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Recombinant human soluble thrombomodulin ameliorates cerebral ischemic injury through a high-mobility group box 1 inhibitory mechanism without hemorrhagic complications in mice

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ABSTRACT

Background: It has been reported that recombinant human soluble thrombomodulin (rhsTM) has a high-mobility group box (HMGB)1 inhibitory effect. Some investigators reported that HMGB1 is associated with ischemic stroke. However, there have been no previous studies to determine whether rhsTM can ameliorate cerebral ischemic injury through its HMGB1 inhibitory mechanism in ischemic stroke. We investigated the effects of rhsTM on cerebral ischemic injury in a 4-h middle cerebral artery occlusion (MCAO) murine model.
Methods: rhsTM (1 or 5 mg/kg, i.v.) was administered immediately after 4-h MCAO. Infarct volume, motor coordination, plasma HMGB1 level, and hemorrhage volume were evaluated 24 h after 4-h MCAO.
Results: The infarct volume ($P < 0.05$) was reduced by rhsTM in mice subjected to 4-h MCAO in a dose-dependent manner. Moreover, rhsTM (5 mg/kg) significantly improved motor coordination determined by the rotarod test ($P < 0.05$), and significantly decreased plasma HMGB1 level compared with vehicle-treated controls ($P < 0.001$). In addition, there was no difference in hemorrhage volume between vehicle-treated controls and the rhsTM treatment group.
Conclusions: This represents the first report that rhsTM ameliorates cerebral ischemic injury through an HMGB1 inhibitory mechanism without hemorrhagic complications in mice. Taken together, these observations indicate a palliative effect of rhsTM and suggest new therapeutic possibilities for treatment of ischemic stroke via inhibition of HMGB1.

1. Introduction

Stroke is the leading cause of morbidity and the third leading cause of mortality in the USA [1]. Approximately 80% of acute strokes are ischemic, with the rest being hemorrhagic (20% are caused by intracerebral or subarachnoid hemorrhage) [2]. About 25%–35% of stroke cases present with large vessel occlusion [3].

High-mobility group box (HMGB)1 is widely expressed in various tissues, including the brain. The level of HMGB1 is elevated in the plasma of stroke patients, and is released from ischemic brain tissue in a mouse model of cerebral ischemia [4]. In addition, HMGB1, a non-histone DNA-binding protein, has been reported to be released in large quantities into the extracellular space immediately after ischemic insult and to induce neuroinflammation and microglial activation in the postischemic brain [5]. These results suggest that HMGB1 may be a clinically useful biochemical marker for ischemic stroke as well as a target for therapeutic interventions.

Thrombomodulin (TM) is a cell-surface glycoprotein that is widely expressed in a variety of cell types. TM acts as a thrombin receptor on the surface of vascular endothelial cells; binding of TM to the thrombin receptor significantly decreases the effect of thrombin in conversion of fibrinogen to fibrin, activation of coagulation factors V and VIII, and platelets, and its D1 (lectin-like) domain has potent antiinflammatory effects through a variety of molecular mechanisms [6]. It has been reported that the D1 domain of TM bound to HMGB1 has anti-inflammatory properties [7] as one of the antiinflammatory mechanisms of action of TM. In addition, recombinant human soluble TM

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(rhsTM) was reported to associate with HMGB1 in some animal models, such as acute lung distress syndrome, sepsis, heatstroke, and hyperalgesia [8–12]. In addition, the commercially developed rhsTM preparation, Recomodulin, was approved for the treatment of disseminated intravascular coagulation (DIC) resulting from infection and cancer in 2008 in Japan [13–17]. rhsTM is widely used for septic DIC in Japan. Moreover, Solulin [18], another rhsTM preparation, has been reported to reduce infarct volume by promoting reperfusion in mice subjected to middle cerebral artery occlusion (MCAO) induced by photothrombosis [19,20]. However, there have been no investigations to evaluate the therapeutic usefulness of rhsTM in ischemic stroke through mechanisms involving HMGB1 in mice subjected to 4-h MCAO. It remains unclear whether rhsTM can improve neurological impairment in this murine ischemic stroke model. The present study was performed to investigate whether rhsTM can ameliorate cerebral ischemic injury and neurological impairment through its inhibitory effect on HMGB1 in mice subjected to 4-h MCAO.

2. Materials and methods

2.1. Animals

Male ddY mice (25–35 g; Kiwa Experimental Animal Laboratory, Wakayama, Japan) were kept under a 12-h light/dark cycle (lights on from 07:00 to 19:00) in an air-conditioned (23 °C ± 2 °C) room with food (CE-2; Clea Japan, Tokyo, Japan) and water available ad libitum. All procedures regarding animal care and use were performed in compliance with the regulations established by the Experimental Animal Care and Use Committee of Fukuoka University.

2.2. Focal cerebral ischemia

Focal cerebral ischemia was induced according to the method described in our previous reports [21–23]. The mice were re-anesthetized with isoflurane (Escain; Pfizer, Osaka, Japan) 4 h after occlusion, and reperfusion was established by withdrawal of the filament. MCAO was confirmed by examining forelimb flexion after awakening from anesthesia. In this 4-h MCAO mouse model, 14-day survival rate was approximately 30% [21], and regional cerebral blood flow decreased to approximately 80% from baseline after 4-h MCAO [24].

