Glycyrrhizin inhibits traumatic brain injury by reducing HMGB1-RAGE interaction

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Abbreviations: Glycyrrhizin, GL; High mobility group box-1, HMGB1; Traumatic brain injury, TBI.

ABSTRACT

Glycyrrhizin (GL) is a major constituent of licorice root and has been suggested to inhibit the release of high mobility group box-1 (HMGB1), a protein considered representative of damage-associated molecular patterns. We found that GL bound HMGB1 but not RAGE with a moderate equilibrium dissociation constant value based on surface plasmon resonance analysis. This complex formation prevented HMGB1 from binding to RAGE in vitro. The effects of glycyrrhizin on traumatic brain injury (TBI) in duced by fluid percussion were examined in rats or mice in the present study. GL was administered intravenously after TBI. Treatment of rats with GL dose-dependently suppressed the increase in BBB permeability and impairment of motor functions, in association with the inhibition of HMGB1 translocation in neurons in injured sites. The beneficial effects of GL on motor and cognitive functions persisted for 7 days after injury. The expression of TNF-α, IL-1β and IL-6 in injured sites was completely inhibited by GL treatment. In RAGE -/- mice, the effects of GL were not observed. These results suggested that GL may be a novel therapeutic agent for TBI through its interference with HMGB1 and RAGE interaction.

Keywords: Traumatic brain injury ; Glycyrrhizin ; HMGB1 ; RAGE ; Brain edema

1. Introduction

Glycyrrhizin (20β-carboxyl-11-oxo-30-norolean-12-en-3β -yl-2-*O*-β-D-glucopyranuronosyl-β-D-glucopyranosiduronic acid, GL) is present in large quantities in licorice root (*Glycyrrhiza radix*), and consists of glycyrrhetinic acid and two molecules of glucuronic acid. GL has been used clinically in East Asia as an anti-inflammatory, anti-allergic, and anti-viral agent (Shamsa et al., 2010; Sharifzadeh et al., 2008). In Japan, GL injections have been used for allergic inflammation since 1948, and for chronic hepatitis since 1979. Additionally, an injection dosage form containing 0.1% cysteine and 2% aminoacetic acid has been developed to control the aldosterone-like effect of GL (Miyaji et al., 2002; Hidaka et al., 2007).

A recent study showed that GL protected rats against ischemia-reperfusion-induced liver injury and derangement of the microcirculatory blood flow in the liver (Mabuchi et al., 2009). Furthermore, it was suggested that GL had neuroprotective effects on the post-ischemic rat brain after middle cerebral artery occlusion (MCAO) and gerbil hippocampi after transient forebrain ischemia (Kim et al., 2012; Hwang et al., 2006). Traumatic brain injury (TBI) is one of the major causes of death and disability in young individuals worldwide; however, efficient therapies for TBI are lacking at present

(Narayan et al., 2002; Shlosberg et al., 2010). Previously we found that high mobility group box-1 (HMGB1), which is recognized as a representative of danger-associated molecular patterns (DAMPs) (Lotze and Tracey, 2005; Andersson and Tracey, 2011), was translocated markedly from neuronal nuclei to the cytosolic and then the extracellular compartment in TBI (Okuma et al., 2012). Extracellular HMGB1 directly induces BBB disruption (Zhang et al., 2011), triggering the inflammatory responses in TBI (Okuma et al., 2012). Systemic injection of anti-HMGB1 monoclonal antibody has been shown to significantly reduce brain edema, inflammatory molecule expression and impairment of sensorimotor functions in TBI (Okuma et al., 2012). Thus, HMGB1 plays important roles upstream of the secondary injury in TBI (Narayan et al., 2002; Shlosberg et al., 2010). We also demonstrated that, among several candidate receptors, RAGE played the predominant role in mediating the effects of HMGB1 (Okuma et al., 2012).

GL has been reported to inhibit the release of HMGB1 from activated or injured cells (Kim et al., 2012; Hwang et al., 2006). Moreover, GL appears to bind HMGB1 and inhibit its cytokine-like activities (Mollica et al., 2007). In the present study, we demonstrated that GL inhibited the interaction between HMGB1 and RAGE by binding to HMGB1 in vitro and that GL exerts its neuroprotective effects on fluid

percussion-induced brain injury through inhibition of HMGB1-induced brain edema, and expression of inflammation-related molecules. GL may be a novel therapeutic agent for TBI with an established safety record due to many years of clinical use for other conditions.

