死亡の危険性がどの程度あるのかを予測する予後予測マーカーとしては、ある程度の評価ができたのではないかと考える。

E. 結論

健常人と比較してSIRS患者ではHRG値が有意に低く、敗血症患者ではより低いという結果が得られた。また、HRG値と死亡とに有意な関連を認め、HRG値の死亡予測に関する感度特異度が共に高いことも示された。HRGは新規重症度マーカーとなり得ることが示唆された。

F. 研究発表

1．論文発表

該当なし

2．学会発表

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G. 知的財産権の出願・登録状況

1．特許取得

該当なし

2．実用新案登録

該当なし

3．その他

該当なし

Fig 1-a HRG値 (ICU Day1) 健常人 vs SIRS患者

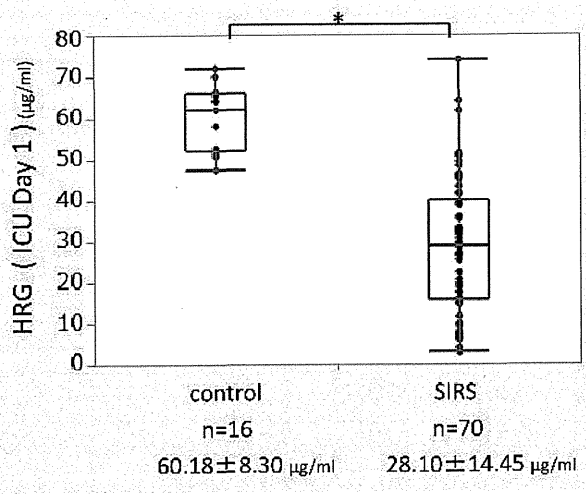


Fig 1-b HRG値(ICU Day1)

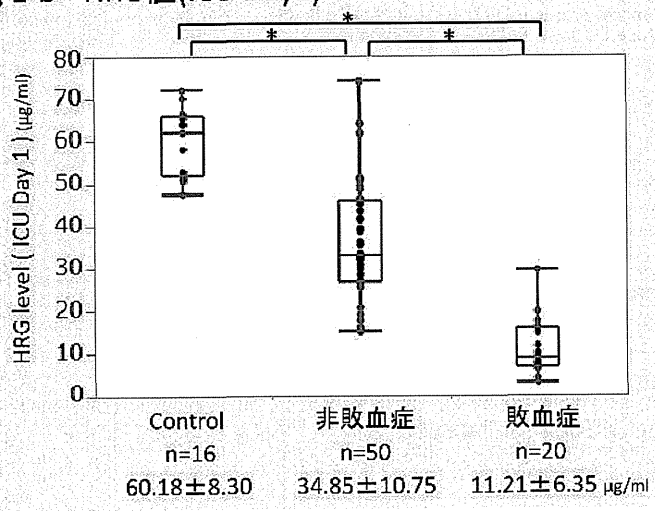


Fig 1-c HRG値(ICU Day1)

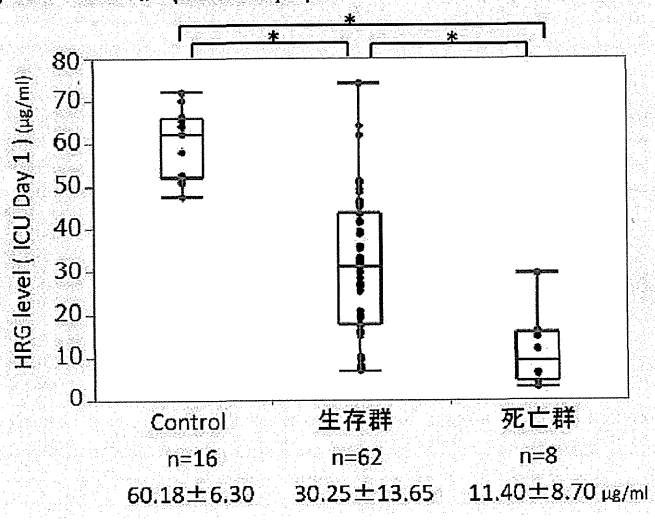


Table 1 A correlation between the HRG level and other markers

	相関係数	P value
HRG level vs		
APACHE Ⅱ score	− 0.41	< 0.01*
SOFA score	− 0.56	< 0.01*
CRP	− 0.43	< 0.01*
WBC	− 0.06	0.59
Plt	0.36	< 0.01*
Fbg	− 0.08	0.52
Lac	− 0.30	< 0.05*
ALB	− 0.00	0.98
T-Bil	− 0.29	< 0.05*

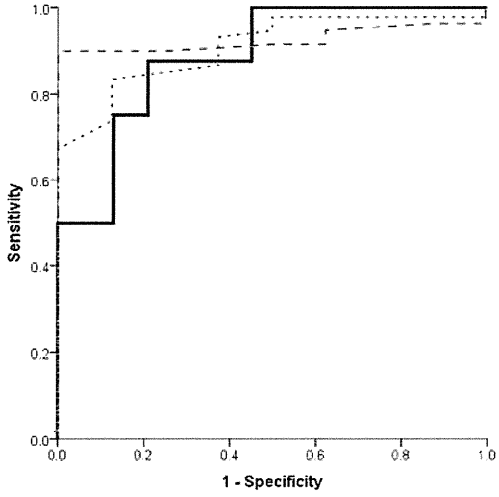
Table 2 Correlations with mortality

	Odd Ratio	95% C.I.	P value
HRG level	0.86	0.76 – 0.94	< 0.01*
APACHE II score	1.22	1.09 – 1.41	< 0.01*
SOFA score	1.41	1.17 – 1.78	< 0.01*
CRP	1.11	1.00 – 1.23	0.034*
Cr	2.05	1.13 – 4.92	0.013*
WBC	0.91	0.75 – 1.05	0.27
Plt	0.97	0.94 – 0.98	< 0.01*
Fbg	1.00	1.00 – 1.01	0.019*
Lac	1.43	1.04 – 2.14	0.043*
ALB	0.62	0.12 – 2.63	0.53
T-Bil	1.21	0.92 – 1.62	0.12

