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主 論 文

Anti-high mobility group box-1 (HMGB1) antibody inhibits hemorrhage-induced brain injury and improved neurological deficits in rats
(抗 HMGB1 抗体はラットにおける出血誘導性脳損傷を抑制し神経障害を改善する)
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副 論 文

Anti-high mobility group box-1 (HMGB1) antibody attenuates delayed cerebral vasospasm and brain injury after subarachnoid hemorrhage in rats
(抗 HMGB1 抗体はラットくも膜下出血後の遅発性脳血管攣縮と脳損傷を緩和する)
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主 論 文

Anti-high mobility group box-1 (HMGB1) antibody inhibits hemorrhage-induced brain injury and improved neurological deficits in rats
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[緒言]

Intracerebral hemorrhage (ICH) accounts for 20–30% of all strokes in Asia; ICH is most commonly attributed to hypertension, and is associated with extremely high rates of mortality, morbidity and disability. Recently, several therapeutic targets were identified and candidate drugs were evaluated in clinical trials. Unfortunately, however, there is still no effective treatment which increases survival or improves the quality of life after ICH. Early surgery may limit the toxic effects of blood clot, but many clinical trials of clot evacuation in ICH have not shown a definitive benefit for surgical removal, which might reflect some of the adverse side effects of surgery. ICH not only causes primary brain injury via its biochemical and mechanical effects, but also induces secondary brain injury, including local inflammatory responses to ICH and the toxic effects of blood breakdown products. Secondary brain injury proceeds over hours to days, and thus it might be possible to intervene therapeutically against it. However, there is also emerging evidence suggesting that inflammation contributes to brain injury during the acute phase of ICH, including breakdown of the blood–brain barrier (BBB) and activation of microglia. Therefore, the suppression of inflammatory responses after ICH might be a novel strategy for reducing the secondary brain injury.

HMGB1 is a ubiquitous and abundant nonhistone DNA-binding protein. HMGB1 is a representative of the damage-associated molecular patterns (DAMPs) family, and exerts an important proinflammatory cytokine-like activity once released into the extracellular space from cellular nuclei. HMGB1 is involved in a diverse range of CNS diseases, including ischemic brain infarction, traumatic brain injury, Parkinson's disease and neuropathic pain. To trigger the inflammation, the secreted HMGB1 stimulates plural receptors i.e., the receptor for advanced glycation end products (RAGE) and toll-like receptor-2 (TLR-2) and TLR-4, which are expressed in peripheral macrophages and vascular endothelial cells as well as microglia and neurons in the central nervous system. Interestingly, the administration of anti-HMGB1 neutralizing mAb has been shown to protect the BBB and to inhibit the inflammation cascade in rat models of middle cerebral artery occlusion/reperfusion-induced infarction and fluid percussion-induced traumatic brain injury. The recent studies also reported the increase in HMGB1 levels in peri-hematomal regions in subacute phase after ICH in rats, however, there was little information about the acute dynamics of HMGB1 in the core area after ICH. Moreover, whether anti-HMGB1 mAb can also provide neuroprotective effects in a rat model of ICH remains to be seen.

In the present study, we demonstrated that anti-HMGB1 mAb remarkably ameliorated ICH injury induced by local injection of collagenase IV in the striatum of rats, and this effect was associated with a decrease in activated microglia and astrocytes and suppression of the expression of

inflammation-related factors. In addition, the treatment with anti-HMGB1 improved neurological function, which may provide a new approach to potentially reduce ongoing edema and improve the neurological outcome after ICH.

[材料と方法]

Animals and treatment groups

Male Wistar rats were randomly divided into 3 groups: (1) an anti-HMGB1mAb group (2) a class-matched control mAb group (3) a sham group.

Surgical induction of ICH and mAb administration

Intracerebral hemorrhage was induced by the local injection of collagenase IV in the striatum of the rats. Briefly, a 30-gauge needle was inserted into the right striatum, 0.2 mm anterior to the coronal suture, 3 mm lateral to the bregma and 6 mm below the skull. Bacterial type IV collagenase (0.03 U) in 2 μl saline was delivered at a constant rate of 0.5 μl/min. In the sham group, we performed the same procedures, except that we used saline injection instead of collagenase IV.

The rats were administered anti-HMGB1 mAb (#10-22, IgG2a subclass, 1 mg/kg) or anti-K*eyhole Limpet hemocyanin* (class-matched control mAb, 1 mg/kg) through the tail vein immediately and 6 h after ICH induction.

