

Assessment of motor function

An accelerated rotarod test (4–40 rpm for a maximum of 5 min; Muromachi, Kyoto, Japan) test was performed at 3, 6 and 24 hours after brain injury. The performance scores at each time were expressed relative to 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.

Histochemical staining

Immunohistochemical staining was performed as described previously.¹⁹ Brain sections were also stained with hematoxylin-eosin and cresyl violet (Nissl stain).

MRI examination

MRI examination was performed as described previously.²⁰ Rat brain water changes were examined in a 3.0-T horizontal-bore magnet (GE Signa EXCITE 3.0 T; GE Healthcare, Buckinghamshire, UK) at 3, 6, and 24 hours after percussion injury (n = 5 per group). MRI parameters were set with TR/TE 5000/115.18 ms, thickness/gap = 2/0.2 mm, field of view = 100 mm × 100 mm, matrix = 480 × 480, number of excitations = 1.5. The body temperature was maintained at 37.0 ± 0.5°C by a heating fan. All MR images were evaluated and interpreted by 2 independent operators experienced in MRI. Processing of the MRI digital imaging and communications in medicine (DICOM) formatted data sets were performed using OsiriX (open-source) for Mac. The volume of the acute lesion was calculated at each time point. The

percentage of edematous high intensity areas in both hemispheres was calculated.

During MRI, data for 5 serial tissue sections (2 mm thick) were obtained.

Evaluation of blood–brain barrier integrity by Evans blue

BBB permeability was assessed by measuring the extravasation of intravenous injected Evans blue.¹⁹ Briefly, 2% solution of Evans blue dye in 0.9% NaCl was administered intravenously over 1 min in a dose of 2 mL/kg at 6 hours after percussion injury, and then allowed to circulate for 3 hours prior to sacrifice. After perfusion with 100 mL of saline via a catheter inserted into the left ventricle of the heart, the brains were immediately removed. The cerebrum of the brain was cut with a blade into the right and left hemispheres along the anatomic midline. The ipsi-lateral percussion side was weighed, placed in 4 ml of 1M KOH and homogenized. Then 1 ml of the homogenized cocktail was placed in 5 ml of the mixture of 0.2 M phosphoric acid and acetone, whose ratio is 5 to 13, and centrifuged (3,000 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 (ng)/ g wet brain weight.

Determination of HMGB1

Plasma levels of HMGB1 were determined by ELISA (Shino-Test Co, Sagamihara, Japan) as described previously.²⁰ Western blotting of HMGB1 in the cerebral cortex from both sides after injury was performed as described previously.²⁰ Brain samples 3 mm square from the injury site were collected 24 hours after injury. β -actin was used as a reference protein.

Real-time PCR

Real-time PCR was performed as described previously¹⁹ with the SYBR Premix EX *Taq* (Takara) in a Light Cycler instrument (Roche) according to the manufacturer's instructions. The sense and antisense primers used for the analysis of mRNA expression are the followings; iNOS: 5'- GCATCCCAAGTACGAGTGGT-3' and 5'-GAAGTCTCGGACTCCAATCTC -3'; TNF- α : 5'- GCCCAGACCCTCACACTC-3' and 5'-CCACTCCAGCTGCTCCTCT -3'; MMP-2: 5'-GCACCGTCGCCCATCA-3' and 5'- GTCTCGATGGTGTCTGGTCAA-3'; MMP-9: 5'-GAGGATCCGCAGTCCAAGAA -3' and 5'-GCACCGTCTGGCCTGTGTA-3'; HIF1 α : 5'-ACAAGTCACCACAGGACAG-3' and 5'-AGGGAGAAAATCAAGTCG-3'; COX-2: 5'-TGTATGCTACCATCTGGCTTCGG-3' and 5'-GTTTGGAACAGTCGCTCGTCATC-3'; VEGF-A₁₈₉: 5'-CTCACCAAAGCCAGCACATA-3' and 5'-CCCTTTCCTCGAACTGAT-3'; VEGF-A₁₆₅: 5'-CTCACCAAAGCCAGCACATA-3' and 5'-TCTGAACAAGGCTCACAGTGA-3'; VEGF-A₁₂₁: 5'-CTCACCAAAGCCAGCACATA-3' and 5'-GCCTTGGCTTGTCACATT-3'; PAI-1: 5'-TGGCTCAGAACAACAAGTTCAAC-3' and 5'-GGCAGTTCCAGGATGTCGTA-3'; Neutrophil elastase: 5'-TCCAGCTCAATGGCTCAGCT-3' and 5'- ACGGAGTTCTGTTACCCAC-3'; IL-8R: 5'-CATCCTGCCTCAGACCTATGG-3' and 5'-AAGACGAGGACCACAGCAAAG-3'; GAPDH: 5'-

AGCCCAGAACATCATCCCTG-3' and 5'-CACCCACCTTCTTGATGTCATC-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.