Table 3 Correlations with mortality
(Multivariate analysis)

	Odd Ratio (Adjusted)	95% C.I.	P value
HRG level	0.90	0.78 – 0.98	0.021*
APACHE Ⅱ score	1.14	1.01 – 1.31	0.021*

Fig 2　ROC curves for detecting non-survivor

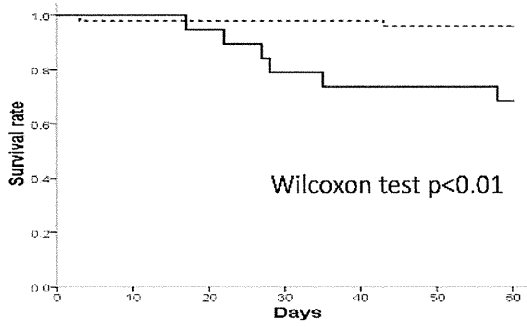


	AUC
HRG	0.88
SOFA score	0.91
APACHE Ⅱ	0.93

Cut off value
HRG 16.01 μg/ml
Sensitivity 0.87
Specificity 0.78

Fig 3　生存時間分析

- Kaplan-Meier curve



- Cox proportional hazard model

Hazard Ratio（低値群 vs 高値群）: 9.18(1.85 – 45.5) (p<0.01)

厚生労働科学研究費補助金（医療技術実用化総合研究事業）
分担研究報告書

HRGによるマスト細胞活性化の制御

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研究要旨

本研究では、HRG がマスト細胞の機能制御に関わる可能性について検証を行った。標準的なモデルであるマウス骨髄由来培養マスト細胞では、HRG を 30 分間前処理することにより、IgE を介する抗原刺激による 20%程度の脱顆粒率が 40%前後に増大することが明らかとなった。また、脱顆粒応答では低分子量 G タンパク質の Rho ファミリーの活性化レベルの低下が生じることを見いだした。HRG の好中球に対する作用を考慮すると、Rho ファミリーの調節を介して HRG が脱顆粒応答を増強するという仮説が考えられる。

A. 研究目的

HRG は血漿タンパク質であり、血管内の細胞や内皮細胞は常時高い濃度の HRG と接していると考えられる。一方、組織側に分布する細胞に関しては、炎症反応や組織傷害といった要因を通じて、接触する HRG の濃度は大きく変化することが予想される。マクロファージが死細胞を取り込む際に HRG が関与するという報告があるが、HRG による組織側の免疫細胞の機能の制御については明らかではない。分担者は、マスト細胞の分化に伴う機能制御を明らかにすることをテーマに研究を進めてきたが、ここでは HRG がマスト細胞の機能制御に関わる可能性について検討を行った。

また、本研究事業において明らかにされている好中球に対する HRG の作用は、HRG の標的分子の下流に低分子量 G タンパク質が関与することを強く示唆している。そこで、HRG による低分子量 G タンパク質の活性制御機構に着目した。

B. 研究方法

1．培養マスト細胞

Balb/c マウス（雄性 8 週齢）の脛骨より骨髄細胞を回収し、IL-3 (10 ng/ml)存在下、約 1 ヶ月培養することにより、FcεRI および c-kit ともに陽性の細胞が 95%以上である骨髄由来培養マスト細胞(BMMC)を得た。

2．マスト細胞の活性化の評価

脱顆粒応答は顆粒酵素である β-hexosaminidase 活性を指標に算出した。脱顆粒応答は PIPES 緩衝液 (25 mM PIPES-NaOH, pH 7.4, 125 mM NaCl, 2.7 mM KCl, 5.6 mM glucose, 1 mM $CaCl_2$, 0.1% BSA)中で検出した。

3．低分子量 G タンパク質の活性化の検出

Rac1 および Cdc42 の GTP 型と特異的に結合する Pak の部分配列を glutathione *S*-transferase (GST)融合タンパク質として大腸菌で発現させ、これを用いて細胞破砕液

中の GTP 型を沈降反応により分離した。イムノブロットにより、全体の低分子量 G タンパク質量、GTP 型の量をそれぞれ定量し、活性化を評価した(Bernard, V et al., ***J. Biol. Chem.*** 1999)。RhoA については、rhotekin の部分配列を利用して同様の評価を行った(Xiang-Dong, R. et al., ***EMBO J.*** 1999)。

(倫理面への配慮)

本研究は岡山大学動物実験委員会の審査を経て、承認されたものである。

C. 研究結果

1. HRG によるマスト細胞の脱顆粒応答の増強

マウス骨髄細胞を IL-3 存在下、1ヶ月培養することにより得られる骨髄由来培養マスト細胞 (BMMC: IL-3-dependent bone marrow-derived mast cell)は、マスト細胞のモデルとしてしばしば用いられる。培養系では通常 10%のウシ胎仔血清(FCS)が添加するため、FCS に含まれるウシ HRG の影響を除いた系で検証する必要性が考えられた。実験では、抗 TNP (trinitrophenol) IgE 抗体を 1 μg/ml で 3 時間感作し、PIPES 緩衝液で二回洗浄して遊離 IgE を除去し、その後ヒト HRG を各濃度で 37°C、30 分間処理した。その後、抗原(TNP-BSA, 3 μg/ml)を添加し、脱顆粒応答を測定した。その結果、ばらつきはあるものの、1 nM 付近の濃度で脱顆粒応答の増強が認められた (図1)。血清中に含まれる HRG の濃度を考慮すると、十分な洗浄を通じて残存する HRG レベルを抑えることが、抑制効果の再現性を得る上では重要であることが推察された。

