Anti-high Mobility Group Box-1 Monoclonal Antibody Protects the Blood–Brain Barrier From Ischemia-Induced Disruption in Rats

Jiyong Zhang, PhD*; Hideo K. Takahashi, MD, PhD*; Keyue Liu, MD, PhD; Hidenori Wake, PhD; Rui Liu, PhD; Tomoko Maruo, MD; Isao Date, MD, PhD; Tadashi Yoshino, MD, PhD; Aiji Ohtsuka, MD, PhD; Shuji Mori, PhD; Masahiro Nishibori, MD, PhD

Background and Purpose—High mobility group box-1 (HMGB1) exhibits inflammatory cytokine-like activity in the extracellular space. We previously demonstrated that intravenous injection of anti-HMGB1 monoclonal antibody (mAb) remarkably ameliorated brain infarction induced by middle cerebral artery occlusion in rats. In the present study, we focused on the protective effects of the mAb on the marked translocation of HMGB1 in the brain, the disruption of the blood–brain barrier (BBB), and the resultant brain edema.

Methods—Middle cerebral artery occlusion in the rat was used as the ischemia model. Rats were treated with anti-HMGB1 mAb or control IgG intravenously. BBB permeability was measured by MRI. Ultrastructure of the BBB unit was observed by transmission electron microscope. The in vitro BBB system was used to study the direct effects of HMGB1 in BBB components.

Results—HMGB1 was time-dependently translocated and released from neurons in the ischemic rat brain. The mAb reduced the edematous area on T2-weighted MRI. Transmission electron microscope observation revealed that the mAb strongly inhibited astrocyte end feet swelling, the end feet detachment from the basement membrane, and the opening of the tight junction between endothelial cells. In the in vitro reconstituted BBB system, recombinant HMGB1 increased the permeability of the BBB with morphological changes in endothelial cells and pericytes, which were inhibited by the mAb. Moreover, the anti-HMGB1 mAb facilitated the clearance of serum HMGB1.

Conclusions—These results indicated that the anti-HMGB1 mAb could be an effective therapy for brain ischemia by inhibiting the development of brain edema through the protection of the BBB and the efficient clearance of circulating HMGB1. (Stroke. 2011;42:1420-1428.)

Key Words: blood–brain barrier ■ brain edema ■ electron microscopy ■ HMGB1 ■ MRI

Disruption of the blood–brain barrier (BBB) is a critical event in the formation of brain edema during the early phase of ischemic brain injury. BBB permeability can be increased by several factors, including cytokines, vascular endothelial growth factor, and nitric oxide. Among these factors, cytokines have been widely described as vital players such as interleukin-1β and tumor necrosis factor-α. High mobility group box-1 (HMGB1) is a ubiquitous and abundant nonhistone DNA-binding protein, which is newly defined as a cytokine that can be passively released from necrotic cells or positively released from immune activated cells under the stimulation of inflammatory signals, thus inducing inflammatory responses in sepsis, acute lung injury, and rheumatoid arthritis. Recently, HMGB1 has received particular attention with respect to its pathological role in cerebral ischemia. In transient middle cerebral artery occlusion (MCAO) in mice and rats, HMGB1 was found to be translocated into the cytoplasmic compartment from nuclei. High levels of serum HMGB1 were observed in patients with stroke compared with healthy control subjects. This early release of HMGB1 into the extracellular space after ischemic injury may contribute to the initial stage of the inflammatory response in the ischemic penumbra.

We previously demonstrated that a neutralizing anti-HMGB1 monoclonal antibody (mAb) remarkably ameliorated brain infarction induced by a 2-hour MCAO in rats and was associated with significantly improved neurological deficits. Although the disruption of the BBB was apparent by the extravasation of
Evans blue dye even at 3 hours after reperfusion in the control animals, the anti-HMGB1 mAb efficiently inhibited protein leakage and the activation of matrix metalloproteinase-9, which has been suggested to be an initial factor inducing endothelial tight junction (TJ) degradation in BBB disruption. Therefore, we hypothesized that HMGB1 may contribute to BBB disruption during the acute phase of ischemia/reperfusion. The present study was undertaken to further investigate the mechanism of the HMGB1-neutralizing mAb from the aspect of maintaining the BBB functionally and structurally. First, we analyzed the time course of HMGB1 translocation and release from brain cells into the cerebrospinal fluid and bloodstream. Second, we observed the changes in the structure of BBB using transmission electron microscopy and T2-weighted MRI. Third, we used the in vitro BBB system to demonstrate the direct effects of HMGB1 on the components of the BBB. The results strongly indicated that HMGB1 may induce morphological and functional changes in the BBB, whereas the anti-HMGB1 mAb prevented the increase of BBB permeability through the maintenance of its structure and facilitated the clearance of circulating HMGB1.

Methods

MCAO Surgical Procedure

All experimental procedures were conducted in accordance with the guidelines of Okayama University for animal experiments and approved by the university’s committee on animal experimentation. Male Wistar rats (Charles River Laboratory Japan, Yokohama, Japan), weighing 250 to 300 g, were used for all experiments. MCAO was performed as previously described. Briefly, the rats were anesthetized with 2% halothane in a mixture of 50% N2O and 50% O2. An 18-mm-long strand of 4.0 nylon thread coated with silicone was inserted into the right internal carotid artery to occlude the origin of the right middle cerebral artery. The thermocouple needle probe was inserted into temporal muscle to maintain the temperature at 37.0 ± 0.1°C with a heating lamp during surgery. Only the rats that showed paralysis of the contralateral limbs after recovery from anesthesia were used for further experiments. Reperfusion was performed at 2 hours after MCAO. As shown in Supplemental Table 1 (http://stroke.ahajournals.org), basic physiological parameters, including blood gases, pH, hemoglobin, and glucose, were monitored. The rats were randomly assigned into 2 groups after MCAO operation, and an anti-HMGB1 mAb (#10-22, IgG2a subclass, 200 μg/rat) or class-matched control mAb (anti-glial fibrillary acidic protein Ab (Abcam plc), or anti-ionized calcium-binding adaptor molecule 1 Ab (Wako, Inc, Osaka, Japan). The endothelial cells were cultured in combination with anti-microtubule-associated protein 2 Ab (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), anti-glial fibrillary acidic protein Ab (Abcam plc), or anti-ionized calcium-binding adaptor molecule 1 Ab (Wako, Inc, Osaka, Japan). For aquaporin 4 (AQP4) staining, a mouse anti-AQP4 mAb (Abcam plc) was incubated with frozen sections of rat brains as the primary antibody. See details in the Supplemental Methods (http://stroke.ahajournals.org).

