Anti-high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats

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ABSTRACT **The high mobility group box-1 (HMGB1), originally identified as an architectural nuclear protein, exhibits an inflammatory cytokine-like activity in the extracellular space. Here we show that treatment with neutralizing anti-HMGB1 monoclonal antibody (mAb; 200 μg, twice) remarkably ameliorated brain infarction induced by 2-h occlusion of the middle cerebral artery in rats, even when the mAb was administered after the start of reperfusion. Consistent with the 90% reduction in infarct size, the accompanying neurological deficits in locomotor function were significantly improved. Anti-HMGB1 mAb inhibited the increased permeability of the blood-brain barrier, the activation of microglia, the expression of TNF-α and iNOS, and suppressed the activity of MMP-9, whereas it had little effect on blood flow. Intracerebroventricular injection of HMGB1 increased the severity of infarction. Immunohistochemical study revealed that HMGB1 immunoreactivity in the cell nuclei decreased or disappeared in the affected areas, suggesting the release of HMGB1 into the extracellular space. These results indicate that HMGB1 plays a critical role in the development of brain infarction through the amplification of plural inflammatory responses in the ischemic region and could be an outstandingly suitable target for the treatment. Intravenous injection of neutralizing anti-HMGB1 mAb provides a novel therapeutic strategy for ischemic stroke.—Liu, K., Mori, S., Takahashi, H. K., Tomono, Y., Wake, H., Kanke, T., Sato, Y., Hiraga, N., Adachi, N., Yoshino, T., Nishibori, M. Anti-high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats. *FASEB J.* 21, 3904–3916 (2007)**

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In ischemic stroke, interruption of blood flow induces neuronal death in the ischemic core as a result of the inability to maintain membrane ion gradients in neurons, excitotoxicity due to elevated glutamate levels and disruption of the blood-brain barrier (BBB). The penumbra, surrounding the core, receives a relatively low blood supply and develops time-dependent inflammatory responses that can be deleterious to the surviving neurons. It is thought that this region can be reversibly rescued and thus could be a target for drug treatment. A diversity of neuroprotective candidate drugs targeting varieties of factors associated with ischemic insult have been subjected to preclinical and clinical studies (1, 2). Despite these extensive efforts, however, an effective therapy has not yet been successfully established. For fibrinolytic purpose, recombinant tissue plasminogen activator (rt-PA) has been used clinically; however, the narrow therapeutic window and adverse effects of rt-PA, such as brain hemorrhage and injury, limit its clinical usefulness (3).

High mobility group box-1 (HMGB1) is a highly conserved nonhistone nuclear protein that contributes to the architecture of chromatin DNA (4). A neurite promoting factor, amphoterin, purified from perinatal rat brain (5, 6), was found to be identical to HMGB1 (6, 7). Recently, HMGB1 was also recognized as a late mediator in septic shock (8–10) as well as a proinflammatory factor (9, 10). The cytokine profile of HMGB1 has shed new light on the role of nuclear proteins and promoted the studies on roles of this unique factor in different disease conditions that are accompanied by a variety of inflammatory responses (11–15). In the central nervous system, HMGB1 was shown to inhibit glutamate transport by glial glutamate-aspartate transporter 1 using the mouse glial membrane preparation

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(16), suggesting a contribution of HMGB1 to the elevation of excitotoxic glutamate in ischemic brain. Moreover, HMGB1 has affinity for t-PA and accelerates its proteolytic activity (7). Since therapeutically injected (17–19) and endogenous t-PA (20) have been implicated in the activation of matrix metalloproteinase-9 (MMP-9) during ischemic insult, HMGB1 may enhance the disruption of BBB structure through activation of MMP via t-PA.

In the present study, we demonstrated that anti-HMGB1 mAb dramatically ameliorated infarction after MCA occlusion in rats, and this effect was associated with a marked reduction in neurological deficits. The treatment resulted in the inhibition of increased BBB permeability, the activation of microglia, and the production of proinflammatory molecules but it did not improve blood flow. This treatment offers a novel therapeutic strategy for ischemic stroke.