Transmission electron microscopic examination

Transmission electron microscopic examination was performed as described previously.²⁰ Briefly, 6 hours after percussion, TBI rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) and perfused through the left ventricle with 50 mL of saline followed by 100 mL of 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/L cacodylic acid buffer (pH 7.3). The fixed brain was dehydrated, embedded in epoxy resin and cut into ultrathin sections. The sections were mounted on copper grids, stained in uranyl acetate and citric acid lead, and then observed under a transmission electron microscope (H-7100; Hitachi Ltd, Tokyo, Japan) equipped in the central laboratory of Okayama University. To quantify astrocyte endfeet swelling, NIH Image J software was used to calculate the ratio of the total area of swollen astrocyte endfeet surrounding against the area of the corresponding capillary lumen. Five rats were analyzed for each group treated with control Ab or anti-HMGB1 mAb.

Zymography

Gelatin zymography was performed as described previously¹⁹ with slight modification. Brain samples (50 mg) from the bilateral cerebral cortex were homogenized and

centrifuged, and the resultant supernatant (20 μ g protein) was used for gelatin zymography without further purification by gelatin-agarose. The results were quantified with NIH Image J software.

Statistical analyses

Statistical significances were performed using ANOVA followed by Student's *t*-test or Dunnett's test. Mann-Whitney U-test was used for statistical analysis for rotarod test and real-time PCR experiments. P values less than 0.05 were considered to be significant.

Results

Translocation of HMGB1 at Injured Site and Effect of Anti-HMGB1 mAb

In the fluid percussion-induced injury model, translocation of HMGB1 from the nuclei to the cytosol was remarkable in MAP2-positive neurons (Fig 1A) but not in GFAP-positive astrocytes or Iba1-positive microglia (data not shown) at the site below percussion 3-6 hours after injury in control rats treated with anti-*keyhole limpet* hemocyanin (1 mg/kg, i.v.). The neuronal translocation of HMGB1 occurred throughout the cerebral cortex in control rats in a time- and space-dependent manner. No such translocation was observed on the contralateral side in control rats. HMGB1 immunoreactivity disappeared from neurons at the primarily lesion site at 24 hours after percussion injury. In some neurons, cytosolic granule-like structures were evident 6 hours after injury.

The administration of anti-HMGB1 mAb (1 mg/kg, i.v.) significantly inhibited the translocation of HMGB1 in neurons and maintained the immunoreactivity in the nuclei (Fig 1A). The translocation pattern of HMGB1 and the suppressive effects of anti-HMGB1 mAb were similar to those occurring in ischemic brain regions in previous reports^{19,20}. In accordance with the results of immunohistochemical staining, western blotting revealed that anti-HMGB1 mAb suppressed the disappearance of HMGB1 from the site of injury (Fig 1B, C).

Effect of Anti-HMGB1 mAb on Neuronal Death and BBB Permeability

Representative coronal sections fixed at 6 hours after percussion injury and stained with hematoxylin-eosin and cresyl violet (Nissl stain) are shown in Fig 2A. In the cerebral cortex of control rats, necrosis developed under the percussion site accompanied by the loss of Nissl-positive pyramidal neurons whereas neurons in anti-HMGB1-treated rats remained intact. We confirmed that there were no differences between the anti-HMGB1 mAb-treated and control mAb-treated groups with respect to the intensity of percussion impulses, blood gasses, biochemical parameters and body temperature 30 minutes after fluid percussion injury (Table 1).