Enzyme-Linked Immunosorbent Assay of HMGB1

For HMGB1 determination in serum samples, blood samples (1 mL) were collected through the inferior vena cava under deep anesthesia with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) followed by centrifugation at 1500 g for 10 minutes. The supernatants were removed to clean Eppendorf tubes and stored at −20°C before use. Cerebrospinal fluid samples were collected from the cistern magna according to the previously reported method. Samples contaminated by blood were excluded. HMGB1 was determined using an HMGB1 enzyme-linked immunosorbent assay kit (Shino-Test Co, Sagamihara, Japan) according to the manufacturer’s protocol.

Brain Water Content Measurement

Ten rats were allocated to 3 groups: the sham group (n=4), control mAb-injected group (n=8), and anti-HMGB1 mAb-injected group (n=9). All animals except the intact group were subjected to surgery for MCAO. Three hours after reperfusion, the animals were anesthetized deeply with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). The brains were rapidly removed and dissected into 4 regions: the hippocampus, striatum, hypothalamus, and cerebral cortex in each hemisphere. After weighing, the tissues were dried at 80°C for 8 hours. The water content in each region was calculated according to the following equation: % water content=100×(wt weight−dry weight)/wt weight.

Transmission Electron Microscopic Examination

Three hours after reperfusion, MCAO 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 through an ethanol series 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 the astrocyte end feet swelling, National Institutes of Health image J 1.42q software was used to calculate the ratio of the total swollen astrocyte end feet surrounding against the area of the corresponding capillary lumen. Eight rats were analyzed for each group treated with control Ab or anti-HMGB1 mAb. Seven capillaries in each area were evaluated.

In Vitro BBB Permeability Assay

An in vitro BBB kit (RBE-12; PharmaCo-Cell Co Ltd, Sakamoto, Japan) composed of rat brain vascular endothelial cells, pericytes, and astrocytes was used to assess the effects of recombinant human HMGB1 (RHMGB1) and the mAb to the BBB unit according to the instructions of the manufacturer. The endothelial cells were cultured on the bottom of the polyester membrane of the insert well. The pericytes were present below the membrane of the insert well. The astrocytes were cultured on the bottom of the lower chamber. Endotoxin-free RHMGB1 (see details in Supplemental Methods) alone or together with the anti-HMGB1 mAb was added into the lower chamber, which is supposed to be the brain side. Transendothelial electric resistance and leakage of the Evans blue–albumin complex were measured thereafter. F-actin was stained to observe the morphological changes of endothelial cells and pericytes. See detail in Supplemental Methods.
In Vivo Brain Microdialysis

Rats were anesthetized using the same condition of the MCAO procedure and placed in a stereotaxic apparatus. Guide cannulae were implanted into the striatum (posterior, 0.25 mm; lateral, 4.0 mm from bregma; below the skull surface, 7.0 mm). Microdialysis probes (Eicom Co, Kyoto, Japan) were inserted through the guide cannulae. The probe was perfused with Ringer solution, and the microdialysis samples were collected. Glutamate in the samples was detected using a Shimadzu high-performance liquid chromatography system (Shimadzu Co, Kyoto, Japan) equipped with a C18 column (TSKgel ODS-100V; Tosoh Bioscience, Tokyo, Japan).17 Before experiments, the in vitro recovery of each microdialysis probe was determined and all probes had 14% recovery at a flow rate of 4 μL/min. See detail in Supplemental Methods.

Statistical Analysis

Statistical significance was evaluated using 2-way analysis of variance followed by the Student t test. The statistical significance of the microdialysis experiments for glutamate was evaluated using 1-factor repeated-measures analysis of variance. The statistical significance of in vitro BBB experiments was evaluated using analysis of variance followed by Dunnett test. A probability value of <0.05 was considered to be significant.