2. 低分子量 G タンパク質の活性化の測定

HRG は好中球の形態変化の誘導や、活性酸素の産性抑制等の作用を示すことから、細胞骨格を制御する低分子量 G タンパク質 Rho ファミリーの活性化レベルを変化させる可能性が考えられた。そこで、マスト細胞において脱顆粒応答を伴う活性化刺激を与えた際の、Rho ファミリーの活性化を検討した。

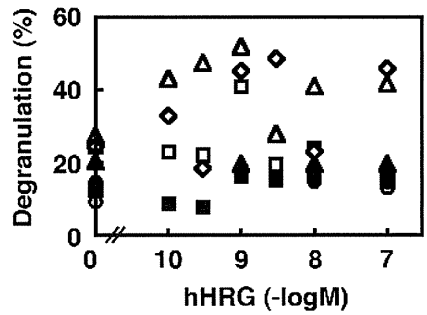

図1　マウス骨髄由来培養マスト細胞の脱顆粒応答のヒト HRG による増強

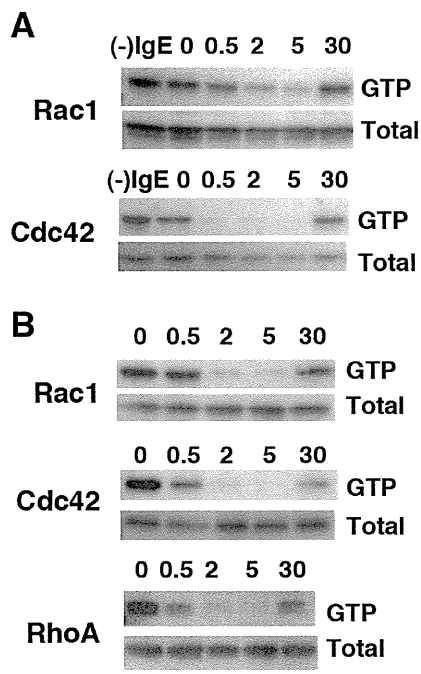

図2　脱顆粒刺激に対する Rho ファミリーの活性化レベルの変化

A：BMMC を抗 TNP IgE 抗体(1 μg/ml)を 12 時間感作後、PIPES 緩衝液で洗浄、抗原(TNP-BSA, 100 ng/ml)を添加し、0.5、2、5、30 分後にそれぞれ細胞を回収した。

B：BMMC を thapsigargin (300 nM)で刺激し、0.5、2、5、30 分後にそれぞれ細胞を回収した。

抗原刺激では、速やかに Rac1、Cdc42 の GTP 型の減少が認められた。一方、小胞体 Ca^{2+}-ATPase 阻害剤である thapsigargin 処理においても同様に速やかな GTP 型の減少が認められ、これは RhoA についても同様であった。これらの結果は、脱顆粒応答時には Rho ファミリーの不活性化が速やかに生じることを示すものである。

D. 考察

比較的低い濃度域で HRG の作用が認められたが、血清から残存するウシ由来 HRG の影響を抑制することにより、さらに明瞭な結果が得られる可能性がある。好中球における正球化は、HRG によって Rho ファミリーの活性レベルの低下が生じる可能性を示唆しているが、マスト細胞の脱顆粒応答では Rho ファミリーの活性化レベルが低下することが明らかとなった。即ち、HRG による脱顆粒応答の増強は Rho ファミリーの活性化レベルの調節を介する可能性が考えられた。この点について、さらに HRG 単独刺激における Rho ファミリーの活性化レベルの検討を行う予定である。

マスト細胞は組織に分布することから、通常は低いレベルの HRG に接していることが予想されるが、炎症応答時には血管透過性の亢進により高レベルの HRG に曝露される可能性が考えられる。即時型アレルギーでは抗原刺激によりマスト細胞が脱顆粒応答を起こし、遊離されたヒスタミン等の作用により血管透過性が亢進する。このとき、HRG がマスト細胞に作用することにより、脱顆粒応答が強化され、より強い炎症応答につながるというメカニズムが考えられる。HRG の中和抗体、あるいは RNAi による血中レベルの低下を誘導した際に、即時型応答がどのような影響を受けるかは興味深い問題である。

E. 結論

ヒト HRG はマウス骨髄由来培養マスト細胞の抗原刺激による脱顆粒応答を増強した。

F. 研究発表

1．論文発表

該当なし

2．学会発表

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該当なし

2）国内学会

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G. 知的財産権の出願・登録状況

1．特許取得

該当なし

2．実用新案登録

該当なし

3．その他

該当なし

厚生労働科学研究費補助金（医療技術実用化総合研究事業）
分担研究報告書

実験的自己免疫性脳脊髄炎に対するHRG効果の検討

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研究要旨

ヒト多発性硬化症の動物モデルである実験的自己免疫性脳脊髄炎（experimental autoimmune encephalomyelitis:EAE）を用いてHRG投与効果を検討する。EAEは、中枢神経組織由来のmyelin oligodendrocyte glycoprotein(MOG)由来のペプチドMOG35-55でマウスを免疫することにより活性化されるTh17タイプの抗原特異的CD4T細胞を介して発症するが、神経組織を攻撃する最終エフェクター細胞は好中球であると考えられている。CD4T細胞の樹状細胞によるプライミングと活性化、Th17への分化、そしてTh17による好中球の活性化と中枢神経局所への遊走という一連の過程を経て発症する当該モデルにおいて、HRG投与が如何なる効果をもたらすのかを検討し、その分子メカニズムの解析を行なう。

