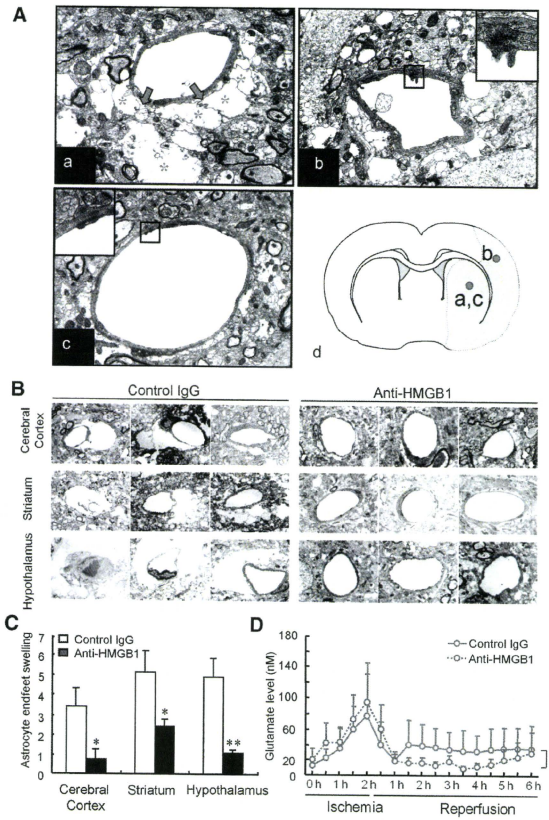

Figure 6. Ultrastructure of the blood–brain barrier (BBB) in the ischemic hemisphere obtained using transmission electron microscopy. **A**, Representative images of the coronal sections from rat brain treated with control IgG (**a**, **b**) or anti-high mobility group box-1 (HMGB1) monoclonal antibody (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 shows 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.

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.[22,23] Thus, 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

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 rHMGB1 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.

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Disclosures

None.

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