2. Materials and Methods

2.1 Surface plasmon resonance analysis (BIAcore)

Analysis of glycyrrhizin binding to HMGB1 or sRAGE was conducted using a BIAcore T100 instrument (GE Healthcare, USA). HMGB1 (40 μg/ml) or sRAGE (53 μg/ml) diluted by sodium acetate buffer (pH 5.0) was immobilized in a CM5 chip, giving a response unit of 5494 (HMGB1) or 1086 (sRAGE). An adjacent vacant flow-cell was activated with equal amounts of 0.2 M N-ethyl-N-[3-diethylamino-propyl]-carbodiimide and 0.05 M N-hydroxysuccinimide under the same conditions as a negative control. HBS-EP＋buffer（10 mmol/l HEPES , 0.15 mol/l NaCl, 3 mmol/l EDTA, and 0.05% surfactant P-20, pH 7.4）was used for sample dilution and analysis. GL at various concentrations was passed over the sensor chip at a flow rate of 30 μl/min for 2 min, and then the dissociation was allowed by application of HBS-EP buffer. The sensor chips were regenerated by washing with 10 mM glycine-HCl (PH 2.5) for 60 s at a flow rate of 10 μl/min. Results were calculated after subtraction of the control values using BIAcore evaluation T100 software (BIAcore) (He et al., 2011; Schiraldi et al., 2012).

2.2 HMGB1-sRAGE binding assay

The effects of GL on HMGB1-sRAGE binding were determined by use of an in vitro binding assay system as described previously (Liu et al., 2009). Briefly, HMGB1 (6 μg/ml) was immobilized on 96-well plates (Sumitomo Bakelite, Tokyo, Japan). Then, sRAGE (25μg/ml) was added to each well in combination with different concentrations of GL (0, 5, 10, 50, 100, and 200 μM). The incubation was continued for 24 h at 4°C. After incubation, the amount of bound sRAGE was determined by binding of Ni-NTA HRP to sRAGE. The absorbance was measured at 415 nm in a microplate reader (Model 680) from BioRad Laboratories (Hercules, CA, USA). The results were expressed as the percentage of binding compared with that in the absence of GL.

2.3 Animals and surgical procedures

All experimental procedures were approved by the committee on animal experimentation at our university. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to in vivo techniques, if available. Adult male Wister rats, 9-11 weeks old and weighing 250–350 g, were used for the experiments. The procedures for fluid percussion injury have been described previously (Okuma et al., 2012; Otani et al., 2002). Under anesthesia with 3.0%

isoflurane in a mixture of 50% oxygen and 50% nitrous oxide gas, a 4.8-mm craniectomy was performed on the right parietal cortex (3 mm posterior and 3 mm lateral from the bregma). A plastic cylinder 4.8-mm in diameter was fixed at the craniectomy site. The dura was left intact during this procedure. On the following day, the rats were subjected to fluid percussion injury of moderate severity (2.2–2.6 atm, 16 ms in duration) using a Dragonfly fluid percussion device (model HPD-1700; Dragonfly R&D, Silver Spring, MD), while maintaining the rectal temperature at 37.0 ± 0.5°C with a heating pad. Basic physiological parameters were monitored.

The rats were randomly assigned to 2 groups after the TBI operation, and GL (0.25, 1.0 or 4.0 mg/kg) or control vehicle was administered intravenously at 5 min after injury. In all experiments, stronger neo-minophagen C^R (Minophagen Pharmaceutical, Tokyo, Japan) containing 0.2% GL with 0.1% cysteine and 2% aminoacetic acid was administered intravenously as GL. The control vehicle contained 0.1% cysteine and 2% aminoacetic acid. Sham control rats were subjected to all of the same procedures except for the actual insult.