A. 研究目的

実験的自己免疫性脳脊髄炎（EAE）を用いてHRG投与による効果を検討し、その細胞及び分子メカニズムの解析を行う。

B. 研究方法

フロイト完全アジュバント（CFA）、*Mycobacterium tuberculosis* H37Ra死菌、MOG35-55ペプチドをエマルジュン化し、C57BL/6マウスの皮下に注射する（day 0）。同時に*Pertussis* toxinをday 0, day 2に投与する。この操作により、10日頃から尾部の麻痺が始まり後脚麻痺→前脚筋力低下と病状が進行する。臨床スコア（0～5）を毎日記載することにより、中枢神経麻痺の進行或は回復具合を観察する。Day 2からHRG（50μg）を2日間隔で静脈内投与し、臨床スコアの推移を観察した。

（倫理面への配慮）

本研究は岡山大学動物実験委員会の審査を経て、承認されたものである。

C. 研究結果

予備実験としてHRG（50μg）を2日間隔で4回投与した。臨床症状は30日に渡って観察を行なった。その結果、HRG投与により発症そのものは抑制できなかったが、HRG非投与群に比べ、臨床スコアの軽減が認められた（各群のN数は10）。

D. 考察

HRG投与により、EAEの症状を軽減できる可能性が示唆された。1回当たりのHRG投与量、投与回数の増加により、さらなる症状の軽減をもたらすことが期待される。

E. 結論

HRG 投与により、EAE の症状を軽減できる可能性があると結論づけた。実験結果の再現性を担保した上で、HRG 効果のメカニズム解析を行なう必要性があると考えられた。

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G. 知的財産権の出願・登録状況

１．特許取得

① 免疫評価方法とその評価 された免疫活性化剤
特願 2014-166593（2014.8.19 出願）
鵜殿平一郎、榮川伸吾、豊岡伸一

２．実用新案登録

該当なし

３．その他

該当なし

研究成果の刊行に関する一覧表

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OkumaY.,et al.	Glycyrrhizin inhibits traumatic brain injury by reducing HMGB1-RAGE interaction.	***Neuropharmacology***	85	18-26	2014
Hanakawa H.,et al.	Does HMGB1 predict occult neck lymph node metastasis in early tongue carcinoma? A case-control study of 26 patients.	***J Laryngol Otol***	128	926-31	2014
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Mannitol enhances therapeutic effects of intra-arterial transplantation of mesenchymal stem cells into the brain after traumatic brain injury

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HIGHLIGHTS

- We hypothesized that glycerol or mannitol might enhance the therapeutic benefits of cell transplantation in a rat TBI model.
- Compared to PBS or glycerol, the administration of mannitol resulted in increased BBB disruption.
- More MSCs were observed in the injured brain tissues of mannitol-treated rats than in glycerol or PBS-treated rats at 24 h after transplantation.
- Intra-arterial transplantation of MSCs combined with mannitol is an effective treatment in a TBI model of rats.

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ABSTRACT

Traumatic brain injury (TBI) sustained in a traffic accident or a fall is a major cause of death that affects a broad range of ages. The aim of this study was to investigate the therapeutic effects of intra-arterial transplantation of mesenchymal stem cells (MSCs) combined with hypertonic glycerol (25%) or mannitol (25%) in a TBI model of rats. TBI models were produced with a fluid percussion device. At 24 h after TBI, MSCs (1×10^6 cells/100 μl) with glycerol or mannitol were administered via the right internal carotid artery. Rats were evaluated behaviorally and immunohistochemically, and hyperpermeability of the blood-brain barrier (BBB) induced by hypertonic solutions was explored. Compared to PBS or glycerol, the administration of mannitol resulted in increased BBB disruption. The mannitol-treated rats showed significant improvement in motor function. Intra-arterial transplantation of MSCs caused no thromboembolic ischemia. Immunohistochemically, more MSCs were observed in the injured brain tissues of mannitol-treated rats than in glycerol or PBS-treated rats at 24 h after transplantation. Intra-arterial transplantation of MSCs combined with mannitol is an effective treatment in a TBI model of rats. This technique might be used for patients with diseases of the central nervous system including TBI.

1. Introduction

Traumatic brain injury (TBI) is a serious public health problem worldwide. It is a complex injury associated with a broad spectrum of symptoms and disabilities. TBI contributes to a number of deaths and cases of permanent neuronal disability. At the present time, effective therapies for TBI are lacking and are sorely needed. Stem cell transplantation is considered to be a hopeful treatment for TBI patients since it exerts a therapeutic effect in TBI animal models [6]. Mesenchymal stem cells (MSCs) have many advantages for cell transplantation according to the pluripotency and easy availability without ethical problems [9]. Because of the tight junctions between vascular endothelial cells, however, the blood-brain barrier (BBB) is a major obstacle to stem cell therapy. If intra-arterial transplantation of stem cells is to be an effective approach to the treatment of TBI, BBB permeability must be temporarily increased during administration. In the injured brain, a transient breakdown of the BBB occurs with inflammatory responses, permitting the migration of stem cells from the blood to the parenchyma. Recent studies have reported that the permeability of the BBB can also be manipulated through the use of osmotic agents (mannitol or glycerol) that transiently disrupt the barrier. Moreover, mannitol has been used as a neuroprotective drug for TBI patients [14]. In the

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present study, we hypothesized that glycerol or mannitol might be an effective BBB permeabilizer that would allow for the easier entry of MSCs into the injured brain and enhance the therapeutic benefits of cell transplantation in a rat TBI model.