Results

HMGB1 Translocation and Release

Immunohistochemical staining of HMGB1 showed that HMGB1 was localized solely in the nuclear compartment with the spotted staining of nucleoli throughout the brain from sham-operated rats (Figure 1Aa). After the onset of ischemia, HMGB1 was translocated to the cytoplasmic compartment with the “lotus root-like structure” and was partially lost in the ischemic core at 2 hours after reperfusion (Figure 1Af). Double immunostaining of HMGB1 and 2-3-5-Triphenyl tetrazolium chloride staining of an ischemic rat brain 12 hours after reperfusion was shown to indicate the positions were the images (a–f) were obtained. The red asterisk in g indicated the places where the images (a–d) were obtained, which indicates the ischemic core in the striatum. The brain region surrounded by dashed line indicates the area where the typical translocation of HMGB1 was observed at a time point of 4 hours after reperfusion. HMGB1 translocated occurs in neurons but conserved in astrocytes and microglia cells even in the later phase of reperfusion. Sections were double stained with anti-HMGB1 antibody (Ab) and antimmicrotubule-associated protein 2 (MAP2) Ab, antiglial fibrillary acidic protein (GFAP) Ab, or anti-ionized calcium-binding adaptor molecule 1 (Iba1) Ab. 4′,6-Diamidino-2-phenylindole (blue) staining was used to show nuclear localization. All images were obtained from the cerebral cortex of the rat brain. Scale bars≈50 μm.
microtubule-associated protein 2 revealed that the translocation mostly occurred in neurons (Figure 1B). In contrast, HMGB1 immunoreactivities in astrocytes and microglial cells were retained in nuclei even in the late phase of reperfusion (Figure 1B). Interestingly, in the ischemic core of the cerebral cortex, we observed a granule-like staining pattern of HMGB1 aligned on the neuronal cell soma, especially in the late phase of reperfusion (Figure 2Aa–b). In addition, the considerable number of small granule-like HMGB1 staining was observed surrounding the capillaries, especially in the hypothalamus of the ischemic hemisphere (Figure 2Ac). Figure 2B shows that at 12 hours after reperfusion, HMGB1 staining was almost completely lost in most of the cells in the ipsilateral side of the brain when compared with the rats treated with control IgG. In contrast, treatment with the anti-HMGB1 mAb inhibited the translocation and disappearance of HMGB1. Consist with the results of immunostaining, we found that the HMGB1 level in the ischemic core area of the rat brain 12 hours after reperfusion significantly decreased to 40% and 44% in the striatum and the cerebral cortex, respectively, compared with the sham-operated rat (Supplemental Figure I).

Determination of HMGB1 using enzyme-linked immunosorbent assay showed that HMGB1 appeared in the cerebrospinal fluid as early as 2 hours after reperfusion and increased to 30 ng/mL 12 hours after reperfusion. Treatment with anti-HMGB1 mAb significantly decreased the HMGB1 levels in cerebrospinal fluid (Figure 2C). Western blot analysis also supported that HMGB1 time-dependently increased in the cerebrospinal fluid (Supplemental Figure II). The increase of serum HMGB1 levels after ischemia was also observed in rats treated with control IgG, but the serum levels of HMGB1 were suppressed in anti-HMGB1 mAb-treated rats to the same level as in sham-operated rats (Figure 2D). To clarify whether the therapeutic anti-HMGB1 mAb affected the HMGB1 enzyme-linked immunosorbent assay results, we coincubated the anti-HMGB1 mAb with HMGB1 in rat brain homogenate or rHMGB1 and examined the incubated samples in the enzyme-linked immunosorbent assay plate. The results clearly show that the therapeutic mAb had no effects on the enzyme-linked immunosorbent assay (Supplemental Figure III). Meanwhile, in the anti-HMGB1 mAb-treated MCAO rats, levels of plasma 4-HNE adducts were also significantly reduced by 61% compared with that in control IgG-treated MCAO rats (Supplemental Figure IV).

**Figure 2.** Translocation and release of high mobility group box-1 (HMGB1) from neurons and its inhibition by anti-HMGB1 monoclonal antibody (mAb). Coronal sections were prepared 12 hours after reperfusion. 4′,6-Diamidino-2-phenylindole staining was performed to show the cell nuclei. A, HMGB1 was stained as a granule-like structure. a, HMGB1 (red) was stained as a granule-like structure aligning the neuron cell soma in the cerebral cortex. b, A representative cell stained both by anti-HMGB1 (green) and anti-NeuN Ab (red). c, HMGB1 staining (green) surrounding capillaries (asterisks) in the hypothalamus of the ischemia hemisphere. d, A 2-3-6-triphenyltetrazolium chloride staining of an ischemic rat brain 12 hours after reperfusion was used to indicate the position where the corresponding images were obtained. The brain region surrounded by dashed line indicates the areas where the typical granule-like staining of HMGB1 were observed. Scale bars=20 μm. B, Representative confocal images showing the inhibitory effects of the anti-HMGB1 mAb on HMGB1 translocation (red, HMGB1; green, microtubule-associated protein 2 [MAP2]). Scale bars=50 μm. C, The HMGB1 levels in cerebrospinal fluid were determined using enzyme-linked immunosorbent assay (ELISA). The results are mean±SEM of 10 (sham), 8 (2 hours in control IgG-treated group), 9 (12 hours in control IgG-treated group), and 9 (anti-HMGB1 mAb-treated group) rats. D, Serum levels of HMGB1 at 12 hours after reperfusion were determined using ELISA. The results are mean±SEM of 8 (sham), 8 (control IgG-treated group), and 9 (anti-HMGB1 mAb-treated group) rats. **P<0.01 compared with sham control. #P<0.05 and ##P<0.01 compared with the group treated with control IgG (reperfusion).

**Alleviation of Brain Edema**

Brain edema is defined as an abnormal accumulation of fluid within the brain parenchyma, producing a volumetric enlargement of brain cells or tissue, which is one of the primary causes of clinical deterioration and a leading cause of death.
after ischemia/reperfusion. In the sham group, the brain water content formed 76% to 78% of the total weight. Three hours after reperfusion, in the rats treated with control IgG, the brain water contents in the cerebral cortex, striatum, hypothalamus, and hippocampus of the ischemic hemisphere had significantly increased to 82.8%, 83.3%, 80.0%, and 82.3%, respectively. In contrast, the anti-HMGB1 mAb attenuated the increase in water content by 3.2%, 5.7%, 2.3%, and 2.85% in each region, respectively (Figure 3A). The hippocampal edema occurred not only in the ischemic side, but also in the nonischemic side that was also inhibited by treatment with the anti-HMGB1 mAb (Figure 3B).

AQP4 is a prominent biological marker to determine the permeability of brain capillaries in brain edema. Figure 3C shows that in the MCAO rats (3 hours after reperfusion) treated with control IgG, the immunoreactivity of AQP4 in the cerebral cortex and striatum was significantly increased. It appears that AQP4 was strongly expressed in capillary vessels, probably on the astroglial membranes that had direct contact with the lamina propria. The results of AQP4 immunohistochemistry were quantitatively determined by counting the AQP4-positive blood vessels (Figure 3D). The treatment with anti-HMGB1 mAb remarkably inhibited the expression of AQP4 both in the cerebral cortex and striatum.