The procedures for fluid percussion injury in mice were described previously (Okuma et al., 2012). A 2.7-mm craniectomy was performed on the right parietal cortex (1.5 mm posterior and 1.5 mm lateral from the bregma). A plastic cylinder 2.7

mm in diameter and 4 mm in height was fixed at the craniectomy site using cyanoacrylate and dental cement. The remaining procedures were the same as used for the rats described above. RAGE-knockout (-/-) mice were produced as described previously (Myint et al., 2006). RAGE-/- mice backcrossed to C57BL/6J (Charles River Japan) for 8 generations were used. Mice at the age of 8-10 weeks were used for the experiments.

2.4 Assessment of motor function

An accelerated rotarod test (4–40 rpm for a maximum of 5 min; Muromachi, Kyoto, Japan) was performed at 3, 6 and 24 h after brain injury. The performance scores at each time point were expressed relative to the pre-injury performance. Forelimb use before and after TBI was also analyzed following observation of the rats in a transparent cylinder (20 cm in diameter and 40 cm in height) (limb-use asymmetry cylinder test). All functional tests were performed by investigators blinded to the treatment.

2.5 Histochemical staining

Immunohistochemical staining was performed for injured brain sections as

described previously (Liu et al., 2007). For double-immunostaining, the coronal brain sections were incubated with the mouse anti-HMGB1 mAb (R&D Systems, Inc.) and rabbit anti-MAP2 Ab or rabbit anti-glial fibrillary acidic protein Ab (Abcam Plc), followed by AlexaFluor 555-labelled and AlexaFluor 488-labelled secondary antibodies.

2.6 Evaluation of blood–brain barrier integrity by Evans blue

BBB permeability was assessed by measuring the extravasation of intravenously injected Evans blue dye into brain tissue (Okuma et al., 2012; Liu et al., 2007). Evans blue was administered over 1 min at a dose of 40 mg/kg in a volume of 1 ml/kg at 6 h after percussion injury, and then allowed to circulate for 3 h prior to sacrifice. Data were expressed as Evans blue (ng)/(g) wet brain weight.

2.7 Determination of HMGB1

Plasma levels of HMGB1 were determined by ELISA (Shino-Test Co., Sagamihara, Japan) as described previously (Okuma et al., 2012). Western blotting of HMGB1 in the cerebral cortex from both sides after injury was performed as described previously (Zhang et al., 2011). Brain samples from a 3mm square portion of the injury site were collected 24 h after injury. After homogenization with phosphate-buffered saline, the

brain samples were treated with sample buffer for SDS-PAGE. β-actin was used as a reference protein.

2.8 Real-time PCR

Real-time PCR was performed as described previously with the SYBR Premix EX *Taq* (Takara) in a Light Cycler instrument (Roche) according to the manufacturer's instructions. Brain samples from an injury site 3 mm square were collected 6 h after injury. The sense and antisense primers used for the analysis of mRNA expression were as follows: for iNOS, 5'-GCATCCCAAGTACGAGTGGT-3' and 5'-GAAGTCTCGGACTCCAATCTC -3'; for TNF-α, 5'-GCCCAGACCCTCACACTC-3' and 5'-CCACTCCAGCTGCTCCTCT-3'; for IL-1β, 5'- CACCTTCTTTTCCTTCATCTTTG-3' and 5'-GTCGTTGCTTGTCTCTCCTTGTA-3'; for IL-6, 5'-CAAGAGACTTCCAGCCAGTTGC-3' and 5'-TGTTGTGGGTGGTATCCTCTGT-3'; and for GAPDH, 5'- AGCCCAGAACATCATCCCTG-3' and 5'-CACCACCTTCTTGATGTCATC-3'. The expression of GAPDH was used to normalize cDNA levels. The PCR products were analyzed by a melting curve to ascertain the specificity of amplification.

2.9 Long-term effects of GL

Long-term beneficial effects of GL on impairment of motor activity and memory disturbance were determined up to 7 days after injury in TBI rats. After fluid percussion injury, the rats received intravenous injection of GL or control vehicle twice, once at 5 min and once at 6 h. Then the same dose of GL was administered daily up to 3 days after TBI. The rotarod test was performed at 1, 3 and 7 days after injury.