2. Materials and methods

2.1. Animals and surgical procedures (Fig. 1)

Adult male Sprague-Dawley rats (n = 58, 9–11 weeks old, 200–250 g, Charles River, Yokohama, Japan) and adult male transgenic rats expressing enhanced GFP (n = 8, 200–250 g, Sprague-Dawley rats) were used for the experiments. All animals were used according to the approved Guidelines of the Institutional Animal Care and Use Committee of Okayama University.

Procedures for fluid percussion injury have been described previously [13]. Under general anesthesia with 3% isoflurane in a mixture of 50% oxygen and 50% nitrous oxide, a craniectomy (4.8 mm) 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. 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 (HPD-1700; Dragonfly R&D, MD, USA), maintaining the rectal temperature at 37.0 ± 0.5 °C with a heating pad. Basic physiological parameters were monitored. Sham-operated rats were subjected to the skull-opening operation alone and no brain injury.

2.2. Evaluation of the blood–brain barrier integrity by Evans blue

Rats (n = 12) were infused with PBS (n = 4), glycerol (25%) (n = 4) or mannitol (25%) (n = 4), immediately followed by 2% Evans blue dye in a dose of 2 ml/kg via the right common carotid artery. To allow the dye to circulate, the rats were then allowed to live for 3 h before sacrifice. After perfusion with saline, the brains were removed. The right hemisphere of the brain was placed in 1 M potassium hydroxide and homogenized. Then 1 ml of the homogenized cocktail was placed in 5 ml of a mixture of 0.2 M phosphoric acid and acetone at a ratio of 5:13 and centrifuged (3000 rpm, 30 min). The supernatant solution was transferred to a microcuvette, and the absorbance was measured at 620 nm. Data were expressed as Evans blue wet (ng/g) brain weight.

2.3. MSCs culture

GFP-expressing transgenic rats (n = 8) were euthanized by sodium pentobarbital (200 mg/kg) with subsequent removal of the femoral bones. After the femoral bone marrow was flushed, cells were cultured in DMEM (Life Technologies, CA, USA) supplemented with 10% fetal calf serum and 1% (v/v) penicillin/streptomycin. Cells were cultured at 37 °C with 10% CO_2. The MSCs were isolated on the basis of their ability to adhere to the culture plates.

2.4. Assessment of motor function

Rats (n = 24) were randomly assigned to three groups after the TBI operation, and PBS (n = 8), glycerol (25%) (n = 8) or mannitol (25%) (n = 8) was administered intravenously, first at 5 min and again at 6 h after injury. Sham control rats (n = 10) were subjected

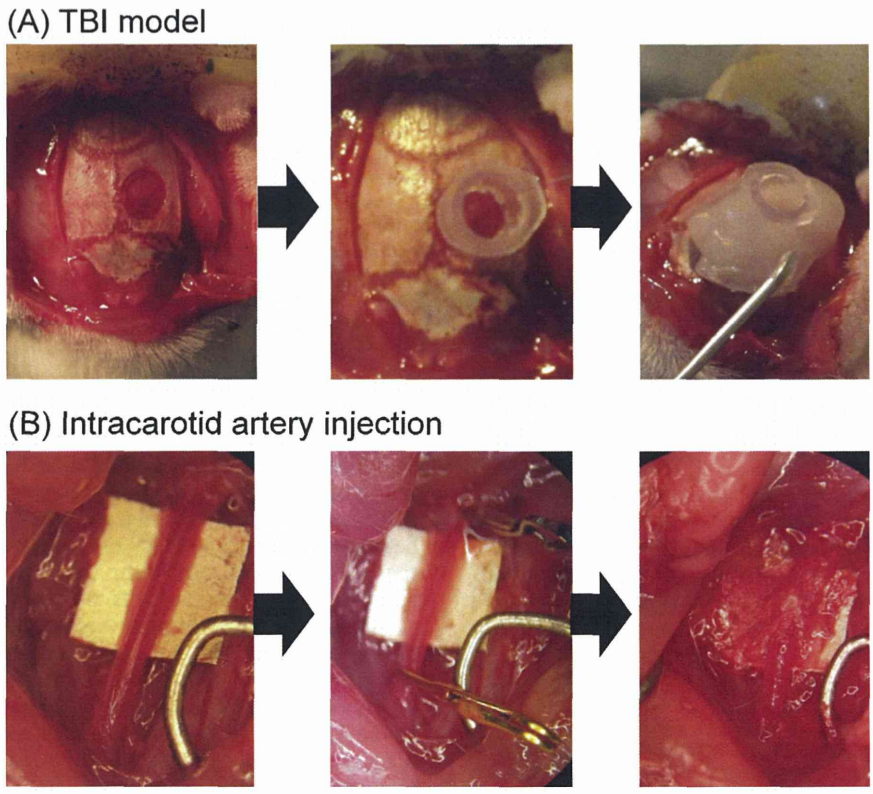

Fig. 1. TBI models and intracarotid artery injection. (A) TBI model: a 4.8 mm craniectomy was performed on the right parietal cortex, which was located 3 mm posterior and 3 mm lateral from the bregma (left panel). A plastic cylinder 4.8 mm in diameter was fixed at the craniectomy site (middle panel) and bone cement was placed (right panel). (B) Intracarotid artery injection: after exposing the carotid sheath, the common, internal, and external carotid arteries were dissected (left panel). Temporary clips were used to clamp the external carotid and the proximal common carotid artery (middle panel). Hemostasis was achieved by applying manual pressure after each injection.

to all of the same procedures except for the actual insult. 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 TBI. The performance scores at each time were expressed as percentages relative to pre-injury performance. All functional tests were performed by investigators blinded to the treatment.