MRI Studies
MRI is considered the most promising and noninvasive tool for recognizing brain edema formation in real time. T2-weighted MRI is frequently used to determine the presence or absence of edema, especially vasogenic edema induced by reperfusion. Consistent with the data for brain edema determined by the water content, T2-weighted MRI clearly showed time-dependent changes in brain edema in the ischemic areas containing the striatum, cerebral cortex, and hippocampus in the control rats. The representative data from 5 rats are shown in Figure 4A. Three hours after reperfusion, striatal edema was evident in the control Ab group. At 6 hours after reperfusion, the edematous areas had expanded to the cerebral cortex. Treatment with the anti-HMGB1 mAb inhibited the increase in the intensity of the images in the ischemic areas (Figure 4A). Figure 4B summarizes the quantitative analysis of MRI based on intensity analysis of the images in the ischemic hemisphere against those in the contralateral hemisphere. At 24 hours after reperfusion, the high intense T2 signal in the control rats may contain a necrotic area. Before Ab treatment, the physiological parameters such as the neurological scores and Rotorod test scores were not different...
In Vitro BBB Permeability Assay

Using an in vitro BBB system composed of rat brain vascular endothelial cells, pericytes, and astrocytes, the direct effects of rHMGB1 on the permeability of BBB were examined (Figure 5). The rHMGB1 generated from inset cells (free of lipopolysaccharide) was added into the brain side (lower chamber) at a concentration of 1 μg/mL and 5 μg/mL, and the incubation continued for a total of 60 minutes. The addition of rHMGB1 concentration-dependently decreased the transendothelial electric resistance, and mAb antagonized the reduction of transendothelial electric resistance induced by rHMGB1, whereas the transendothelial electric resistance levels before stimulation were the same among groups (Figure 5A). The stimulation with rHMGB1 concentration-dependently increased the permeability of the Evans blue–albumin complex. The mAb significantly inhibited the enhanced permeability of BBB indicated by Evans blue–albumin leakage (Figure 5B).

Consistent with the results of the dye leakage and the reduction of transendothelial electric resistance, the stimulation with rHMGB1 induced the morphological changes in pericytes and endothelial cells. In pericytes, the F-actin staining reduced after the stimulation with rHMGB1 concentration-dependently. Also, the vascular endothelials seemed to shrink longitudinally, leading to the intercellular space formation. These morphological effects of rHMGB1 were antagonized by the addition of anti-HMGB1 mAb. In contrast, there was no difference in astrocyte shape among groups examined (Figure 5C).

Electron Microscopic Observation of the BBB

Images from transmission electron microscopy clearly identified the BBB unit composed of endothelial cells, basal lamina, pericytes, and astrocyte end feet. Figure 6A shows representative images of the BBB unit in the striatum. In the control IgG rat brains fixed at 3 hours after reperfusion, it is evident that the astrocyte end feet were swollen to various extents in the ischemic regions (Figure 6Aa–b). In many cases, the intracellular organelles were absent or scarce in such swollen astrocyte end feet labeled by asterisks in Figure 6Aa. In addition to the enlargement of astrocyte end feet, the detachment of the end feet plasma membrane from the basal lamina was observed (arrows...
in Figure 6Aa). Electron-dense TJ structures between the capillary endothelial cells were deformed and the gap clefts were seen in the control IgG-treated rats (Figure 6Ab). Treatment with the anti-HMGB1 mAb remarkably protected the BBB structures from the enlargement of the astrocyte end feet, detachment of the end feet plasma membrane from the basal lamina, and the deformation of the TJ (Figure 6Ac). To quantitatively evaluate the effect of the anti-HMGB1 mAb on astrocyte swelling in the different brain regions, we measured the ratio of the area of the swollen astrocyte end feet against a capillary luminal area. Figure 6B shows 3 representative images of the calculated astrocyte end feet from the relative brain regions. The results indicate that astrocyte end feet swelling was significantly inhibited by treatment with the anti-HMGB1 mAb (Figure 6C).

High levels of extracellular glutamate can lead to disruption of the BBB and consequently to vasogenic edema.22,23Thus, we detected the extracellular levels of glutamate in the striatum using a microdialysis—high-performance liquid chromatography technique. We found that extracellular glutamate decreased immediately after blood reflow induced by reperfusion. However, at 1 hour after reperfusion, a secondary increase of glutamate was detected persisted for up to 6 hours after reperfusion, which may be induced by the secondary injury induced by reperfusion.24,25 Impressively, treatment with the anti-HMGB1 mAb significantly inhibited the sustained elevation of extracellular glutamate (Figure 6D).

**Discussion**

In the present study, we provided further evidence for the therapeutic effects of anti-HMGB1 mAb on brain edema and BBB disruption induced by brain ischemic insult. Electron microscopic observation in the 2-hour MCAO rat model