MATERIALS AND METHODS

Animals and experimental procedures

All experimental procedures were conducted in accordance with the Okayama University guidelines for animal experiments and were approved by the University's committee on animal experimentation. Male Wistar rats (Charles River, Yokohama, Japan), weighing 250–300 g, were used for all experiments. MCA occlusion was performed as described previously (21). The rats were anesthetized with 2% halothane in a mixture of 50% N_2O and 50% O_2 using a face mask. The root of the right MCA was occluded by insertion of a silicone-coated 4.0 nylon thread from the bifurcation of the internal and external carotid arteries. The tip of the thread was placed 18 mm distal from the bifurcation. 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. After the surgical incision was sutured, the rats were allowed to recover from anesthesia. All rats showed paralysis of the contralateral limbs after recovery from anesthesia. The rats were anesthetized again 5 min before reperfusion. After the skin was reopened, the cerebral blood flow was restored 2 h after MCA occlusion by pulling the thread by 5 mm. Anti-HMGB1 mAb (200 μg) or class-matched control mAb (IgG2a) against *Keyhole Limpet* hemocyanin was administered i.v. immediately and 6 h after reperfusion. For i.c.v. injection of HMGB1 (5 μg in 5 μl of 10 mM Tris-buffered saline, pH 7.4), the injection cannula was inserted into the right ventricle just after reperfusion under anesthesia. HMGB1 was immunoaffinity-purified from HMGB1/HMGB2 mixture (Wako, Osaka, Japan). Body temperature in control and anti-HMGB1-treated rats was within 37.2 ± 0.2°C throughout the experiments and no difference was found between 2 groups.

Evaluation of neurological deficits

Neurological deficits were evaluated by two methods. In the rota-rod test, rats were conditioned for 3 days before MCA occlusion on an accelerating rota-rod cylinder at 5 to 15 rpm. Rats that could stay on the rotating rod at 15 rpm for 180 s were subjected to MCA occlusion. After MCA occlusion, each rat was subjected to trials conducted at 3 different speeds (5, 10 and 15 rpm), and the mean duration of three trials at each speed on the rota-rod was recorded. The other test was a neurological scoring method, essentially as described by Bederson *et al.* (22). The signs of hemiparesis and postural reflex were analyzed as follows: *1)* Forelimb flexion: rats were held by the tail on the flat surface. Paralysis of forelimbs was evaluated by the degree of flexion. *2)* Hind-limb paralysis: both hind limbs were pulled caudally, and the difference in resistance was measured. *3)* Torso twisting: rats were held by the tail on a flat surface, and the degree of body rotation was evaluated. *4)* Lateral push: rats were pushed laterally, and resistance was evaluated. *5)* Posture: the slope of the body axis was evaluated. The scores were categorized according to four grades (0, normal; 1, moderate; 2, considerable; 3, severe). The neurological evaluation was performed by an investigator blind to the treatment condition.

Triphenyltetrazolium chloride (TTC) staining

Coronal sections of the brain 2 mm thick were incubated with 2% TTC at 37°C for 30 min with gentle shaking and then were fixed with 10% formalin in PBS. The stained slices were photographed, and the size of the infarct was quantified using NIH image software.

Histological study

For the histological study, rats were anesthetized with an i.p. injection of sodium pentobarbital at a dose of 50 mg/kg and perfused through the left ventricle with 100 ml of saline, followed by 200 ml of 10% formalin in 0.01 M phosphate-buffered saline. The fixed brain was embedded in paraffin, and sections 6 μm thick were used for histological or immunohistochemical study. Anti-HMGB1 mAb was labeled with horseradish peroxidase and used for immunohistochemical staining. *Griffonia Simplicifolia* IB_4 lectin-HRP conjugate was obtained from EY Laboratories (San Mateo, CA, USA) and anti-CD68 Ab was from R&D (San Diego, CA, USA). Immunohistochemical staining was performed using a Ventana system (Ventana Japan, Osaka, Japan). To determine the number of infiltrating neutrophils in coronal sections (1.7 mm and 0.7 mm rostral and 0.3 mm caudal to the bregma), cryostat sections were stained with antimyeloperoxidase antibody as described previously (21). The number of neutrophils was calculated as the number of myeloperoxidase-positive cells minus the number of CD68-positive cells.