2.5. Cell transplantation

Intra-arterial MSCs transplantation was performed at 24 h after TBI. Under general anesthesia with 3% isoflurane in a mixture of 50% oxygen and 50% nitrous oxide, the right carotid artery was dissected. The external carotid and the proximal end of the common carotid artery were clamped with temporary clips. A 27G winged needle (Terumo, Tokyo, Japan) was inserted upward into the common carotid artery. One million MSCs with either PBS ($n=4$), glycerol + PBS (25%) ($n=4$) or mannitol + PBS (25%) were injected into the internal carotid artery in 1 min. The needle and clips were then removed under compression and bleeding was stopped by manual pressure at each injection site.

2.6. Detection of transplanted MSCs

At 24 h after MSCs transplantation, rats were euthanized and brains were removed. Coronal sections were cut at a thickness of 10 μm with a freezing microtome. The sections were incubated with 4′,6-Diamino-2-phenylindole (DAPI) (Sigma–Aldrich, Tokyo, Japan) for 30 min. Finally, stained sections were analyzed with fluorescence microscopy (Keyence, Osaka, Japan).

2.7. Cytotoxicity assay of glycerol and mannitol for MSC in vitro

MSCs were seeded in a 24-well culture plate (BD Falcon, NJ, USA) and incubated with glycerol (25%), mannitol (25%) or PBS. At 24 h after treatment, MSCs were fixed with 4% paraformaldehyde and incubated with DAPI solution for 30 min. Finally, DAPI-stained cells were analyzed with fluorescence microscopy (Keyence).

2.8. Statistical analysis

Statistical significance was assessed using ANOVA followed by Student's *t*-test or Dunnett's test. *P* values less than 0.05 were considered to be significant.

3. Results

3.1. Localization of transplanted MSCs

In this TBI model, we found that the majority of transplanted MSCs were localized to the injured cortex (Fig. 2), although some

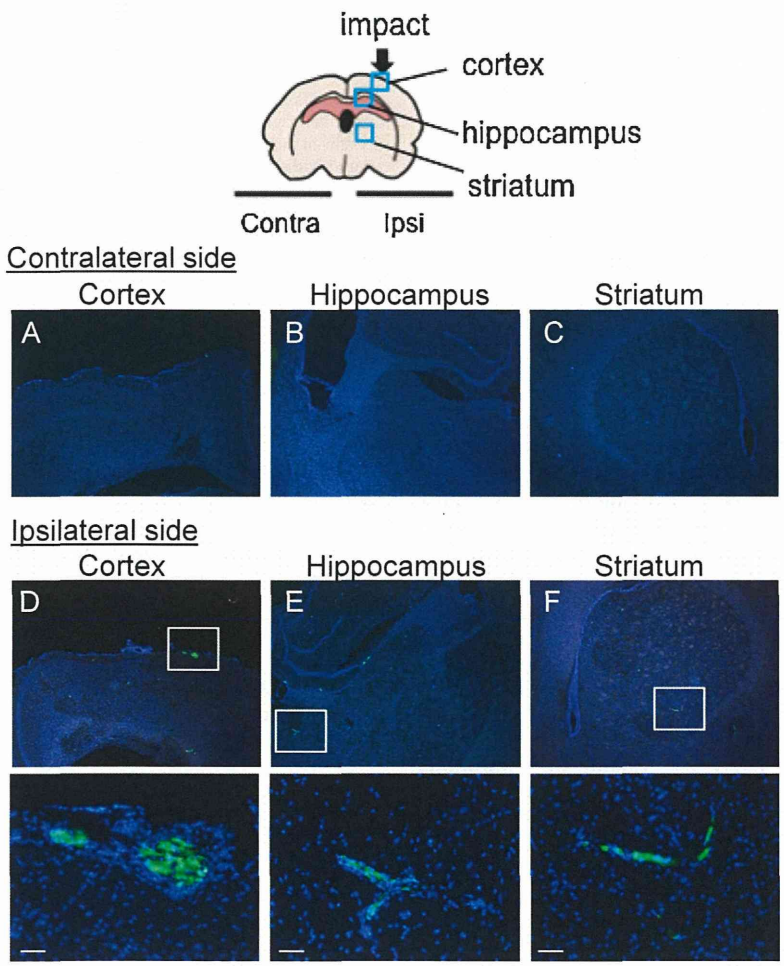

Fig. 2. Localization of transplanted MSCs in rat model of TBI. (A–C) No cells could be observed in the contralateral side. (D–F) Transplanted cells were observed in the ipsilateral cortex, hippocampus and striatum at 24 h after transplantation. Green: GFP-positive MSCs, Blue: DAPI-positive nuclei. Scale bars: 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

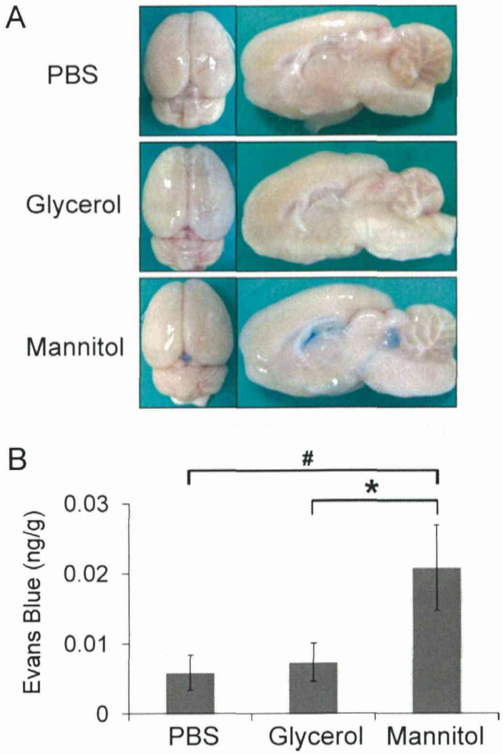

Fig. 3. Effects of mannitol on BBB permeability in rat model of TBI. (A) Three representative brains are shown. The ipsilateral hemisphere and ventricle were stained by Evans blue dye in the rats receiving mannitol. (B) The administration of mannitol resulted in increased BBB disruption, compared to that of PBS or glycerol. Results are expressed as the mean value ± SEM of five rats. $^{\#}p < 0.05$ compared with the PBS group. $^{*}p < 0.05$ compared with the glycerol group.

