Therapeutic effects of anti-HMGB1 monoclonal antibody on pilocarpine-induced status epilepticus in mice

(抗HMGB1モノクローナル抗体によるピロカルピニン誘導性癲癇マウスの病態改善効果の検討)

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(Epilepsy is a disabling neurological disorder affects around 60 million people of all ages worldwide. Temporal lobe epilepsy (TLE) is the most common form of partial epilepsy, affecting at least 20% of all seizure patients. Pilocarpine-induced epilepsy is a well-established animal model for status epilepticus (SE) and has also been reported as a good candidate for human TLE. HMGB1, a non-histone DNA-binding protein, has been suggested to play important roles in the regulation of gene expression, DNA repair, and maintenance of chromatin structure. HMGB1 appears to be a mediator of the neurovascular unit, and excessive HMGB1 release may be associated with brain injury and dysfunction. Therefore, we hypothesized that HMGB1, as a pro-inflammatory cytokine-like molecule, might be involved in the development of epileptogenesis, especially through BBB disruption and induction of inflammatory processes. This study was designed to determine whether HMGB1 is involved in the pathogenesis of epilepsy or the ability of anti-HMGB1 mAb to protect BBB permeability, neurons and inflammation)

Experimental animals
Female C57BL/6 N mice weighing 18 to 23 g were used.

Model of pilocarpine-induced epileptic state
Mice were pre-treated with methyl-scopolamine bromide (1 mg/kg, i.p.) followed by pilocarpine (350 mg/kg or 300 mg/kg, i.p.) 30 min later. Anti-HMGB1 mAb (1 mg/kg, i.v.), class-matched control IgG mAb (1 mg/kg, i.v.), or PBS was administered intravenously 1 min after first stage 5 seizure.

Measurement of BBB permeability
To determine the BBB permeability under acute status epilepticus condition, mice were under deep anesthesia with sodium pentobarbital (50 mg/kg) and were perfused via the left ventricle with 30 ml of cold saline (0.9% NaCl). Brains were immediately removed and weighed, and the cerebrum was dissected. The whole cerebrum was placed in 1 ml of 1 M potassium hydroxide, then kept overnight at 4°C and homogenized. A 0.5 ml aliquot of the homogenized suspension was mixed with 1 ml of a mixture of 0.6 M phosphoric acid and acetone (5:13), and the resulting solution was centrifuged at 17400 g for 30 min. Finally, the supernatant solution was transferred to a cuvette, and the absorbance
at 620 nm was measured. Data were expressed as Evans blue ng/g wet cerebrum weight.

**Measurement of HMGB1 concentrations in the cerebrum and plasma during the acute phase of status epilepticus.**

Mice were anesthetized deeply with an i.p. injection of sodium pentobarbital (50mg/kg) and perfused by cold saline. Cerebra in each group were homogenized in cold radioimmunoprecipitation assay buffer (RIPA) (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 0.1% Triton X-100, and 50 mM Tris-HCl (pH 8.0)) with cocktail protease inhibitors. The brain homogenates were then centrifuged at 10,000 g for 20 min. The protein concentration in the supernatant was detected by Bio-Rad Protein Assay. β-actin was used as a reference protein. Mouse anti-HMGB1 mAb and mouse anti-β-actin mAb were used as primary antibodies followed by HRP-conjugated goat anti-mouse IgG. After washing in T-TBS, an ECL system was used to visualize the bands of HMGB1 and β-actin.

**Histochemical staining and TUNEL assay**

Samples were collected 4 h after anti-HMGB1, control IgG or PBS injection under acute seizure condition. The brains of the epileptic mice were perfused with 0.9% NaCl from the left ventricle, then fixed with 10% formalin and post-fixed overnight in 10% formalin at 4°C. The samples were then embedded in paraffin and cut at a thickness of 5 µm. Brain sections were stained with hematoxylin-eosin. Apoptotic cells were measured by the in situ terminal deoxynucleotidyl transferase-mediated dUTP-biotinnick end-labeling (TUNEL) method using an in situ Apoptosis Detection Kit. Morphological changes of nuclei were observed under a BZ-X700 All-In-On fluorescence microscope.