Spectrophotometric assay for hemoglobin

Brain hemorrhage was detected by quantifying the hemoglobin content with Drabkin's reagent as described previously. Briefly, after removing the blood through transcardial perfusion, the ipsilateral brain tissue containing the hemorrhage region was obtained at 24 h after ICH, and PBS (3 ml) was added to each hemisphere, followed by homogenization for 1 min, sonication on ice with an ultrasonicator, and centrifugation for 30 min. The supernatant was reacted with Darbkin's solution for 15 min. After that, the optical density (OD) was measured at 540-nm wavelength.

Western blot analysis

Brain samples were collected from the striatum hematoma core and homogenized for 5 min in RIPA lysis buffer. The protein samples were homogenized and separated by electrophoresis, then transferred onto nitrocellulose membranes. After blocking with 10% skim milk, the membranes were incubated at 4°C overnight with anti-HMGB1 antibody. β-actin, as a reference protein, was probed with a mouse anti-β-actin mAb followed by goat anti-mouse Ab.

Enzyme-linked immunosorbent assay

To determine HMGB1 levels in plasma, blood samples were collected through the rat heart under deep anesthesia, then centrifuged for 15 min at 800 g. HMGB1 was detected by using an ELISA kit, according to the manufacturer's instructions.

Evaluation of BBB integrity

To evaluate BBB permeability, Evans blue extravasation was measured as described previously. Briefly, 2% Evans blue dye was injected intravenously at 6 h or 3 days after ICH. Three hours later the rats were perfused with 100 ml of saline via the left ventricle of the heart under deep anesthesia. The brains were immediately removed and the ipsilateral hemispheres were homogenized in 1 M

potassium hydroxide. Then the homogenized mixture was placed in 50% trichloroacetic acid and centrifuged at 800 g for 30 min. The absorbance of the supernatant solution was measured at 620 nm.

Brain water content

Brain edema of the ipsilateral brains was determined by the wet–dry weight ratio method at 72 h after ICH, as described previously. Brain water content was calculated as [(wet weight − dry weight)/wet weight] × 100%.

Immunohistochemical studies

The primary antibodies used in the experiments included anti-HMGB1 Ab, anti-AQP4 Ab, anti-IL-1β Ab, anti-MAP2 Ab, anti-GFAP Ab, anti-Iba1 Ab and MPO Ab. In order to investigate the cellular source as well as the localization of IL-1β, double immunohistochemical staining was carried out with cell marker antibodies including MAP2, GFAP, iba1 or MPO antibodies and an antibody against IL-1β. The sections were then incubated with secondary Abs conjugated with Alexa-488, Alexa-555 or Alex594. Finally, the sections were mounted and observed under an LSM 780 confocal microscopic system.

Quantitative real-time PCR

The brain samples for RT-PCR were obtained from peri-hematomal regions. The expressions of the following IL-1β, IL-8R, TNF-α, iNOS, MMP-2, MMP-9, VEGF-A189, VEGF-A121, COX-2, AT-1R, PAR-1, α1R, V1R, eNOS, TxA2, RAGE and GAPDH. GAPDH expression was used as an internal control to normalize cDNA levels. Fold changes in expression levels were calculated by the comparative cycle threshold method ($2^{-\Delta\Delta CT}$).

Assay of reactive oxygen metabolites and antioxidant potency

Derivatives of reactive oxygen metabolites (d-ROM) and biological antioxidant potency (BAP) were measured in plasma at 24 h after ICH using a free radical electron evaluator. In brief, the plasma was added to an acetate buffer with $FeCl_2$ at 37°C, followed by the addition of a chromogenic mixture including aromatic alkyl-amine. After incubation at 37°C for 5 min, the colored radical derivative was measured at 505 nm. For BAP assay, the plasma was dissolved in a colored solution that was prepared previously by mixing $FeCl_3$ with a thiocyanate derivative. After 5 min of incubation at 37°C, the chromatic change was read at 505 nm.

TUNEL staining and analysis

Paraffin-embedded brain sections were fixed at 24 h after ICH, then processed for terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling assay. Briefly, the sections were treated with proteinase K for 20 min, and then an FITC-labeling reaction mixture was applied to the slide and allowed to react for 90 min in a 37°C humidified chamber. TUNEL-positive cells were analyzed by fluorescent microscopy.

Assessment of motor function

A grip strength test was performed before injury and at 6, 24 and 48 h after brain injury. Briefly, the rats were held by their body, and allowed to grip the grid with the contralateral forelimb of the

hemorrhaged side. Then, the rats were gently pulled downwards and the peak of the grip strength was recorded. For the forelimb use asymmetry test, forelimb use was analyzed by observing the rats in a transparent cylinder before and after ICH.