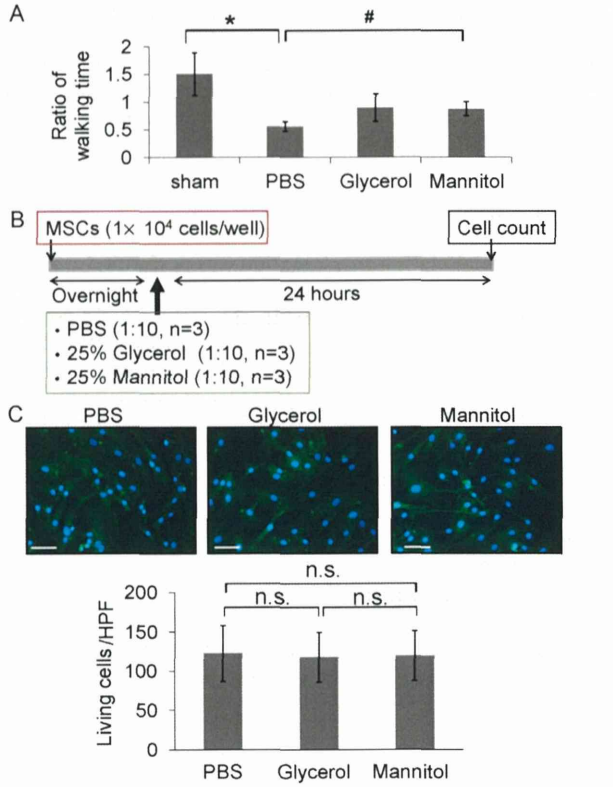

Fig. 4. Effects of glycerol and mannitol in vivo and in vitro. (A) Mannitol-treated rats exhibited significantly better motor function compared with PBS-treated rats. The results of the rotarod test are expressed as mean ± SEM of 8 or 10 rats. $^{*}p < 0.05$ compared with the sham-operated group. $^{\#}p < 0.05$ compared with the PBS group. (B) Time course of the cytotoxic assay of glycerol and mannitol for MSCs. (C) Counting under a microscope the number of healthy living cells in five random high-power fields (HPF). There were no significant differences among the three groups. The results were expressed as mean ± SEM of three wells. Scale bars: 50 μm.

cells were observed in the ipsilateral hippocampus. In the brains of the sham-operated rats, in contrast, no MSCs were found (data not shown).

3.2. Effects of mannitol on BBB permeability

Leakage of Evans blue dye was measured to assess the extent of BBB disruption. The ipsilateral hemisphere and ventricle were stained after administration of mannitol, while there was almost no staining in the rats receiving glycerol or PBS (Fig. 3). Thus the administration of mannitol as opposed to PBS or glycerol resulted in increased BBB disruption and a consequent greater than three fold increase in the leakage of Evans blue dye.

3.3. Effects of mannitol and glycerol on motor activity

Impairment of coordinated locomotor activity after injury was evaluated using a rotarod test (Fig. 4). Walking time durations before and after brain injury were measured and expressed as a ratio. In the sham-operated rats, a learning tendency was observed. In PBS-treated rats, the ratios were significantly lower than those in sham-operated rats at 24 h after percussion injury (PBS: 0.556 ± 0.0872, Sham:1.51 ± 0.383, $^{*}p < 0.05$). In glycerol-treated rats, a tendency toward recovery was observed, though the ratio was not significantly different from that in PBS-treated rats (glycerol: 0.896 ± 0.249). In contrast, the difference between PBS-treated and mannitol-treated rats at 24 h after injury was significant (mannitol: 0.879 ± 0.124, $^{\#}p < 0.05$).

3.4. Cytotoxicity of glycerol and mannitol for MSCs

MSCs were stained with DAPI solution. DAPI-stained cells were counted using Dynamic Cell Count software BZ-HIC (Keyence). There were no significant differences among the groups (Fig. 4).

3.5. Blood brain barrier disruption favorably affects MSC transplantation

GFP-positive cells were counted using Dynamic Cell Count BZ-HIC software (Keyence). The majority of transplanted MSCs were localized to the injured cortex, although some cells were observed in the ipsilateral hippocampus. Substantially more GFP-positive cells were observed in the injured cortex of the mannitol group than in those of the PBS or glycerol groups. This result suggests that mannitol effectively potentiated MSCs to migrate into injury sites (Fig. 5).

4. Discussion

4.1. MSC transplantation is an effective option for TBI

TBI contributes to almost half of all trauma-related deaths. Given the large societal burden that results from the morbidity and mortality associated with TBI, this disease entity has been the focus of extensive research. Yet despite decades of basic and clinical research, TBI remains poorly understood [1]. Recent studies have revealed that MSC transplantation has neuroprotective and