Measurement of BBB leakage

To determine changes in vascular permeability, Evans blue dye (2% in saline, 2 ml/kg; Wako, Japan) was administered to rats via the tail vein immediately after the treatment with antibody. After 3 h, the rats were perfused via the left ventricle with 150 ml of saline, and the brains were removed, weighed, and dissected into hemispheres after removal of the brain stem and cerebellum. The hypothalamus and the remaining brain of 2 mm thick from rostral (no. 1) to caudal (no. 5) were dissected in each hemisphere, homogenized in 1 M KOH, and kept overnight. Brain homogenate was then mixed with 0.6 M H_3PO_4-acetone (5:13) at a 1:9 ratio. After centrifugation, the supernatant was discarded, and 50% trichloroacetic acid was added to the pellet to extract Evans blue. After centrifugation, the resultant supernatant was diluted with ethanol (1:3), and the dye concentration was determined spectrophotometrically at 620 nm. The tissue concentration of Evans blue was quantified from a standard curve that was derived from a known amount of the dye mixed with brain homogenate, and was normalized to tissue weight.

Zymography

Gelatin zymography was performed as follows. Briefly, the brain hemisphere was homogenized with 0.1 M Tris-HCl, pH 7.4, containing 5 mM $CaCl_2$ and 0.05% Brij-35, and the homogenate was centrifuged at 10,000 *g* for 30 min. The resultant supernatant (40 μg protein) was mixed with 100 μl of gelatin-agarose for 1 h at 4°C with gentle shaking. The agarose beads were washed twice with 800 μl of 50 mM Tris-buffered saline, and then the bound proteins were eluted with 10% DMSO in PBS. The eluate was mixed with the sample buffer to give a final concentration of 2.5% glycerol, 5% SDS, and 125 mM Tris-HCl (pH 6.8) in the absence of reducing agent, and incubated at 25°C for 10 min. Protein samples were then electrophoresed on 10% SDS-polyacrylamide gel containing 1 mg/ml gelatin. After electrophoresis, the gels were incubated twice in 2.5% Triton X-100 at room temperature for 30 min to remove SDS from the gel. The gels were next incubated in 0.05 M Tris-buffered saline (pH 7.4) containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$, and 0.035% Brij-35 for 10 min at room temperature and then sandwiched between 3 pieces of Whatman 3MM chromatography paper that had been saturated with buffer. The reaction was continued for 40 h at 37°C, and the gels were subsequently stained with Coomassie Brilliant Blue R-250 and appropriately destained.

Determination of cerebral blood flow

Cerebral blood flow (CBF) was measured on-line using a flexible-fiber probe linked to a Doppler blood flow device TBF-LC1 (Unique Medical Inc., Osaka, Japan). The scalp was incised at the midline and the skull was exposed. Probes were attached to the skull surface on both lateral sides 7 mm inferior from the bregma. CBF signals from both hemispheres were simultaneously monitored throughout the experiments while anesthesia was maintained with 2% halothane in a mixture of 50% N_2O and 50% O_2.

RT-PCR

The rats were treated with 50 mg/kg sodium pentobarbital (i.p.) 6 h after reperfusion and then perfused transcardially with 50 ml of ice-cold saline before decapitation. The striatum and cerebral cortex corresponding to the core infarct were dissected. Total RNA was isolated with BioRobot EZ1 (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed with Avian Myeloblastosis virus reverse transcriptase XL and Oligo dT-primers (Takara RNA PCR Kit; Takara Biomedicals, Shiga, Japan). PCR was performed using Takara Ex taq HS DNA polymerase (Takara). The sense and antisense primers used for the analysis of expression of HIF-1α, VEGF, iNOS, MMP-2, MMP-9 and β-actin were as follows. HIF-1α: 5′-GCAGCACGATCTCGGCGAAGCAAA-3′ and 5′-GCACCATAACAAAGCCATCCAGGG-3′ (235 bp). VEGF: 5′-GCTCTCTTG GGTGCACTGGA-3′ and 5′-CTTCTCTTGGGTGCACTGGA-3′ (635 bp). iNOS: 5′-GCATCCCAAGTACGAGTGGT-3′ and 5′-GAAGGCGTAGCTGAACAAGG-3′ (700 bp). MMP-2: 5′-GATCTGCAAGCAAGACATTGTCTT-3′ and 5′-GCCAAATAAACCGATCCTTGAA-3′ (77 bp). MMP-9: 5′-GTAACCCTGGT CACCGGACTT-3′ and 5′-ATACGTTCCCGGCTGATCAG-3′ (80 bp). β-actin: 5′-CAGAGCAAGAGAGGCATCCT-3′ and 5′-GGCAGCTCATAGCTCTTCTC-3′ (535 bp). The annealing temperature was 55°C for all primer pairs.