**Immunofluorescence staining**

Mice were anesthetized deeply with an i.p. injection of sodium pentobarbital (50mg/kg) and perfused by cold saline followed with 10% formalin. Brains were post-fixed overnight under 4°C condition in 10% formalin and paraffin-embedded. Brain sections were cut at a thickness of 5 µm. Antigen retrieval was performed by incubating the section with 10 mM sodium citrate buffer (pH 6.0) for 10 min at 120°C. Incubation with 10% normal goat serum and 1% bovine serum albumin (BSA) in 10 mM TBS for 2 h at room temperature was used to block nonspecific binding. Brain sections were stained with mouse anti-HMGB1 mAb, rabbit anti-MAP2 pAb, rabbit anti-GFAP pAb, rabbit anti-Iba-1 Ab or rabbit polyclonal anti-IL-1β antibody as the primary antibody overnight. Alexa 555-labeled anti-mouse IgG and Alexa 488-labeled anti-rabbit IgG were used as the secondary antibodies and incubated for 1 h at room temperature. VectorShield Hard Set Mounting Medium with DAPI was used for mounting. Stained sections were observed under an LSM 780 confocal imaging system. The number of specific cells was counted in four randomly chosen squares.

**Cell morphology and cell counting quantification**

The H&E staining was used for identifying the pyknotic cells. The apoptotic cells were defined as those with nuclear staining with a dark-brown color. Reactive astrocytes usually shown as cell swelling with fine spongiform process with a greater number of main process extending from the soma and the GFAP immunoreactivity exhibited a distinct increase. We set two criteria to identify activated astrocytes: 1) the thickness of main process were over 2.5 µm; 2) the length of processes from soma was more than 15 µm. Astrocytes met both criteria were identified as activated astrocyte and counted. Under resting conditions, microglia in the CNS were ramified. The resting microglial cells were
characterized by a small cell soma and several branching thin processes that extend in all directions. However, when brain insult causes a transformation from ramified into activated microglia even an amoeboid form, these processes retracted microglia cell processes and become fewer and much thicker. At the same time, the size of the cell bodies will increase. Then, the criteria for activated microglia were 1) the thickness of process was over 3 µm; 2) the cell soma was oval shape and the longest diameter was longer than 12 µm; 3) the visible processes were shorter than 5 µm from the soma. Microglia cells met these three criteria were identified as activated microglia and counted. The highly IL-1β-immunoreactive cells were identified as those having more than four times higher intensity of fluorescence accompanied with sham average control cells in the same area.

Real-time polymerase chain reaction

For analyzing the expression of inflammation-related molecules, samples were taken from the hippocampus and cerebral cortex 24 h after the treatment with anti-HMGB1 mAb, control IgG mAb or PBS under an epileptic state. Total RNA was extracted from the mouse hippocampus and cerebral cortex by using an RNeasy mini kit according to the manufacturer’s protocol. The RNA was then reverse-transcribed to cDNA by a reverse transcriptase kit-RNA PCRT M Kit (AMV) Ver 3.0. The real-time polymerase chain reaction analysis using SYBR Premix EX Taq in a Light Cycler instrument according to the manufacturer’s instructions. The expression of GAPDH was used to normalize cDNA levels. PCR products were analyzed using a melting curve to ascertain the specificity of amplification.

Behavior and latency test

This experiment, we divided this part into two sections, firstly, mice were treated with PBS, control IgG (1 mg/kg) or anti-HMGB1 mAb (1 mg/kg) immediately after pilocarpine injection. The time to the onset, first latency to stage 5 and the frequency of stage 5 were all recorded in an observation period of 60 min. Secondly, mice were pretreated with anti-HMGB1 mAb (1 mg/kg), control IgG (1 mg/kg) or PBS 4 h before pilocarpine (350 mg/kg) injection. After the injection of pilocarpine, the time to the onset, first latency to stage 5, and the time to death were all recorded in an observation period of 60 min. All the data were counted by an examiner who was blinded to the experimental protocol. Mice under Racine stage 5 were excluded from this experiment.

【結果】
Effects of anti-HMGB1 mAb on BBB integrity under an acute epileptic state

Pilocarpine-induced acute status epilepticus dramatically increased the leakage of Evans blue, especially into the thalamus and hypothalamus regions, However, after anti-HMGB1 mAb treatment the concentration of Evans blue dye decreased during pilocarpine-induced acute status epilepticus, while the control IgG group did not show any reduction in BBB leakage.