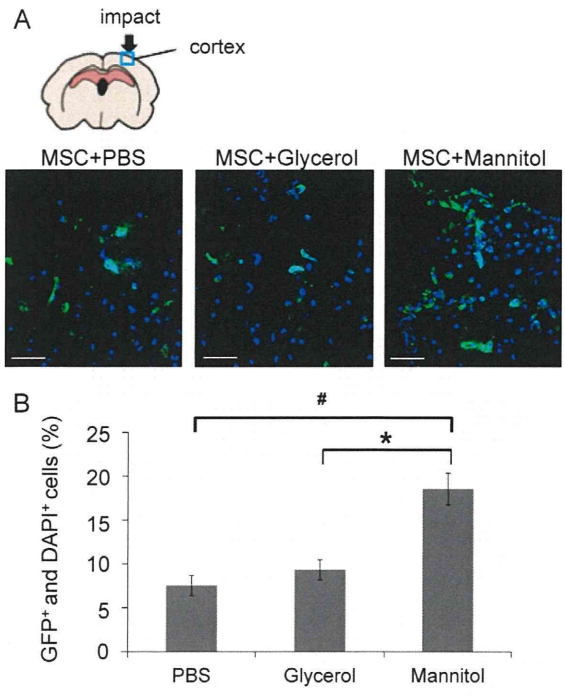

Fig. 5. Comparison of mannitol and glycerol for MSC localization. (A) Transplanted MSCs were observed in the cortex at 24 h after transplantation. Representative photos are shown. Green: GFP-positive MSCs, Blue: DAPI-positive nuclei. Scale bars: 50 μm. (B) The percentages of GFP-positive cells (transplanted MSCs) were counted in three fields in the cortex. Transplanted MSCs were clearly observed in the mannitol group. Results are expressed as mean ± SEM of four rats. $^{\#}p < 0.05$ compared with the PBS group. $^{*}p < 0.05$ compared with the glycerol group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

nursing effects in TBI animal models. The major mechanism of functional recovery exerted by cell transplantation is neuroprotection by neurotrophic factors in the injured brain [22]. MSCs secrete neurotrophic factors, including vascular endothelial growth factor, glial cell line-derived neurotrophic factor and brain-derived neurotrophic factor, which are known as strong neuroprotectants for central nervous system (CNS) disorders [20]. The potential use of MSCs for brain repair and regeneration has been reported based on animal models of various CNS diseases [11]. Moreover, various cell concentrations and routes of transplantation have been tested to demonstrate the therapeutic efficacy of MSC treatment for neural injury [10]. Our previous study indicated that the BBB in TBI rats is damaged due to brain edema subsequent to injury and contrast enhancement in magnetic resonance imaging [13]. In general, the distribution and number of transplanted MSCs are greater in the traumatic brain than in the normal brain. Indeed, we found many more surviving MSCs in our TBI-model rats than in sham-operated rats.

4.2. Intra-arterial injection might be better than intravenous injection

Several injection routes, namely, intravenous, intraventricular, intracerebral and intra-arterial, have been employed for transplantation of MSCs in animal models of CNS disorders [5]. In particular, we found that many studies have used the intravenous and intra-arterial routes. Compared to intravenous injection, intra-arterial injection resulted in less entrapment of MSCs in filtering organs like the lungs or spleen and exhibited a higher capacity for engraftment of MSCs in the brain. Intra-arterial transplantation is a promising method for targeted delivery of MSCs to the injured region. Some studies have suggested that intra-arterial transplantation of MSCs yields more favorable effects than intravenous transplantation does [19]. Furthermore, our study suggests the possibility that the beneficial effects of intra-arterial transplantation of MSCs might be enhanced by pretreatment using mannitol.

4.3. Mannitol has been used for osmotic BBB disruption

The BBB is one of the limiting factors obstructing MSCs from reaching injured regions of the brain [17]. MSCs delivered by intra-arterial injection need to pass through the BBB before they can successfully migrate and survive into an injured site [18]. BBB disruption with mannitol, an alcohol derivative of the sugar mannose, was introduced in 1961 [21]. Mannitol has been used to reduce cerebral edema or elevated intracranial pressure in clinical practice [14]. The agent has previously been demonstrated to increase intracerebral drug levels and to allow for easy passage of MSCs to damaged regions in animals [18,4]. The treatment of human umbilical cord blood coinfused with mannitol significantly increased brain levels of neurotrophic factors, which correlated with improved behavioral functions in neonatal hypoxic-ischemic rats [2,22,3]. As an adjunctive treatment, mannitol has frequently been used to decrease brain edema or intracranial pressure in cases of massive brain lesions [12,16]. Moreover, mannitol has also been used to open the BBB by temporarily shrinking the tightly coupled endothelial cells that make up the barrier, thus allowing drugs to be delivered directly to the brain [8]. Although this practice is still somewhat controversial, mannitol is widely used to control elevated intracranial pressure following brain injury. A survey of the critical care management of head-injured patients in the United States performed in 1995 showed that 83% of centers used osmotic diuretics for more than half of severely head-injured patients [7].

In addition to its effects on BBB permeability, mannitol might improve the results of MSCs treatment for TBI by other mechanisms. Administration of mannitol ameliorated clinical outcomes of patients with TBI in a recent study [15]. The cerebral blood flow to hypoperfused brain regions was improved after TBI. This effect cannot be explained solely by the increased BBB permeability induced by mannitol. We previously reported that secondary injury in TBI was more severe than expected [13]. Additionally, we found here that the cytotoxicity levels of glycerol and mannitol against MSCs are very low.

One limitation of this study is the fact that we observed transplanted MSCs for only up to 24 h. In order to demonstrate the therapeutic effects of intra-arterial transplantation of MSCs for TBI with certainty, long-term assessment would be needed.

5. Conclusions

Here, we demonstrated that mannitol improved the survival of transplanted MSCs in a rat model of TBI. In addition, we found that mannitol is safe for MSCs and effective in treating TBI; accordingly, we propose BBB disruption with mannitol as a useful technique for intra-arterial transplantation of MSCs.

Conflict of interest

The authors have declared that no conflict of interest exists.

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Glycyrrhizin inhibits traumatic brain injury by reducing HMGB1–RAGE interaction

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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) induced 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.

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

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

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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 (25 μg/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-labeled and AlexaFluor 488-labeled 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 3 mm 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 two 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.