Real-time PCR

Real-time PCR was performed 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 the expression of iNOS, TNF-α, and β-actin were the following. Inducible NOS: 5′-GCATCCCAAGTACGAGTGGT-3′ and 5′-GAAGTCTCGGACTCCAATCTC-3′ (154 bp). TNF-α: 5′-GCCCAGACCCTCACACTC-3′ and 5′-CCACTCCAGCTGCTCCTCT-3′ (99 bp). β-Actin: 5′-CCCGCGAGTACAACCTTCT-3′ and 5′-CGTCATCCATGGCGAACT-3′ (72 bp). The expression of β-actin was used to normalize cDNA levels. The PCR products were analyzed by a melting curve to ascertain the specificity of amplification.

Production of HMGB1-specific monoclonal antibody

Rats were immunized with HMGB1/HMGB2 (Wako, Osaka, Japan) emulsified with Freund's complete adjuvant and the booster injection with incomplete adjuvant was administered 3 wk later. After confirming elevation of anti-HMGB Ab levels, hybridomas were produced as described previously (23). The epitope recognized by each monoclonal antibody was determined by dot blotting using synthetic overlapping peptides derived from human HMGB1 sequence 15 amino acids in length. The clone (#10–22, subclass IgG2a) recognizing the C-terminal sequence of the HMGB1 molecule (DEDEEEE) and specific for HMGB1 but not for HMGB2 was used for the experiments. A monoclonal antibody (IgG2a subclass) against *Keyhole Limpet* hemocyanin was produced in the same way and was used as the control antibody.

Statistics

Statistical significance was evaluated by ANOVA followed by Dunnett's test for multiple comparison, or by Student's *t* test for comparisons between 2 groups. *P* values less than 0.05 were considered to be significant.

RESULTS

Characterization of mAbs against HMGB1

Three clones (#10–22, #4–1, and #11–19) producing anti-HMGB1 Ab were obtained. The epitope recognized by each mAb was determined using synthetic overlapping peptides derived from human HMGB1 sequence 15 amino acids in length. **Figure 1*A*** and ***B*** show that mAb (#10–22, subclass IgG2a) recognized the C-terminal peptide sequence no. 41 (EDEEDEDEEEDDDE) of the HMGB1 molecule that was specific for HMGB1 but not for HMGB2. Since no. 40 peptide (DEEEEEDEEDEEE) was not recognized by mAb (#10–22) at all, the epitope was estimated to reside in the latter half of peptide no. 41. The other two mAbs (#4–1 and #11–19) recognized no. 30 and no. 31 peptides, indicating that the epitopes were overlapping sequence of both peptides (LKEKYEKDIA) that was also included in HMGB2. Therefore, we used mAb (#10–22) in the following experiments. The mAb detected a single band of 29 kDa on Western blotting using rat brain (Fig. 1*C*). HMGB1 concentration-dependently (0.01–10 μg/ml) stimulated the expression of ICAM-1 on the surface of CD-14-positive monocytes in human peripheral mononuclear cell culture when the expression was determined 24 h after the start of

