1-RAGE interaction in vitro. An in vitro binding assay of HMGB1 to sRAGE in the presence of different concentrations of GL to sRAGE was performed. The IC_{50} value of GL was estimated to be 6 μM from the inhibition curve. The results are expressed as the mean ± SEM of 3 wells. $^{\#}P<0.05$ compared with the control value in the absence of GL. (**C**) Translocation of HMGB1 in neurons in the rat cortex and hippocampus in the ipsilateral hemisphere after TBI. Five minutes after fluid percussion injury on the right temporal cortex (top panel), the rats received an intravenous injection of GL (4.0 mg/kg) or control vehicle, and at 6 h after percussion the brains were fixed. The coronal brain sections were double-immunostained with

anti-HMGB1 and anti-MAP-2 antibodies, followed by AlexaFluor 555-labelled and AlexaFluor 488-labelled secondary antibodies, respectively. Nuclei were stained by DAPI. The indicated areas from cerebral cortex and hippocampus CA1 were shown both at lower (upper panels) and higher (lower panels) magnifications. White arrows indicate the HMGB1-immunoreactivities in the extranuclear space. Scale bars: 50 μm (white) and 5 μm (yellow). (**D**) Decrease in HMGB1 levels in the TBI region. Brain samples from both sides of the cerebral cortex (3 mm square) were collected 24 h after injury for western blotting. β-actin was used as the internal control. (**E**) Quantitative analysis was performed using NIH Image J software. Results are expressed as the mean ± SEM of 5 rats. *P<0.05 compared with the control rats. (**F**) Plasma levels of HMGB1 were determined by ELISA in rats with TBI 6 h after injury. Results are expressed as the mean ± SEM of 10 rats. *P<0.05, *P<0.01 compared with non-injured rats (Sham). $^{\#\#}$P<0.01 compared with the injured control vehicle-treated rats.

Fig. 2.

(**A**) Effect of GL on BBB permeability in rats with TBI induced by fluid percussion. The rats received intravenous injection of different doses of GL (0.25, 1.0 and 4.0 mg/kg) or control vehicle at 5 min after injury. The permeability of brain capillary

vessels was examined by intravenously injecting Evans blue (40 mg/kg) at 6 h after injury and then measuring the leakage of Evans blue-albumin into the brain parenchyma at 3 h after Evans blue injection. (**B**) After extraction from brain tissue, the amount of Evans blue was determined in both control and GL (4.0 mg/kg) groups. The results are expressed as the mean ± SEM of 5 rats. **P<0.01 compared with the control vehicle-treated rats. (**C**) The percentage reduction of extravasated Evans blue was calculated in each GL dosage group. ##P<0.01 compared with control vehicle-treated rats. (**D**) Expression of inflammation-related molecules in rats with TBI. The rats received an intravenous injection of GL (4.0 mg/kg) or control vehicle 5 min after injury. Brain samples from both sides of the cerebral cortex were prepared 6 h after injury as described in the Methods section. Real-time quantitative PCR was performed for the determination of inflammation-related molecule expression. The results were normalized to the expression of GAPDH. Results are expressed as the mean ± SEM of 5 rats. *P<0.05, **P<0.01 compared with the contralateral side. #P<0.05 compared with control rats treated with vehicle.

Fig. 3.

(**A**) Effects of GL on the impairment of motor functions in TBI rats. The rats received intravenous injection of GL (4.0 mg/kg) or control vehicle 5 min after injury. The