Evans blue leakage was measured to assess extravasation of serum albumin from capillary vessels in the TBI model. Increased BBB permeability was apparent 6 to 9 hours after percussion injury above the injured hemisphere beyond the primary lesion site (Fig 2B, C). Anti-HMGB1 treatment significantly inhibited Evans blue leakage by 88%, restricting the leakage area to the primary lesion site. The immunostaining of rat albumin in coronal brain sections showed that BBB permeability was increased throughout the cortical layer in control rats, whereas staining was minimal in anti-HMGB1-treated rats (Fig 2D).

Evaluation of Brain Edema by T2-Weighted MRI

Brain edema was detected by T2-weighted MRI serially up to 24 hours after percussion injury. As shown in Fig 3A, brain edema centered around the percussion site was evident as early as 3 hours after injury in control rats and the edema persisted for 24 hour. In contrast, the high intensity area was restricted to a very narrow region on the surface of the cerebral cortex in anti-HMGB1-treated rats. Brain edema did not

increase at all in anti-HMGB1-treated rats even at 24 hours post-injury (Fig 3B). The distribution and size of edematous regions in the brain detected by T2-weighted MRI were consistent with the results of Evans blue leakage.

Effects of anti-HMGB1 mAb on Motor Activity

Impairments of coordinated locomotor activity and lateral dominance of forelimb movement after percussion injury were evaluated using rotarod and cylinder tests, respectively (Fig 4A, B). Walking time before and after brain injury was measured by the rotarod test and expressed as a ratio. In control rats, the ratios were significantly reduced compared with sham controls at 3, 6 and 24 hours after percussion injury. Although the ratio at 3 hours after percussion injury in the anti-HMGB1-treated group was lower than that of the sham-control group, there was no difference between the sham control and anti-HMGB1-treated groups at 6 and 24 hours after injury. Conversely, there were significant differences between control IgG-treated and anti-HMGB1-treated groups at 6 and 24 hours after injury. Cylinder testing to evaluate the right-left dominance of forelimb movement showed a time-dependent decrease in contralateral forelimb in control rats while no lateral dominance of forelimb movement was noted in anti-HMGB1-treated rats throughout the observation periods.

BBB Disruption in TBI and its Protection by Anti-HMGB1 mAb

Electron microscopic observations revealed that there were marked structural changes in the capillary microvasculatures in the ipsilateral cerebral cortex of control rats. Swelling of astrocytic endfeet surrounding vascular endothelial cells was apparent at 6

hour after injury (Fig 5A). Detachment of the cytoplasmic membrane of astrocyte endfeet from the lamina propria of capillary vessels, as well as the deformation in vascular endothelial cells were often observed in control rats. Quantification of astrocyte endfeet swelling in the cerebral cortex clearly showed that anti-HMGB1 therapy significantly inhibited the swelling (Fig 5B). Zymography revealed that MMP-9 and MMP-2 activity in injured cerebral cortex of control rats was increased considerably whereas the increase in the anti-HMGB1-treated group was significantly suppressed (Fig 5C, D). This was also true for the contralateral side although the increase in activity of MMPs was relatively small.

Expression of Inflammatory Molecules

To analyze the anti-inflammatory mechanism for the effects of anti-HMGB1 mAb, we examined the expression of inflammation-related molecules on both sides of the cerebral cortex using real-time PCR (Fig 6). Rapid and transient expression of TNF- α and iNOS was observed after percussion injury, and the expression of both was significantly suppressed following treatment with anti-HMGB1. The expression of HIF-1 α , COX-2, VEGF-A₁₈₉ and VEGF-A₁₆₅ was up-regulated in both hemispheres, suggesting the presence of induction mechanism mediated by neural connections between hemispheres or diffusible humoral factors. The expression of PAI-1, IL-8R and neutrophil elastase was also up-regulated in the ipsilateral side of the control rats, indicating the activation of vascular endothelial cells and the presence of neutrophils in the vasculatures in the injured side. These changes were also suppressed by treatment with anti-HMGB1 mAb.

Effects of Recombinant HMGB1 on TBI

Intravenous injection of recombinant HMGB1 (0.04, 0.2 and 0.4 mg/kg) dose-dependently enhanced the increase in BBB permeability induced by moderate intensity percussion (2.0-2.2 atm) (Fig 7A, B). In addition, intravenous administration of recombinant HMGB1 produced dose-dependent exacerbation of motor impairments as measured by the rotarod test (Fig 7C). Thus, circulating HMGB1 may impair BBB integrity and facilitate the breakdown of the BBB. Determination of plasma levels of HMGB1 in rats treated with the percussion intensity of 2.2–2.6 atm (Fig1-6) showed that a 10-fold increase in HMGB1 levels occurred 6 hours after percussion injury (Fig 7D). This increase was almost completely inhibited by intravenous administration of anti-HMGB1 mAb which did not interfere with ELISA assay for HMGB1²⁰.