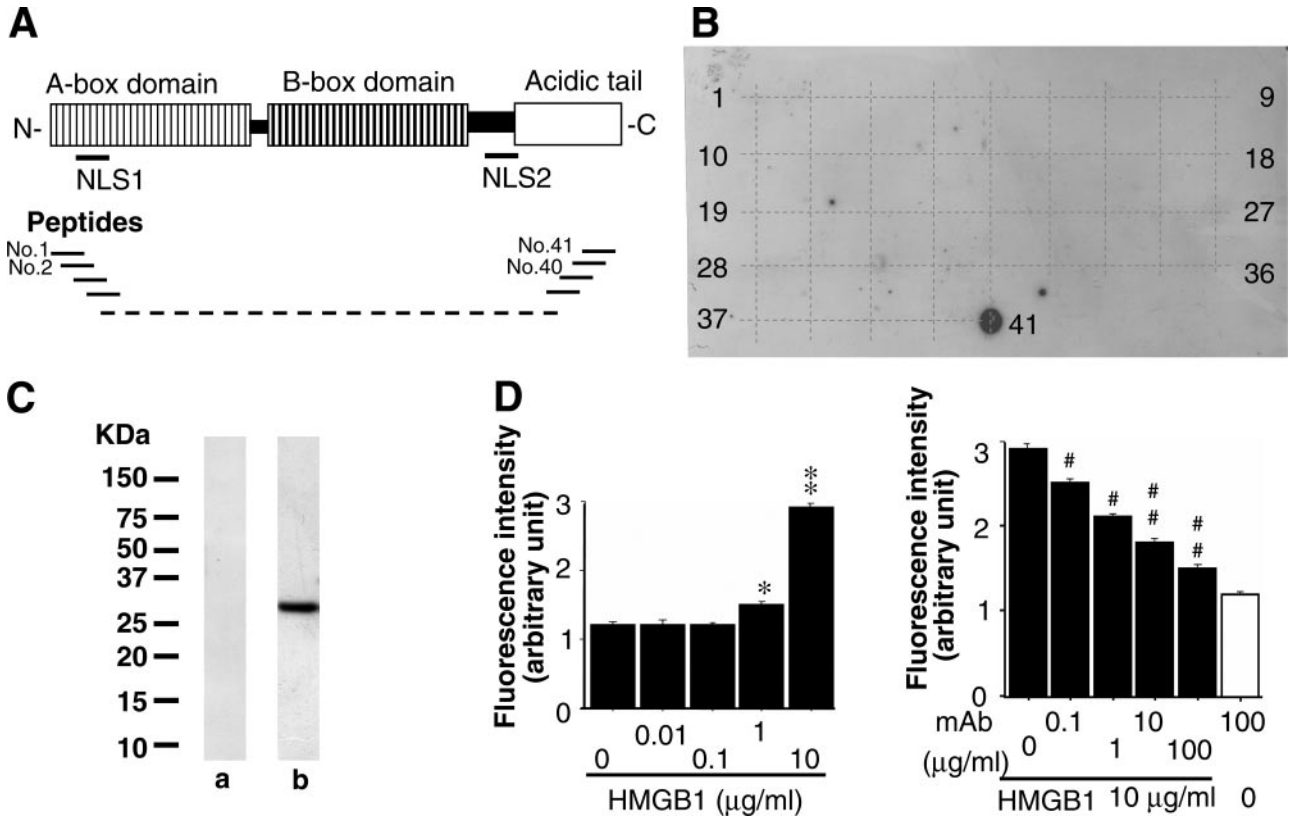

Figure 1. Epitope determination and characterization of rat mAb against HMGB1. Monoclonal Abs were produced as described in Materials and Methods. *A)* The domain structure of HMGB1 and the synthetic overlapping peptides 15 amino acids in length (nos. 1–41) for determining epitopes were shown. NLS; nuclear localization signal. *B)* The epitopes of anti-HMGB1 mAbs were determined by dot blotting. The anti-HMGB1 mAb (clone #10–22) used in the present study, recognized the C-terminal sequence (No. 41 peptide, DEDEEEE) of the HMGB1 molecule and was specific for HMGB1 but not for HMGB2. *C)* Detection of rat brain HMGB1 by Western blotting. The whole rat brain was homogenized with 50 mM Tris-HCl buffer, pH 7.5, containing 1% Triton-X100. After centrifugation at 100,000 *g* for 30 min, the resultant supernatant was subjected to SDS-PAGE under reducing condition. The proteins were blotted with anti-HMGB1 mAb (clone #10–22) (lane b) with the secondary antirat IgG goat IgG-HRP conjugate. The chemiluminascence detection was performed. In lane a, the primary Ab was omitted. *D)* Neutralization of ICAM-1-inducing activity of HMGB1 by anti-HMGB1 mAb (clone #10–22). Human PBMC (1×10^5 cells) were stimulated with HMGB1 in the presence of different concentrations of anti-HMGB1 mAb, and the expression of ICAM-1 on CD14-positive monocytes was determined by FACS analysis 24 h after stimulation. $^*P < 0.05$, $^{**}P < 0.01$ compared with the value in the absence of HMGB1. $\#P < 0.05$, $\#\#P < 0.01$ compared with the value in the absence of anti-HMGB1 mAb.