Detection of HMGB1 dynamics after pilocarpine injection

Acute status epilepticus led to a significant reduction of the amount of HMGB1 in the cerebrum due to the HMGB1 translocation. However, treatment with anti-HMGB1 mAb partially inhibited the HMGB1 translocation and increased the amount of HMGB1 in mice in an acute status epilepticus compared with the PBS and control IgG groups. At the same time, the plasma concentrations of HMGB1 were significantly higher in the PBS and control IgG groups than in the sham and anti-HMGB1 mAb-treatment groups, indicating an increase in HMGB1 translocation into the peripheral circulation. Anti-
HMGB1 mAb significantly suppressed the HMGB1 levels in plasma.

**Evaluation of HMGB1 translocation in the CA1 hippocampal and cerebral cortex regions by immunofluorescence staining.**

Treatment with anti-HMGB1 mAb not only inhibited the distribution pattern of histogram to the left direction by pilocarpine but also the number of small granules outside nuclei. Similar results were found in the cerebral cortex neurons. HMGB1 translocation and release occurred in the CA1 and cerebral cortex regions under an acute epileptic state, and anti-HMGB1 mAb treatment could inhibit the HMGB1 translocation into the cytoplasm and extracellular space.

**Histochemical staining**

Acute epileptic state increased the number of pyknotic and shrunk cells, but anti-HMGB1 mAb treatment significantly reduced the number of pyknotic cells. The pyknotic cells are indicated by the black arrows in. The number of pyknotic cells was significantly decreased after anti-HMGB1 mAb treatment. Anti-HMGB1 mAb conferred protective effects on CA1 hippocampal neurons under an acute epileptic state.

**Morphological changes of astrocytes and microglial cells in the CA1 and dentate gyrus hippocampal regions by immunofluorescence staining**

The number of activated astrocytes and microglia had increased in the CA1 region of epileptic mice after treatment with PBS or in the Control IgG mice. Anti-HMGB1 mAb treatment significantly reduced the number of astrocytes and microglial cells while simultaneously maintaining or recovering their original cell shape. A similar trend was seen in the dentate gyrus region. The neutralization of HMGB1 by anti-HMGB1 mAb significantly suppressed the acute status epilepticus-caused astrocytes and microglia cells activation.

**Evaluation of IL-1β expression in the CA3 region and thalamus under an acute status epilepticus**

We found that IL-1β was expressed at very low levels in the nuclei of CA3 pyramidal cells and thalamic cells in intact mice. At the same time the intensity of staining in the PBS and control IgG mice was much higher than in the sham group. Anti-HMGB1 treatment also remarkably inhibited the number and the fluorescence intensity of highly IL-1β-immunoreactive cells in both the CA3 and thalamus.

**Determination of inflammation-related molecules in the hippocampus and cerebral cortex under an acute epileptic state**

The expressions of hippocampal MCP-1, CXCL-1, TNF-α, HIF-1α, and TLR-4 were significantly upregulated in the PBS and Control IgG-treatment groups, whereas anti-HMGB1 antibody treatment suppressed the hippocampal expression of these inflammation-related factors. A similar trend was observed in the cerebral cortex region.

**Behavior and latency test**

Pretreatment of anti-HMGB1 mAb 4 h before pilocarpine treatment reduced the Racine stage 5 frequencies, and increased both the latency to stage 5 and the time to death. Moreover, anti-HMGB1 mAb treatment immediately after pilocarpine injection increased the latency to onset and to the stage
5, while at the same time lowering the stage 5 frequency.

【考察】

The cholinergic agonist pilocarpine has been widely used experimentally to mimic SE in rodents. Pilocarpine-induced epilepsy is triggered primarily by the activation of M1 muscarinic receptors in the central nervous system (CNS), and the seizures are maintained by N-Methyl-D-aspartate (NMDA) receptor activation. Systemic administration of pilocarpine in rodents reproduces the principle features of human TLE, i.e., limbic seizures, secondary generalized seizures and SE that lasts for several hours.