Evaluation of Receptor Involvement Using Gene Knockout Mice

To investigate the receptors involved in the action of HMGB1, we performed percussion experiments using RAGE^{-/-}, TLR-4^{-/-}, TLR-2^{-/-} and corresponding wild-type mice. In wild-type mice, inhibition of BBB permeability by anti-HMGB1 treatment was evident, as was observed in the rat experiments (Fig 8A). In RAGE^{-/-} mice, the increase in BBB permeability was lower than that in wild-type mice after the same intensity of percussion injury. Treatment of RAGE^{-/-} mice with anti-HMGB1 mAb did not produce any inhibitory effects on BBB permeability, suggesting that RAGE plays a crucial role in mediating the effects of HMGB1. In contrast, the increase in BBB permeability in TLR4^{-/-} or TLR-2^{-/-} mice induced by the same intensity of

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percussion was not different from that of wild-type mice. However, the inhibitory effects of anti-HMGB1 in TLR4^{-/-} and TLR-2^{-/-} mice was not statistically significant. Moreover, anti-HMGB1 mAb did not antagonize the motor impairment induced by percussion injury in RAGE ^{-/-} mice, although the Ab significantly ameliorated motor impairment in wild-type mice (Fig 8B). In TLR4^{-/-} and TLR-2 ^{-/-} mice, the effects of anti-HMGB1 were intermediate but were not statistically significant.

Discussion

In the present study, we observed the marked translocation of HMGB1 from the nucleus to cytoplasm in lesion sites within the cerebral cortex after fluid percussion injury in rats. The time-dependent pattern and the neuron-dominant translocation were quite similar to those observed in ischemic rat brain in previous studies.^{20, 23} Thus, the translocation of HMGB1 in neurons appears to be phenomena common to both ischemic and traumatic insults. Alternatively, the secondary brain ischemia due to brain edema in TBI^{24, 25} might be a primary factor involved in the induction of neuronal translocation of HMGB1. The reduction in HMGB1 levels in the lesion site and the significant elevation of plasma levels of HMGB1 after percussion injury strongly support the notion that neuronal HMGB1 is released into extracellular space and diffuses into surrounding areas including the blood stream.²⁰ The strong inhibitory effects of anti-HMGB1 mAb on the neuronal translocation of HMGB1 suggests the existence of a vicious cycle for extracellular HMGB1-induced facilitation of HMGB1 translocation in TBI as well as in ischemic brain injury.²⁰

We clearly demonstrated in the present study that the treatment of TBI rats with anti-HMGB1 neutralizing mAb dramatically inhibited acute brain edema, as detected by T2-weighted MRI, associated with the concomitant decrease in BBB permeability, structural changes in capillary blood vessels, upregulation of MMP-2/9 activity, and expression of inflammatory molecules.^{19, 20} Direct effects of recombinant HMGB1 on reconstituted cultured BBB *in vitro* were shown in a previous study²⁰; therefore, it is possible that HMGB1 released from the TBI lesion caused the protein leakage by

directly affecting the microvasculature in surrounding areas. Judging from the marked increase in the permeability of the BBB at 6-9 hours after percussion injury and the considerable ultrastructural changes in capillary vessels observed at 6 hours, it was strongly suggested that the BBB breakdown occurred up until 6 hours after injury in the present model. In fact, T2-weighted MRI detected localized brain edema clearly at 3 hours and beyond, indicating that the BBB breakdown started at least from this time point onwards in control rats. Similar ultrastructural observations showing astrocyte swelling and endothelial degeneration were reported at an earlier time point after fluid percussion injury.²⁶ Thus, BBB breakdown appears to be a very early event after percussion injury. In contrast, brain edema detected by T2-weighted MRI in rats treated with anti-HMGB1 mAb was minimal throughout the observation periods (Fig 3A). In parallel with the marked effects of anti-HMGB1 mAb on brain edema, this treatment reduced the increase in BBB permeability, MMP2/9 activity and expression of major inflammatory molecules including TNF- α and iNOS. Induction of MMP-2/9²⁷,²⁸ and iNOS,^{29,30} facilitating the digestion of tight junction-associated protein complex and extracellular matrix including basal lamina and the inflammatory response, respectively, has been suggested to be involved in TBI. Moreover, the inhibition of MMPs or iNOS by specific inhibitors was shown to result in the reduction of TBI to some extent.³⁰⁻³² Thus, the regulation of both MMP-2/9 and iNOS may contribute significantly to the protective effects of anti-HMGB1 mAb. The pharmacological effects of anti-HMGB1 therapy include protection of the BBB structurally and functionally, as well as inhibition of the expression of major inflammatory molecules and suppression of MMP-2/9 activity. These diverse effects connecting and depending