---

**Figure 6.** Ultrastructure of the blood–brain barrier (BBB) in the ischemic hemisphere obtained using transmission electron microscopy. A, Representative images of the coronal sections from rat brain treated with control IgG (a, b) or anti-high mobility group box-1 (HMGB1) monoclonal antibody (mAb; c) at 3 hours after reperfusion. a, Astrocyte end feet swelling and detachment. Asterisks indicate the swollen astroglial end feet. Arrows indicate the detachment of astrocyte end feet from the basal lamina. b, Tight junction deformation. The inserts show a higher magnification of detached tight junction. c, A BBB unit in the anti-HMGB1 mAb-treated rat brain. The insets show an intact tight junction. d, A scheme showing the places where the images (a–c) were obtained. B, Representative images for the quantitative analysis of the astrocyte end feet swelling in the rats treated with control IgG or anti-HMGB1 mAb. C, Quantitative results of astrocyte end feet swelling in each region were determined as described in the “Methods.” The results are mean±SEM of 7 capillaries in each area of 8 rats from each group. *P<0.05 and **P<0.01 compared with the control group (original magnification ×8000). D, Extracellular levels of glutamate in microdialysis samples during and after brain ischemia. The rats were treated with control IgG or anti-HMGB1 mAb immediately after reperfusion. Each point represents mean±SEM of 4 rats. The statistical analysis was performed by comparing the time course changes of each group at 1 hour after reperfusion up to 6 hours using 1-factor repeated-measures analysis of variance. *P<0.05 compared with the control group.
demonstrated the swelling of astrocyte end feet, the detachment of the plasma membrane astrocyte end feet from the basal lamina, and the loosening of the TJ between capillary endothelial cells occurred at 3 hours after reperfusion. T2-weighted MRI at 3 hours after reperfusion was consistent with the electron microscope findings. This may indicate that the therapeutic approach to an ischemic brain injury should contain an aspect to control this rapid and drastic disruption of the BBB. In fact, anti-HMGB1 mAb therapy efficiently ameliorated these changes to the BBB structure. Thus, the decrease in protein leakage identified in our previous study was due to the effects of the anti-HMGB1 mAb on BBB maintenance.9

Using an in vitro BBB system, we clearly demonstrated that HMGB1 increased the vascular permeability of the BBB to the Evans blue–albumin complex associated with the morphological changes in endothelial cells and pericytes. Thus, the significant inhibitory effects of anti-HMGB1 mAb on rHMGB1 action in vitro probably reflect the in vivo condition after ischemic insult. HMGB1 once released into the extracellular space surrounding capillary vessels therefore may induce BBB disruption directly by acting on its component cells. Further work is necessary to determine whether HMGB1 affects both endothelial cells and pericytes at the same time or in order.

In the present study, we clearly showed that HMGB1 translocation was time-dependent and cell type-specific. In the case of neurons, the time-dependent translocation of HMGB1 can be summarized into 3 steps: (1) its redistribution inside the nucleus; (2) the translocation of HMGB1 from the nucleus into the cytosolic compartment; and (3) the release of HMGB1 into the extracellular space. The typical translocation of HMGB1 from the nucleus to the cytoplasm was observed at 2 to 4 hours after reperfusion, which is consistent with the findings obtained by other groups using the mouse MCAO model.10–11 Thus, the intranuclear and cytosolic translocation and then extracellular release of HMGB1 probably take place in the majority of neurons under ischemic conditions. The translocation of HMGB1 may be the result of the chemical modification of HMGB1 such as acetylation, phosphorylation, or methylation.26–28 There may be such modifications under ischemic conditions, although the signaling cascades required to trigger the activation of the necessary enzymes remain to be determined.

Another interesting finding in this study is that we observed a special granule-like structure of HMGB1 aligned on the neuronal cell soma in the ischemic core area in the cerebral cortex. It appears that HMGB1 may be taken up into vesicular structures in the cytoplasm. These vesicular structures may bind to the plasma membrane judging from their distribution pattern depicting cell soma. At this time, we do not know the nature of the vesicular structures, that is, endosome, lysosome, mitochondria, or fused synaptic vesicles. Further study is needed to identify the vesicles using double immune staining of HMGB1 and specific markers of vesicles to clarify this point. In the present study, we used a transient occlusion model; however, permanent MCAO may show a different pattern of HMGB1 translocation and BBB disruption. Therefore, the effects of anti-HMGB1 mAb on a permanent MCAO model should be examined experimentally.

Using a microdialysis–high-performance liquid chromatography technique, we detected a rapid elevation of glutamate during ischemia. The second elevation of glutamate was detected at 1 hour after reperfusion. Interestingly, treatment with the anti-HMGB1 mAb significantly inhibited the second increase in glutamate. It was also reported that glutamate can induce the release of HMGB1 from neuronal cells in vitro.10 The released HMGB1 probably facilitated the disruption of BBB as demonstrated in the present study, leading to brain edema. The resultant increase in brain tissue pressure as well as gas-diffusion barrier formation will exacerbate the neuronal damage, leading to glutamate release. Therefore, neutralization of HMGB1 by mAb will reduce the BBB damage, glutamate release, and HMGB1 translocation at the same time.

The rapid increase in HMGB1 levels in the cerebrospinal fluid as well as the HMGB1 translocation demonstrated by immunohistochemistry supported that a considerable amount of HMGB1 was released into the extracellular space. Moreover, we observed a marked increase in the serum levels of HMGB1 after the ischemic brain insult. This finding was consistent with the data reported by others.8,29 We also determined the effect of the anti-HMGB1 mAb on serum HMGB1 levels and found that the therapy dramatically reduced serum HMGB1 levels to those observed in sham rats. To examine the possibility that the therapeutically administered anti-HMGB1 mAb interfered with the sandwich enzyme-linked immunosorbent assay, we added the therapeutic mAb to this assay together with the standard HMGB1 preparation. The anti-HMGB1 mAb as well as an anti-KLH mAb had no influence on enzyme-linked immunosorbent assay (Supplemental Figure III). Therefore, we concluded that the intravenously injected anti-HMGB1 mAb binds to circulating HMGB1 and facilitates its clearance from the bloodstream.