stimulation (Fig. 1*D*), as shown by FACS analysis. Addition of the mAb to culture medium concentration-dependently (0.1–100 μg/ml) inhibited the up-regulation of monocyte ICAM-1 expression induced by HMGB1 (10 μg/ml) (Fig. 1*D*). Thus, anti-HMGB1 mAb (#10–22) was demonstrated to be a neutralizing activity for HMGB1.

Effects of Anti-HMGB1 mAb on brain infarction

Middle cerebral artery (MCA) occlusion for 2 h in rats produced massive infarction in the fronto-parieto-temporal regions of the cerebral cortex as well as the striatum in the ischemic hemisphere when the infarct areas were determined with tritetrazolium chloride (TTC) staining 24 h after reperfusion (**Fig. 2*A***). Treatment with i.v. injection of anti-HMGB1 mAb (200 μg) twice (immediately and 6 h after reperfusion) reduced the infarct volumes dramatically (Fig. 2*A*). The infarction volumes were quantified in the cerebral cortex and the striatum, respectively, and the results are summarized in Fig. 2*B*. Anti-HMGB1 mAb reduced the infarction volumes in the cerebral cortex and the striatum equally, leading to the 90% and 75% reduction at 24 and 48 h, respectively.

Consistent with the reduction in infarct volumes, treatment with i.v. injection of anti-HMGB1 mAb markedly improved the neurological deficits observed on the rota-rod test (Fig. 2*C*). In particular, mAb treatment significantly ameliorated the deficits observed in control rats under the high-speed load (15 rpm). Moreover, high neurological deficit scores in the control rats persisted for up to 48 h; however, the scores in the anti-HMGB1 mAb treated-group time-dependently decreased and reached a considerably lower level at 48 h (Fig. 2*D*). These findings as a whole indicated that anti-HMGB1 mAb dramatically ameliorated infarct formation and improved the accompanying neurological symptoms significantly. Furthermore, we found that intracerebroventricular (i.c.v.) injection of HMGB1 (5