Memory impairment was assessed using a Morris water maze test, as described previously (Morris, 1984; Ohshima et al., 2005). Briefly, using a water maze pool (Muromachi, Kyoto, Japan), rats were given 2 consecutive training trials per day for a total of 7 days, or 5 days before TBI plus 2 days after TBI. If a rat failed to locate the platform within 60 s on any given trial, it was led there by the investigators. After completion of the training period, the rats were administered 2 consecutive tests per day. During each test and the last trial before TBI, the time required to find the hidden platform was recorded with a CompACT VAS/DV video-tracking system (Muromachi, Kyoto, Japan). After selecting the best time, the performance scores for each time point were expressed relative to the pre-injury performance. All functional tests were performed by investigators blinded to the treatment.

2.10 Statistical analyses

Statistical significance was determined using ANOVA followed by Student's *t*-test or Dunnett's test. Repeated measures two-way ANOVA was used for the statistical analysis for the rotarod test. P values less than 0.05 were considered to be significant.

3. Results

3.1 GL binding to HMGB1

The BIAcore sensograms of GL to immobilized HMGB1 showed a rapid increase in response units (RU) indicating binding of GL to the immobilized HMGB1 on the chip followed by a decrease of RU resulting from the dissociation of binding molecules upon washing. The binding of GL to HMGB1 was concentration-dependent (Fig. 1A). The equilibrium dissociation constant (Kd) was determined to be 4.03 μM. On the other hand, the GL binding to immobilized sRAGE was minimal (Fig. 1A).

The in vitro binding assay of HMGB1 to sRAGE showed that GL could block HMGB1 binding to sRAGE concentration-dependently (Fig. 1B). The IC50 value of GL was estimated to be 6 μM from the inhibition curve. Thus these results suggested that GL inhibited the interaction between HMGB1 and RAGE by binding to HMGB1.

3.2 Translocation of HMGB1 at the injured site and effect of GL

In the fluid percussion-induced injury model, translocation of HMGB1 from the nuclei to the cytosol was remarkable in MAP2-positive neurons in the cerebral cortex and dorsal hippocampus (arrows in Fig. 1C) but not in GFAP-positive astrocytes or Iba1-positive microglia as reported previously (Okuma et al., 2012) at the site below

percussion 6 h after injury in control rats. No such translocation was observed on the contralateral side in the control rats. In both the cerebral cortex and hippocampus, some neurons had evident cytosolic HMGB1-immunoreactive granule-like structures at 6 h after injury, whereas HMGB1 immunoreactivity disappeared from the other neurons at the primary lesion site.

The administration of GL (4 mg/kg, intravenously) significantly inhibited the translocation of HMGB1 in neurons and maintained the immunoreactivity in the nuclei (Fig. 1C). In accordance with the results of immunohistochemical staining, western blotting revealed that GL suppressed the reduction of HMGB1 from the site of injury (Fig. 1D, E).

Determination of the plasma levels of HMGB1 in rats treated with a percussion intensity of 2.2–2.6 atm showed that an 8-fold increase in HMGB1 levels occurred 6 h after injury. This increase was inhibited by half by intravenous administration of GL (4.0 mg/kg, intravenously) (Fig. 1D).

3.3 Effect of GL on BBB permeability

Evans blue leakage was measured to assess the extravasation of serum albumin from capillary vessels in the TBI model as reported previously (Okuma et al., 2012). The

Evans blue dye was injected intravenously 6 h after TBI, and the brain concentrations of Evans blue in the injured hemisphere were determined 3 h thereafter. After fluid percussion injury, the rats received three different doses of intravenous injection of GL (0.25, 1.0 or 4.0 mg/kg) at 5 min. GL dose-dependently inhibited BBB permeability (Fig. 2A, B, C), realizing 43% inhibition at 4.0 mg/kg.

3.4 Real-time PCR of inflammatory molecules

In the previous study, we found a marked up-regulation of inflammation-related molecules including TNF-α and iNOS in the ipsilateral injured cerebral cortex (Okuma et al., 2012). Therefore, we here examined the effect of GL (4.0 mg/kg) on the expression of inflammatory molecules on both sides of the cerebral cortex using real-time PCR (Fig. 2D). The expression of TNF-α, IL-1β, IL-6 and iNOS was up-regulated in the injured hemisphere after TBI. The treatment with GL (4.0 mg/kg) almost completely suppressed the enhanced expression of TNF-α, IL-1β, and IL-6 in the injured side (Fig. 2D). Also, there was no significant increase in the expression of TNF-α, IL-1β, IL-6 and iNOS in the injured hemisphere in GL-treated rats.