[結果]

Treatment with neutralizing anti-HMGB1 mAb (1 mg/kg, i.v. twice) remarkably ameliorated ICH-injury induced by local injection of collagenase IV in the striatum of rats. Administration of anti-HMGB1 mAb inhibited the release of HMGB1 into the extracellular space in the peri-hematomal region, reduced serum HMGB1 levels and decreased brain edema by protecting blood-brain barrier integrity, in association with decreased activated microglia and the expression of inflammation-related factors at 24 h after ICH. Consequently, anti-HMGB1 mAb reduced the oxidative stress and improved the behavioral performance of rats. These results strongly indicate that HMGB1 plays a critical role in the development of ICH-induced secondary injury through the amplification of plural inflammatory responses. Intravenous injection of neutralizing anti-HMGB1 mAb has potential as a novel therapeutic strategy for ICH.

[考察]

HMGB1 was released mainly from neuronal nuclei and partly from astrocytes in the peri-hematomal areas in the acute phase of ICH, yielding a diverse range of secondary inflammation responses that was inhibited by anti-HMGB1 mAb. As a result, the treatment with anti-HMGB1 mAb ameliorated the neurological symptoms. To the best of our knowledge, these findings are the first to demonstrate that intravenous treatment with a neutralizing anti-HMGB1 mAb had neuroprotective effects on this ICH model.

A recent clinical study reported that the serum levels of HMGB1 were dramatically increased in patients with acute ICH, and this increase was significantly correlated with stroke severity. In related studies, ICH animal models were used to evaluate two pharmacological inhibitors of HMGB1 secretion, ethyl pyruvate and glycyrrhizin. The administration of ethyl pyruvate improved the functional outcome, reduced brain edema and decreased the number of apoptotic cells in the rat intracerebral hemorrhage and rat traumatic brain injury models. Similarly, glycyrrhizin suppressed brain edema and improved behavioral performance in ICH rats, probably by binding to HMGB1 and thereby inhibiting the interaction between HMGB1 and RAGE. Together, these findings suggest that HMGB1 may be one of the key inflammatory factors in ICH pathology and that therapeutic targeting for HMGB1 produces beneficial effects in ICH. In the present study, anti-HMGB1 mAb was shown to bind to HMGB1 directly and suppress the secondary brain injury efficiently. Thus, administration of intravenous anti-HMGB1 mAb may be a quite effective therapy for ICH in addition to brain ischemia, brain trauma, and neuropathic pain.

Three receptors of HMGB1 (RAGE, TLR-2 and TLR-4) have been reported in earlier studies. Although ICH was shown to significantly up-regulate the expression of all three receptors of

HMGB1, only treatment with the RAGE antagonist (FPS-ZM1) significantly reduced ICH-induced infiltration of inflammatory cells and the expressions of IL-1β and MMP-9 in the peri-hematomal region. In contrast, TLR2/4 antagonists had no influence on the ICH-induced infiltration of inflammatory cells, even though they did slightly reduce the expressions of IL-1 and MMP-9. In the present experiments, the mRNA level of RAGE was also decreased by treatment with anti-HMGB1 mAb after ICH. Taken together, these results suggest that RAGE may be the predominant receptor for HMGB1 in the pathogenesis of inflammation after ICH, and anti-HMGB1 mAb might exert its effects through the HMGB1/RAGE signaling pathway.

Cytokines can increase the production of other cytokines via a positive feedback loop. For example, HMGB1 acting as an early pro-inflammatory cytokine promotes the production of many cytokines during ischemic brain injury, including IL-1β and TNF-α. Conversely, increased levels of cytokines such as IL-1β and TNF-α have also been shown to stimulate HMGB1 secretion in different types of cells in vitro experiments. Here, we reported an up-regulated mRNA expression of proinflammatory molecules such as TNF-α, IL-1β, IL-6, IL-8R, iNOS and COX-2 in the peri-hematomal region after ICH induction, and a significant reduction in the expression of these molecules in the peri-hematomal region at 24 h after intravenous injection of anti-HMGB1 mAb. Hence, HMGB1 release may be one of the causative factors to enhance the expression of these proinflammatory molecules after ICH. Up-regulation of these inflammatory molecules probably contributes to astroglial proliferation and neutrophil recruitment in the region surrounding the hematoma, and causes BBB damage and brain edema as in cerebral ischemia, traumatic brain injury, and neurodegenerative disorders. In addition, a recent study showed that IL-1β was bound to HMGB1 with high affinity and the association of HMGB1 with IL-1β further potentiated the inflammatory responses, as the cellular activation induced by the HMGB1-IL-1β complex was significantly greater than that found with equivalent amounts of IL-1β or HMGB1 alone. In regard to the protein expression of IL-1β, we clearly showed that it was strongly up-regulated in control ICH rats, and that this up-regulation was considerably inhibited by anti-HMGB1 mAb therapy. It is therefore possible that anti-HMGB1 mAb, which has ability to neutralize released HMGB1, diminished the synergistic proinflammatory effects between HMGB1 and IL-1β. In addition, COX-2 inhibition treatment by celecoxib can reduce the infiltration of inflammatory cells and brain edema, resulting in a reduction of TUNEL-positive peri-hematomal cell death. Notably, anti-HMGB1 mAb therapy may suppress plural pathways in the secondary inflammatory responses after ICH.