HMGB1 released in circulation may increase the inflammatory response to the endothelial cells of the vulnerable BBB.30 It is well established that oxidative stress during focal cerebral ischemia is one of the major contributors to the disruption of BBB and secondary brain damage.31–33 The serum HMGB1 released from the ischemic brain may stimulate the production of proinflammatory cytokines in monocytes or activate vascular endothelial cells, which may produce high amounts of reactive oxygen species.30,34 The reactive oxygen species in turn induces protein and lipid oxidation (Supplemental Figure IV) in the blood. The elimination of HMGB1 from circulation by the anti-HMGB1 mAb must be another important mechanism for the effects of mAb therapy. At this time, we do not know the relative contribution of this effect of the anti-HMGB1 mAb to the total beneficial effects of the Ab; however, it is possible that clearance of the HMGB1 antigen may inhibit the procoagulant effect of HMGB1, the activation of vascular endothelial cells, and monocyte activation.9,34,35,36

In conclusion, mAb treatment against HMGB1 may provide a new strategy for brain infarction by inhibiting important inflammatory responses in addition to thrombolytic tissue plasminogen activator.
Acknowledgments
We thank Dr Kazushi Kinugasa for his discussion on the article and Mr Hiroshi Okamoto and Mr Masahiro Narasaki for their technical assistance.

Sources of Funding
This work was supported by grants from the Scientific Research for Promotion of Industry. This work was also supported by grants from the Ministry of Health, Labor and Welfare of Japan, from the Scientific Research Assistance.

Mr Hiroshi Okamoto and Mr Masahiro Narasaki for their technical assistance.

We thank Dr Kazushi Kinugasa for his discussion on the article and Mr Hiroshi Okamoto and Mr Masahiro Narasaki for their technical assistance.

Disclosures
None.

References
Anti-high Mobility Group Box-1 Monoclonal Antibody Protects the Blood–Brain Barrier From Ischemia-Induced Disruption in Rats
Jiyong Zhang, Hideo K. Takahashi, Keyue Liu, Hidenori Wake, Rui Liu, Tomoko Maruo, Isao Date, Tadashi Yoshino, Aiji Ohtsuka, Shuji Mori and Masahiro Nishibori

*Stroke*. 2011;42:1420-1428; originally published online April 7, 2011; doi: 10.1161/STROKEAHA.110.598334

*Stroke* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/42/5/1420

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2011/04/27/STROKEAHA.110.598334.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Stroke* can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to *Stroke* is online at:
http://stroke.ahajournals.org//subscriptions/
Supplemental Materials

Supplemental Methods

Recombinant human HMGB1 (rHMGB1)

rHMGB1 was produced in Sf9 cells to obtain LPS-free HMGB1. In brief, full-length human HMGB1 DNA was amplified by PCR using Cap Site cDNA dT from human microvascular endothelial cells (Nippon Gene, Tokyo, Japan) and primers (forward 5’-GCA GAA TTC ATG GGC AAA GGA GAT CCT A-3’, reverse 5’-CAT CTC GAG TCA TTA TTC ATC ATC ATC ATC-3’). The fragment was digested with EcoRI and XhoI and cloned into the pFastBacHTA (Invitrogen, Carlsbad, CA, USA) expression vector. The transfection of the Sf9 cells with the pFastBacHTA-HMGB1 bacmid was performed according to the manufacturer’s instructions (Bac-to-Bac Baculovirus Expression System, Invitrogen). The infected SF9 cell extract containing His-tagged HMGB1 protein was applied to Ni-NTA agarose (Qiagen, Hilden, Germany) and incubated for 3 hours at room temperature. After extensive washing, rHMGB1 was eluted with imidazole buffer. The rHMGB1 was collected and dialyzed over night at 4 °C against PBS. Purified rHMGB1 protein was identified by SDS-PAGE and Western blotting with anti-HMGB1 mAb. (#10-22). The final HMGB1 preparation contained LPS of less than 2.0 pg/µg protein.

Immunohistochemistry staining

Rats were anesthetized deeply with an i.p. injection of sodium pentobarbital (50mg/kg) and transcardially perfused by cold saline and then 10% formalin. Brains were post-fixed overnight in 10% formalin and paraffin-embedded. Brain sections were cut at a thickness of 6 µm. An antigen retrieval procedure was performed in a 0.1 M sodium citric acid buffer (pH 6.0) by heating in autoclave at 120 °C for 10 min. After washing with tris-buffered saline (TBS), the sections were immersed in 10% normal goat serum (Sigma-Aldrich Co., St. Louis, MO, USA) in TBS containing 1% bovine serum albumin (BSA) for 2 h to block nonspecific binding. HMGB1 was stained using a mouse anti-HMGB1 mAb (R&D systems, Inc., Minneapolis, MN, USA) or a rabbit anti-HMGB1 Ab (Abcam plc, Cambridge, UK). For double immuno staining, the sections were incubated overnight with anti-HMGB1 mAb (R&D systems, Inc., Minneapolis, MN, USA) in combination with anti-microtubule associated protein 2 (MAP2) Ab (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-gli fibrillary acidic protein (GFAP) Ab (Abcam plc, Cambridge, UK), or anti-ionized calcium-binding adaptor molecule 1 (Iba1) Ab (Wako, Inc, Osaka, Japan) as the primary antibodies at 4 °C. Alexa-555 labeled anti-mouse IgG (Invitrogen Co., Branford, CT, USA) and alexa-488 labeled anti-rabbit IgG (Invitrogen Co., Branford, CT, USA) were used as the secondary Abs. Sections were incubated with the secondary Ab at room temperature for 1 h and mounted using VECTORSHIELD Hard Set Mounting Medium with DAPI (Vector Laboratories, Inc., Burlingame, CA, USA). Stained sections were observed under an LSM 510 confocal imaging system (Carl Zeiss, Inc., Jena, Germany). For aquaporin 4 (AQP4) staining, frozen sections of rat brains were prepared. A mouse anti-aquaporin 4 mAb (Abcam
plc, Cambridge, UK) was used as the primary Ab, followed by incubation with HRP-conjugated goat anti-mouse IgG (MBL international, Inc., Woburn, MA, USA). Diaminobenzidine (Sigma-Aldrich Co., St. Louis, MO, USA) and H$_2$O$_2$ were used as the substrates for the reaction.