rotarod test was performed before and after TBI on each rat. The results are the ratio of the walking time at each time point against the pre-injured value. The results of the rotarod test were expressed as the mean ± SEM of 5 rats. *P<0.05 compared with the sham control at each time point. #P<0.05 compared with the corresponding injured control vehicle-treated rats at the same time point. (**B**) The rats received intravenous injection of different doses of GL (0.25, 1.0 or 4.0 mg/kg) or control vehicle 5 min after injury. The rotarod test was performed at 6 h after injury as in (**A**). The results were expressed as the mean ± SEM of 5 rats. *P<0.05 compared with the injured control vehicle-treated rats. (**C**) Effect of GL on the lateral dominance of motor functions in TBI rats. The results of the limb-use asymmetry cylinder test were expressed as the mean ± SEM of 5 rats. *P<0.05 compared with the pretreatment value. #P<0.05 compared with the injured control vehicle-treated rats. (**D**) Determination of the therapeutic time window of GL treatment in rat TBI. The rats received intravenous injection of GL (4 mg/kg) or control vehicle at 5 min, 3 h or 6 h after fluid percussion injury. The rotarod test was performed 6 h after injury. Results are expressed as the ratio of the walking time to the pre-injured value. The results are the mean ± SEM of 5 rats. *P<0.05 compared with the injured control rats treated with vehicle. (**E**) The permeability of brain capillary vessels was examined by intravenously injecting Evans

blue (40 mg/kg) at 6 h after injury and then measuring the leakage into the brain parenchyma at 3 h after Evans blue injection. Results for 3 representative individuals are shown. (**F**) Results are expressed as the percentage reduction of Evans blue content by GL treatment at different time points. The results are the mean ± SEM of 5 rats. $^{*}P<0.05$, $^{**1}P<0.01$ compared with injured control rats treated with vehicle.

Fig. 4.

The effects of GL in RAGE-knockout and wild-type mice. (**A**) TBI was induced in RAGE-/- and their wild-type mice as described in the Methods section. GL (4.0 mg/kg) or control vehicle was injected at 5 min after percussion injury. BBB permeability was assessed by measuring Evans blue leakage into the brain. Results are expressed as the mean ±SEM of 5 mice. $^{**}P<0.01$ compared with control mice treated with vehicle. $^{\#\#}P<0.01$ compared with wild-type (WT) mice. (**B**) Coordinated motor function was evaluated 6 hours after injury using the rotarod test. Results are expressed as the mean ± SEM of 5 mice. $^{*}P<0.05$, $^{**}P<0.01$ compared with the respective sham control without injury. $^{\#}P<0.05$ compared with the respective control mice treated with vehicle. $^{\$}P<0.05$ compared with wild-type (WT) mice.

Fig. 5.

(**A**) Long-term beneficial effects of GL on motor activity and cognitive function in TBI rats. The rats received intravenous injection of GL (4.0 mg/kg) or control vehicle at 5 min and at 6 h after fluid percussion injury. Then the same dose of GL was administered once a day for 3 days. The rotarod test was performed at 1, 3 and 7 days after injury. Results are expressed as the mean ± SEM of 6 rats. $^{*}P<0.05$, $^{**}P<0.01$ compared with the pre-injured value. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with injured control rats treated with vehicle. (**B**) After mild fluid percussion injury, the rats received intravenous injection of GL (4.0 mg/kg) or control vehicle at 5 min and at 6 h. Then the same dose of GL was administered once a day for 3 days. The Morris water maze test was performed before and after mild TBI. Results are expressed as a ratio of swimming time at each time point against the pre-injured value. The results are the mean ± SEM of 6 rats. $^{*}P<0.05$ compared with the pre-injured value. $^{\#}P<0.05$ compared with injured control rats treated with vehicle. (**C**) Immunohistochemical staining of the injured cortex and dorsal hippocampus 7 days after injury. The rats received GL or control vehicle as in (A). The coronal brain sections were double-immunostained with anti-HMGB1 and anti-GFAP antibodies, followed by AlexaFluor 555-labelled and AlexaFluor 488-labelled secondary antibodies, respectively. Nuclei were stained by DAPI. The indicated areas from cerebral cortex and hippocampus CA1 were shown.

White arrows indicate the strong GFAP-positive astrocytes. Scale bar = 50 μm.

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