each other could explain the remarkable efficacy of anti-HMGB1 mAb therapy in TBI.

In other words, the action of HMGB1 could be present upstream of the secondary responses in TBI, triggering a cascade of events leading to BBB disruption, brain edema, and brain injury.^{1,33}

Consistent with the reduction in brain edema and decrease in the resultant brain lesion observed with anti-HMGB1 mAb, this therapy significantly improved the acute motor impairments induced by percussion injury throughout the observation period, except at 3 hours after injury. Thus, protection of the BBB from disruption as well as the control of inflammatory responses by treatment with anti-HMGB1 mAb accompanied the maintenance of CNS motor function. Hematoxylin-eosin and Nissl staining of brain sections from control rats revealed a considerable loss of neurons in the cerebral cortex whereas the area of neuronal loss was restricted to the surface of the cerebral cortex in anti-HMGB1-treated rats. These histological findings were consistent with the neurological evaluation data.

Intravenously administered recombinant HMGB1 dose-dependently exacerbated the increase in BBB permeability and motor impairment in rats receiving moderate percussion injury. Therefore, it is possible that circulating endogenous HMGB1 plays an important role in the development of BBB disruption and brain inflammation, especially through the stimulation of vascular endothelial cells and blood cells. Indeed, anti-HMGB1 mAb significantly inhibited the expression of plasminogen activator inhibitor-1, a marker of vascular endothelial cell activation, and neutrophil elastase, suggesting that the interaction between vascular endothelial cells and blood cells was reduced^{5,34} through the clearance of circulating HMGB1 by specific mAb (Fig 7D).

These aspects await clarification in future studies.

It has been reported that the effects of HMGB1 are mediated by plural receptors; i.e., receptor for advanced glycation endproducts (RAGE)^{12, 13} and toll-like receptor-2/4 (TLR-2/4).¹⁴⁻¹⁶ To examine the relative involvement of these receptors, we conducted studies using the respective gene knockout- and wild-type mice. From these experiments, it could be concluded that RAGE is predominantly involved in the mediation of HMGB1 effects because the increase in BBB permeability induced by percussion injury was much less in RAGE^{-/-} mice compared with wild-type mice and the inhibitory effects of anti-HMGB1 mAb observed in wild-type mice disappeared in RAGE^{-/-} mice. Concerning the TLR-2/4, the phenotype in knockout mice was intermediate and not so conclusive. Thus, further works in this area is needed.

Judging from the remarkable efficacy of anti-HMGB1 mAb in the therapy of rat TBI, as well its putative mechanisms, this novel therapy could be applicable for clinical use. Since uncontrolled brain edema leading to brain herniation is one of the main causes of death in patients after TBI,¹ early treatment with anti-HMGB1 mAb might be a promising strategy for TBI. In addition, patients with TBI often experiences posttraumatic epilepsy^{35, 36} and impairment in cognitive function.³⁷ Moreover, TBI has been considered as a risk factor for Alzheimer's disease.³⁸ Accordingly, it is possible that anti-HMGB1 mAb therapy might inhibit the secondary brain injury arising from TBI, and thereby greatly improve the prognosis for this condition and reduce the incidence of complications.

In conclusion, anti-HMGB1 mAb efficiently reduced acute brain edema after TBI through protection of the BBB and inhibition of the inflammatory responses. It was

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strongly suggested that endogenous HMGB1 may play plural and crucial roles in the early events of TBI and that the processes mediated by HMGB1 may be present upstream in the sequence of events. We propose mAb therapy as a novel treatment approach for TBI.

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