3.5 Effects of GL on motor activity

Impairments of coordinated locomotor activity after percussion injury were evaluated using a rotarod test. The walking time periods before and after brain injury were measured by the rotarod test and were expressed as a ratio of two determinations. In the sham rats, a learning tendency was observed, but this trend did not reach the level of statistical significance. In vehicle control rats, the ratios were significantly reduced compared with sham controls at 3, 6 and 24 h after percussion injury (Fig. 3A). In GL (4.0 mg/kg)-treated rats, there was no difference in the ratios compared with those in the sham rats at 3, 6 or 24 h after injury. Conversely, there were significant differences between the vehicle-treated and GL-treated groups at 6 and 24 h after injury (Fig. 3A). The beneficial effects of GL on coordinated motor activity were dose-dependent when the performance was determined at 6 h after injury (Fig. 3B).

3.6 Lateral dominancy of forelimb movement

Cylinder testing to evaluate the right-left dominancy of forelimb movement showed a time-dependent decrease in contralateral forelimb movement in vehicle control rats, while no lateral dominancy of forelimb movement was noted in GL (4.0 mg/kg)-treated rats throughout the observation period (Fig. 3C). There were significant differences

between the vehicle-treated and GL-treated groups at both 6 and 24 h after injury.

3.7 Determination of the therapeutic time window of GL

After fluid percussion injury, the rats received intravenous injection of GL (4.0 mg/kg) or vehicle control at 5 min, 3 h or 6 h. Not only at 5 min but also at 3 h, intravenous injection of GL significantly improved the motor impairments as measured by the rotarod test (Fig. 3D). In addition, intravenous administration of GL even at 6 h significantly reduced BBB permeability (Fig. 3E, F). Thus, our data suggested that GL treatment was effective even at 6 h after TBI.

3.8 Evaluation of receptor involvement using gene-knockout mice

In our previous study (Okuma et al. 2012), we demonstrated that RAGE predominantly might mediate the effects of HMGB1 released from neurons in the injured lesion. To confirm the receptors involved, we examined the effects of GL using RAGE-/- and corresponding wild-type mice (Fig. 4A). In the wild-type mice, inhibition of BBB permeability by GL (4.0 mg/kg) treatment was evident, as was observed in the rat experiments. In RAGE-/- mice, the increase in BBB permeability was lower than that in wild-type mice. Treatment of RAGE-/- mice with GL (4.0

mg/kg) did not produce any inhibitory effects on BBB permeability, suggesting that RAGE plays a crucial role in mediating the effects of GL (Fig. 4A). Moreover, GL did not antagonize the motor impairment induced by percussion injury in RAGE -/- mice, although GL significantly ameliorated motor impairment in wild-type mice (Fig. 4B).

3.9 Long-term beneficial effects of GL

The long-term beneficial effects of GL (4.0 mg/kg) were evaluated by the rotarod test at 1, 3 and 7 days after injury (Fig. 5A). In the vehicle-treated rats, the ratio of walking time decreased time-dependently up to 3 days and the reduced ratio remained to 7 days (Fig. 5A). Treatment with GL (4.0 mg/kg) significantly elevated the ratios compared with those in vehicle-treated rats at all time points examined.

We also examined the cognitive and motor function of rats with TBI induced by moderate intensity percussion (2.0-2.2 atm) using a Morris water maze test (Fig. 5B). The swimming time before and after brain injury was measured by the Morris water maze test and expressed as a ratio. In the vehicle control rats, there was marked prolongation of swimming time at 3 and 7 days after injury. In contrast, the swimming time in GL-treated rats at 3 and 7 days was the same as the pre-injury value and significantly different from the vehicle control value.

Long-term observation up to 7 days suggested that GL improves not only coordinated locomotor activity but also cognitive function during the subacute phase of TBI. In accordance with the results of the Morris water maze test and rotarod test, immunohistochemical staining revealed a large number of strong GFAP-positive astrocytes (white arrows in Fig. 5C) with broad processes in the control vehicle-treated rats 7 days after injury in both the cortex and dorsal hippocampus of the injured side (Fig. 5C). The number of these reactive astrocytes was reduced substantially in glycyrrhizin-treated rats.