**Western blotting for HMGB1 in cerebrospinal fluid**

Cerebrospinal fluid samples were collected from the cistern magna according to the previously reported method$^1$. The samples were mixed with SDS-PAGE sample loading buffer under reducing condition. We loaded 15 μL of the samples per lane on SDS-PAGE. After transferring the proteins to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA), the membranes were blocked with TBS containing 0.1% Tween 20 (T-TBS) and 10% skimmed milk, and then probed with the anti-HMGB1 mAb (#10-22) labeled by horseradish peroxidase using a Peroxidase Labeling Kit-NH2 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). After washing in T-TBS, an ECL system (Thermo Fisher Scientific Inc., Rockford, IL, USA) was used to visualize the HMGB1 band.

**Western blotting using brain homogenate samples**

Rat brain samples were collected at the time point of 12 h after reperfusion. Brain slices were cut from 0.5 mm anterior from the bregma with the thickness of 2 mm. The ischemic core area in the striatum and the cortex indicated as Supplemental Figure 1B were cut and homogenized in cold Radio Immuno Precipitation Assay buffer (RIPA) (150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris-HCl, pH 8.0) with cocktail protease inhibitors (Sigma-Aldrich Co., St. Louis, MO, USA). The brain homogenate were then centrifuged at 10,000 g for 20 min. The protein concentration in the supernatant were detected by Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA), and adjusted to 250 μg/mL as the final concentration by Western sample buffer, 10 μL of each sample was loaded to the SDS-PAGE. After transferring the proteins to a nitrocellulose membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA), the membranes were blocked with T-TBS and 10% skimmed milk, and then probed with the anti-HMGB1 mAb (#10-22) labeled by horseradish peroxidase using a Peroxidase Labeling Kit-NH2 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). β-actin was probed with a mouse anti-β-actin mAb (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA ) followed by a HRP conjugated goat anti-mouse Ab. After washing in T-TBS, an ECL system (Thermo Fisher Scientific Inc., Rockford, IL, USA) was used to visualize the bands of HMGB1 and β-actin.

**In vitro BBB permeability assay**

An in vitro BBB kit$^{TM}$ (RBE-12, PharmaCo-Cell Co. Ltd., Sakamoto, Japan) was used to assess the effects of rHMGB1 and the mAb to the BBB unit according to the instruction of the manufacturer$^2$. The BBB system was composed of rat brain vascular endothelial cells, pericytes and astrocytes. The endothelial cells were cultured on the bottom of polyester
membrane (radius, 6 mm; thickness, 10 μm; pore size, 0.4 μm) of the insert well. The pericytes were present below the membrane of the insert well. The astrocytes were cultured on the bottom of the lower chamber. RHMGB1 expressed and purified from Sf9 insect cells. RHMGB1 alone or together with the anti-HMGB1 mAb after pre-incubation for 30 min was added into the lower chamber which is supposed to be the brain side. Trans Endothelial Electrical Resistance (TEER) was measured before and 30 min after stimulation with rHMGB1 using an EVOM resistance meter (World Precision Instruments, Sarasota, FL) as previously reported (Hui et al., 2008). The values are shown as Ω × cm². Thereafter, Evans’ blue (165 μg/mL) bound to 0.1% BSA³ was added to the insert well which is supposed to be the blood vessel side. The plate was further incubated for 30 min at 37°C. The media in the lower compartment was then collected, and the absorbance of Evans blue at 595 nm was detected using a spectrophotometer (U-1500, Hitachi Ltd., Tokyo, Japan) to study the leakage of Evans blue-albumin complex.

The insert wells were washed by PBS, and the cells in the insert wells were fixed by 4% paraformaldehyde for 30 min at room temperature. The fixed cells were then washed by PBS and incubated with 0.1% Triton-X100 for 5 min at room temperature. An alexa-488 labeled phallolidin (Invitrogen Co., Branford, CT, USA) was added to the insert wells for F-actin staining. Cell nuclei were visualized by staining with DAPI (0.3 μM). The endothelial cells and pericytes in the insert wells were examined using a LSM 510 confocal imaging system (Carl Zeiss, Inc., Jena, Germany). The autofluorescence of Evans blue uptaken by astrocytes was observed in a fluorescence microscope (BZ-8000, Keyence Co., Osaka, Japan).

**In vivo brain microdialysis of glutamate**

Rats were anesthetized using the same condition of MCAO procedure. Rats were placed in a stereotaxic apparatus. Guide cannulae were implanted into the striatum (posterior: 0.25 mm, lateral: 4.0 mm from bregma; below the skull surface: 7.0 mm) through a hole drilled in the skull and fixed with two anchor screws and dental cement. One week after surgery, microdialysis probes (Eicom Co., Kyoto, Japan) with a membrane length of 3 mm were carefully inserted through the guide cannulae. The probe was perfused at a flow rate of 4 μL/min with Ringer’s solution (Na⁺: 147 mM, K⁺: 4 mM, Ca²⁺: 4.5 mM, Cl⁻: 155.5 mM). After a stabilization period (approximately 2–3 hours), the microdialysis samples were collected every 15 min before, during and after MCAO. Samples were precisely reacted with OPA working solution (OPA, β-ME, sodium tetraborate) for 4 min at 32°C according to the previously reported method⁴, and detected using a Shimadzu HPLC system (Shimadzu Co., Kyoto, Japan) equipped with a C18 column (TSKgel ODS-100V, Tosoh Bioscience, Tokyo, Japan) and a fluorescence detector (RF-530, Shimadzu Co., Japan). The mobile phase was 0.1 M Na₂HPO₄ containing 25% methanol (pH 6.75 adjusted by H₃PO₄). Before experiments, the in vitro recovery of each microdialysis probe was determined and all probes had 14% recovery at a flow rate of 4 μL/min.