Blood components (e.g., thrombin, hemoglobin, iron) and the inflammatory responses they induce play a major role in ICH-induced BBB dysfunction. Earlier studies showed up-regulation of MMP-2 and MMP-9, as well as acute brain injury, after ICH. Although collagenase IV is itself one of the MMPs, it has no effect on the expression of other MMPs. These findings suggest that the increase in MMP-2 and MMP-9 after ICH in our study probably contributed to further BBB disruption after collagenase. Indeed, the synthetic inhibitors of MMPs (BB-1101 and GM6001) have been shown to reduce BBB permeability and hemorrhage after ICH in animal models. Here, we

demonstrated that anti-HMGB1 mAb achieved a considerable reduction in collagenase IV-induced BBB disruption in the striatum, in association with a decrease of MMP2/9 expression. In addition, the mRNA expression of pro-inflammatory molecules (TNF-α, IL-1β, IL-6, IL-8R, iNOS and COX-2) and the protein expression of IL-1β were significantly increased after ICH induction. The increased levels of these molecules should be associated with a breakdown of BBB and recruitment of neutrophils into the CNS. Since our previous investigations using an in vitro BBB system clearly demonstrated that recombinant human HMGB1 increased the vascular permeability of BBB in association with morphological changes in endothelial cells and pericytes, in the present study, HMGB1 probably facilitated BBB disruption both directly and indirectly through induction of the expression of cytokines, MMPs and chemokines.

[結論]

In conclusion, our findings lend support to the idea that HMGB1 contributes to ICH-induced secondary inflammatory responses, which in turn cause the microglial activation, cytokine expression and BBB disruption. Our results also suggest that anti-HMGB1 mAb could be a valuable neuroprotective agent for the treatment of ICH, even if the treatment is initiated at 3 h after the onset of hemorrhage. We propose that anti-HMGB1 mAb treatment could be a novel therapeutic strategy applicable for three types of stroke: ICH, brain infarction and subarachnoid hemorrhage.

副　論　文

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(抗 HMGB1 抗体はラットくも膜下出血後の遅発性脳血管攣縮と脳損傷を緩和する)

Although delayed cerebral vasospasm (DCV) following subarachnoid hemorrhage (SAH) is closely related to the progression of brain damage, little is known about the molecular mechanism underlying its development. HMGB1 plays an important role as an initial inflammatory mediator in SAH. In this study, an SAH rat model was employed to evaluate the effects of anti-HMGB1 mAb on DCV after SAH. A vasoconstriction of the basilar artery (BA) associated with a reduction of nuclear HMGB1 and its translocation in vascular smooth muscle cells were observed in SAH rats, and anti-HMGB1 mAb administration significantly suppressed these effects. Up-regulations of inflammation-related molecules and vasoconstriction-mediating receptors in the BA of SAH rats were inhibited by anti-HMGB1 mAb treatment. Anti-HMGB1 mAb attenuated the enhanced vasocontractile response to thrombin of the isolated BA from SAH rats and prevented activation of cerebrocortical microglia. Moreover, locomotor activity and weight loss recovery were also enhanced by anti-HMGB1 mAb administration. The vasocontractile response of the BA under SAH may be induced by events that are downstream of responses to HMGB1-induced inflammation and inhibited by anti-HMGB1 mAb. Anti-HMGB1 mAb treatment may provide a novel therapeutic strategy for DCV and early brain injury after SAH.

【主論文との関連性】
◎主論文の内容と副論文の内容との直接的関連性について

Both of two papers strongly indicate that HMGB1 was translocated into extracellular space and HMGB1 plays a critical role in the development of ICH-induced secondary injury and vasospasm after SAH through the amplification of plural inflammatory responses. Intravenous injection of neutralizing anti-HMGB1 mAb provides a novel therapeutic strategy for different types of stroke.

◎論文相互間の引用の有無について

Supplementary research paper is cited in the thesis.