BBB disruption and inflammatory responses have been strongly suggested to be involved in the epileptogenic process for decades. In fact, Evans blue dye leakage after pilocarpine injection in mice was previously reported and was confirmed in the present study. Although there are many candidate molecules for induction of the increase in BBB permeability, the fact that a specific antibody against HMGB1 significantly inhibited the BBB permeability clearly demonstrated that HMGB1 is one such molecule and plays an important role in the increase in BBB permeability in the acute phase of epilepsy. Moreover, we found that the BBB disruption was exacerbated after the administration of exogenous HMGB1, implying the possible influence of systemic inflammation on vascular damage in the brain, which has also been reported in experimental stroke and brain trauma in addition to status epilepticus.

Taken together, the translocation of HMGB1 in neurons, decreased HMGB1 levels in the brain and increased HMGB1 level in plasma in mice with pilocarpine-induced epilepsy support the notion that HMGB1 was translocated and released from neuronal nuclei into the surrounding areas, including the blood stream. The mobilization pattern and dynamics of HMGB1 in mice with pilocarpine-induced epilepsy were quite similar to those observed in ischemia trauma and neurotoxin induced injuries. Moreover, the inhibition of HMGB1 mobilization by anti-HMGB1 was common to different types of brain injuries, suggesting the existence of HMGB1-induced mechanisms of HMGB1 release in all brain injuries examined. In a previous study, our group demonstrated the direct effects of HMGB1 on vascular endothelial cells and pericytes leading to contractile responses of these cells and the BBB breakdown. Based on these findings, it was speculated that both HMGB1 release into the CNS and HMGB1 release into the peripheral blood stream after pilocarpine injection contributed to the induction of BBB breakdown.

The activation of astrocytes and microglia in pilocarpine-induced acute status epilepticus was also confirmed by the increase in the number of cells and the activation-specific cell shape not only in the hippocampal but also the cerebral cortex regions. This activation of cells was significantly inhibited by the systemic administration of anti-HMGB1 mAb. Thus, the treatment of anti-HMGB1 mAb not only decreased BBB breakdown but also suppressed the activity of intrinsic inflammatory cells in the brain. Consistent with these findings, the determination of the expression of a representative pro-inflammatory cytokine, IL-1β, by immunohistochemistry clearly revealed the upregulation of IL-1β in neurons in the hippocampus and thalamus in pilocarpine-induced acute status epilepticus in mice. The up-regulation of IL-1β expression was once again significantly inhibited by the anti-HMGB1 administration. Furthermore, quantitative RT-PCR revealed that the up-regulated expressions of MCP-1, TNF-α, IL-6 and CXCL-1 were all suppressed by anti-HMGB1 mAb treatment. Since these cytokines are suggested to be involved in epileptogenesis through the amplification of inflammation and neural network remodeling in kindling as well as kainate-induced model, it is likely that anti-
HMGB1 treatment can inhibit the downstream processes induced by these cytokines. Among the upregulated cytokines, IL-1β has been reported to form a complex with HMGB1, and the resultant complex increased the affinity of IL-1β to IL-1β receptors. Therefore, it is worth noting that the inhibitory effects of anti-HMGB1 on IL-1β expression are particularly important with regard to the suppression of enhanced and synergistic inflammation by the combination of HMGB1 and IL-1β. Inhibition of inflammatory factors is becoming a novel component in the recently proposed etiologies for epilepsy. However, the simultaneous suppression of several inflammatory cytokines by a single drug would be difficult. Nonetheless, anti-HMGB1 mAb would seem to be a candidate for such a drug therapy, due to its ability to inhibit the expression of a diverse range of inflammation-related molecules.

[結論]

In conclusion, we demonstrated that intravenous treatment with neutralizing anti-HMGB1 mAb conferred protective effects on neuronal apoptosis, in association with an inhibition of HMGB1 release, protection of the BBB and inhibition of inflammation in a very-acute-phase of status epilepticus induced by pilocarpine. These effects of anti-HMGB1 mAb as a whole appear to lead to the prolongation of seizure latency and seizure frequencies in pilocarpine-induced acute status epilepticus. Thus, anti-HMGB1 therapy may provide a novel strategy for controlling the epileptogenesis.