**Determination of 4-hydroxynonenal (4-HNE) adducts**
Plasma samples were prepared from 1 mL of blood collected via the inferior vena cava of sham and MCAO operated rats treated with control Ab or anti-HMGB1 mAb. The levels of 4-HNE adducts in each sample were determined using an OxiSelect™ HNE-His Adduct ELISA Kit (Cell Biolabs, Inc., San Diego, CA, USA) with HNE-BSA as the standard. The determination was performed on triplicate samples.
## Supplemental Table

Table 1. Basic physiological parameters for study groups.

<table>
<thead>
<tr>
<th></th>
<th>Pre</th>
<th>Control IgG</th>
<th>Anti-HMGB1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 min</td>
<td>8 h</td>
<td>10 min</td>
</tr>
<tr>
<td>pH</td>
<td>7.4 ± 0.08</td>
<td>7.4 ± 0.01</td>
<td>7.4 ± 0.02</td>
</tr>
<tr>
<td>PCO₂ (mmHg)</td>
<td>43 ± 2.45</td>
<td>43.2 ± 1.35</td>
<td>46.4 ± 2.67</td>
</tr>
<tr>
<td>PO₂ (mmHg)</td>
<td>162.6 ± 17.6</td>
<td>170.6 ± 6.05</td>
<td>153.4 ± 6.17</td>
</tr>
<tr>
<td>Base excess (mmol/L)</td>
<td>-0.5 ± 1.6</td>
<td>-1.1 ± 0.8</td>
<td>-0.7 ± 0.8</td>
</tr>
<tr>
<td>HCO₃⁻ (mmol/L)</td>
<td>22.4 ± 1.7</td>
<td>23.9 ± 0.7</td>
<td>24.7 ± 0.7</td>
</tr>
<tr>
<td>Hemoglobin (g/dl)</td>
<td>11.2 ± 0.6</td>
<td>12 ± 0.3</td>
<td>12.3 ± 0.9</td>
</tr>
<tr>
<td>Na⁺ (mmol/L)</td>
<td>127 ± 2.4</td>
<td>130.8 ± 1.3</td>
<td>135.6 ± 0.7</td>
</tr>
<tr>
<td>K⁺ (mmol/L)</td>
<td>3.8 ± 0.1</td>
<td>6.5 ± 0.7*</td>
<td>4.0 ± 0.1</td>
</tr>
<tr>
<td>Ca²⁺ (mmol/L)</td>
<td>0.8 ± 0.07</td>
<td>1.0 ± 0.06</td>
<td>1.1 ± 0.02</td>
</tr>
<tr>
<td>Cl⁻ (mmol/L)</td>
<td>95 ± 1.2</td>
<td>102.8 ± 0.5</td>
<td>107.4 ± 0.8</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>158.6 ± 8.7</td>
<td>202.6 ± 13.9*</td>
<td>165.2 ± 9.7</td>
</tr>
<tr>
<td>Lactate (mg/L)</td>
<td>19.4 ± 1.5</td>
<td>18.2 ± 0.5</td>
<td>16 ± 1.2</td>
</tr>
</tbody>
</table>

Control IgG or anti-HMGB1 mAb was administered intravenous under halothane anesthesia, and physiologic variables were determined 10 min and 8 h after mAb administration. Each value represents the mean ± s.e.m of 5 rats. *P<0.05 compared with the Pre value, #P<0.05 compared with the value at the same time point in control IgG group. Pre, pre-ischemia.
Supplemental Figures

A

Supplemental Figure 1

Detection of HMGB1 in the ischemia core area using Western blotting. The rat brain in control IgG-treated group and anti-HMGB1 treated group was perfused by cold saline, collected and homogenized in RIPA solution with protease inhibitors as indicated in supplemental methods. HMGB1 was blotted by a peroxides-labeled anti-HMGB1 mAb (#10-22) and visualized by ECL system. β-actin was used as the internal control. (A) The bands of HMGB1 and β-actin detected by Western blotting from 3 representative brain samples in sham (n=5), control IgG (n=8), and anti-HMGB1 (n=8) groups. (B) A representative image of TTC staining of a brain slice from a MCAO rat 12 h after reperfusion. The squares circled by dash line indicate the positions where the brain samples were collected. S: striatum, C: cortex. (C) Quantitative results of the bands detected by Western blotting using NIH image J 1.42q software. The results are the means ± s.e.m. **P<0.01 compared with sham control, ##P<0.01 compared with control IgG group.
Supplemental Figure 2

Detection of HMGB1 in the cerebrospinal fluid after brain ischemia using Western blotting. Cerebrospinal fluid was collected from the cisterna magna at different time points. Homogenate sample of normal rat brain was used as the positive control. HMGB1 was blotted by a peroxides-labeled anti-HMGB1 mAb (#10-22) and visualized by ECL system as described in supplementary methods. (Re: reperfusion)
Supplemental Figure 3

Effects of anti-HMGB1 mAb (#10-22) on the ELISA. Different concentrations of anti-HMGB1 mAb were added to the incubation mixture with the brain homogenate samples containing HMGB1 (A) and rHMGB1 (B). The brain homogenizing samples were prepared by the rat brain using 50 mM Tris-HCL (pH 8.0), and diluted to 80~100 ng HMGB1 equivalent/mL by the sample buffer provided in the ELISA kit. The results are the means ± s.e.m. of three determinations.
**Supplemental Figure 4**

Plasma 4-HNE adducts levels in control IgG-treated and anti-HMGB1 mAb-treated rats. Plasma samples were collected from ischemic rats at 6 h after reperfusion. 4-HNE adducts in the samples were detected by ELISA using 4-HNE-BSA as the standard. The results are the means ± SEM of 4 (sham), 5 (control IgG- and anti-HMGB1 mAb-treated group) rats. **P<0.01 compared with sham control, ##P<0.01 compared with control IgG group.