2.3. Cerebral infarct volume and hemorrhage volume 24 h after MCAO

The animals were sacrificed by decapitation 24 h after MCAO. The brains were removed and cut into four coronal sections 2 mm thick using a mouse brain matrix. The hemorrhagic area was measured in each slice using an image analysis system (NIH Image, version 1.63; National Institutes of Health, Bethesda, MD), and the hemorrhage volume was calculated. Cerebral infarct volume was also measured by image analysis in slices stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC; Sigma, St. Louis, MO).

2.4. Neurological score

Neurological score [21] was measured 24 h after cerebral ischemia, and divided into five groups: 0 = normal motor function, 1 = flexion of the torso and of the contralateral forelimb on lifting of the animal by the tail, 2 = circling to the ipsilateral side but normal posture at rest, 3 = circling to the ipsilateral side, 4 = rolling to the ipsilateral side, and 5 = leaning to the ipsilateral side at rest (no spontaneous motor activity).

2.5. Rotarod test in MCAO mice

Motor coordination was measured by the rotarod test as described previously [21,22]. Mice were placed on a rod 3 cm in diameter with a nonskid surface rotated at a speed of 10 rpm (Neuroscience Inc., Tokyo, Japan), and the latency to fall was measured for up to 2 min.

2.6. HMGB1 measurements

Blood samples were collected 24 h after MCAO in 4-h MCAO mice. Plasma was obtained after centrifugation (1200 rpm for 10 min at 4 °C). Plasma HMGB1 levels were measured by enzyme-linked immunoadsorbent assay (ELISA; Shino-Test Corporation, Kanagawa, Japan).

2.7. Drug preparation and administration

rhsTM, also known as ART-123 (Recomodulin), was provided by Asahi Kasei Pharma (Tokyo, Japan). rhsTM was dissolved in distilled water, and administered after 4-h MCA occlusion (1 or 5 mg/kg i.v.). Mice were randomized into three groups: vehicle-treated controls, rhsTM 1 mg/kg, and rhsTM 5 mg/kg.

2.8. Statistical analysis

Data are presented as means ± standard error of the mean (SEM). The data were analyzed by one-way analysis of variance (ANOVA) followed by Turkey's post hoc test. In all analyses, $P < 0.05$ was taken to indicate statistical significance. All statistical analyses were performed using JMP® version 10 (SAS Institute, Cary, NC).

3. Results

3.1. Effects of rhsTM on brain infarct volume 24 h after 4-h MCAO

Infarct volume was measured 24 h after 4-h MCAO cerebral ischemia by triphenyltetrazolium chloride staining. The mean infarct volumes were 93.1 ± 7.0 mm^3 in the vehicle-treated group, 76.7 ± 7.3 mm^3 in the rhsTM (1 mg/kg)-treated MCAO group, 64.8 ± 6.4 mm^3 in the rhsTM (5 mg/kg)-treated MCAO group. The cerebral infarct volume was reduced by rhsTM in a dose-dependent manner ($F(2,32) = 4.804$, $P < 0.05$, one-way ANOVA), and the infarct volume was significantly improved at a dose of 5 mg/kg ($P < 0.05$, Tukey's test) compared with the vehicle-treated group (Fig. 1).

3.2. Effects of rhsTM on neurological score and motor coordination in 4-h MCAO

The mean neurological scores were 3.6 ± 0.3 in the vehicle-treated group, 3.6 ± 0.3 in the rhsTM (1 mg/kg)-treated MCAO group, and 2.9 ± 0.3 in the rhsTM (5 mg/kg)-treated MCAO group. rhsTM at a dose of 5 mg/kg showed a tendency to improve the neurological score in comparison with the vehicle-treated controls, but the effect was not statistically significant.

Mean riding times in the rotarod test were 120.0 ± 7.7 s in the sham-treated group, 22.3 ± 12.2 s in the vehicle-treated group, and 66.2 ± 9.9 s in the rhsTM (5 mg/kg)-treated MCAO group. Motor coordination in the rotarod test was significantly impaired in the vehicle-treated group ($F(2,27) = 25.387$, $P < 0.001$, one-way ANOVA). At a dose of 5 mg/kg ($P < 0.05$, Tukey's test), rhsTM significantly improved motor coordination in comparison with the vehicle-treated group (Fig. 2).

3.3. Effects of rhsTM on HMGB1 in the plasma

The mean plasma level of HMGB1 was significantly increased in the vehicle-treated group compared with the sham-treated group (37.0 ± 3.11 ng/mL and 18.2 ± 3.81 ng/mL, respectively, $P < 0.01$, Tukey's test). The mean plasma levels of HMGB1 were 20.1 ± 3.81 ng/mL in the rhsTM (1 mg/kg)-treated MCAO group and 14.9 ± 3.11 ng/mL in