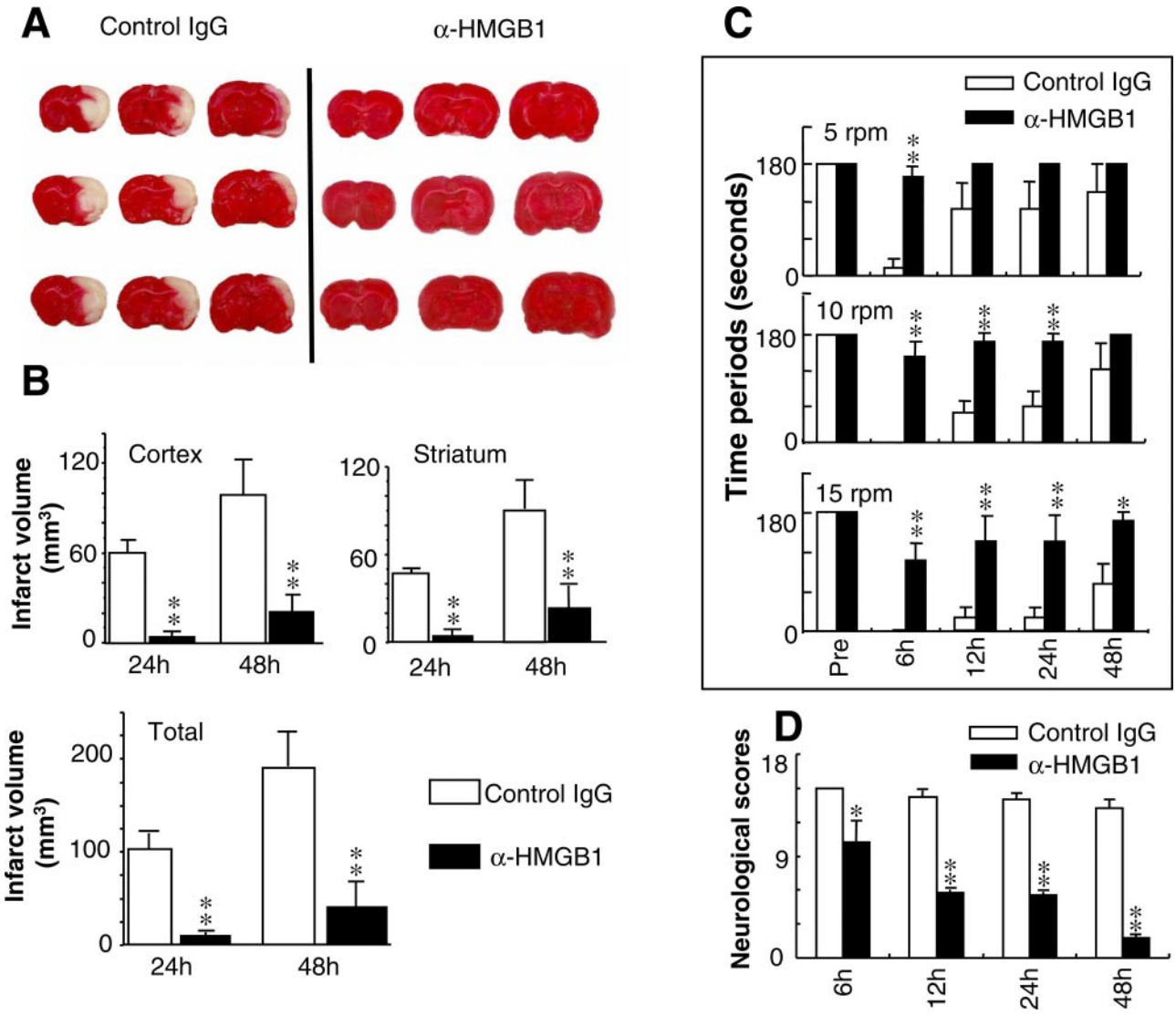

Figure 2. The effects of anti-HMGB1 mAb on brain infarction induced by MCA occlusion in rats. *A)* Brain infarction induced by right MCA occlusion was evaluated by TTC staining of brain slices from rats treated with anti-HMGB1 mAb or control class-matched mAb (anti-*Keyhole Limpet* hemocyanin mAb, IgG2a) 24 h after reperfusion. Three representative cases from each group are shown. *B)* The infarct volumes in the striatum and cerebral cortex 24 or 48 h after reperfusion were quantified using NIH image software. The results are the means ± SEM of 5 animals. $*P < 0.05$, $**P < 0.01$ compared with the control group. *C)* Neurological deficits in rats after MCA occlusion were examined using the rota-rod test and by neurological scoring. In the rota-rod test, trials were performed at 3 different speeds and the time intervals running on the rod were determined for each rat after reperfusion. The results are the means ± SEM of 4 animals. $*P < 0.05$, $**P < 0.01$ compared with the control group. *D)* Neurological scoring was done according to the categories described in Materials and Methods. The results are the means ± SEM of the total score from 4 animals. $*P < 0.05$, $**P < 0.01$ compared with the control group.

μg) immediately after reperfusion aggravated infarction and exacerbated the neurological deficit induced by 1.5-h occlusion of the MCA (**Fig. 3**). These findings were consistent with the effects of neutralizing anti-HMGB1 mAb.

Histological studies on the effects of anti-HMGB1 mAb

Hematoxylin-eosin staining of brain sections from the control MCA-occlusion rats showed that diffuse neuronal cell death occurred 12 h after reperfusion in the cerebral cortex and the striatum regions, and thereafter these regions were designated as the infarct areas (**Fig. 4**). In contrast, these areas appeared to be intact in rats treated with anti-HMGB1 mAb (Fig. 4). Immunohistochemical studies revealed the presence of HMGB1 immunoreactivity in the nuclei of brain cells in the nonischemic side (**Fig. 5*A***). The immunoreactive nuclei were widespread, but not all (arrowheads in Fig. 5*A*). The distribution of HMGB1 immunoreactivity in the intact rat brain was similar to that observed in the nonischemic side (data not shown).

The cytoplasm and neuronal processes were also positive in some neurons. In the ischemic side, strongly

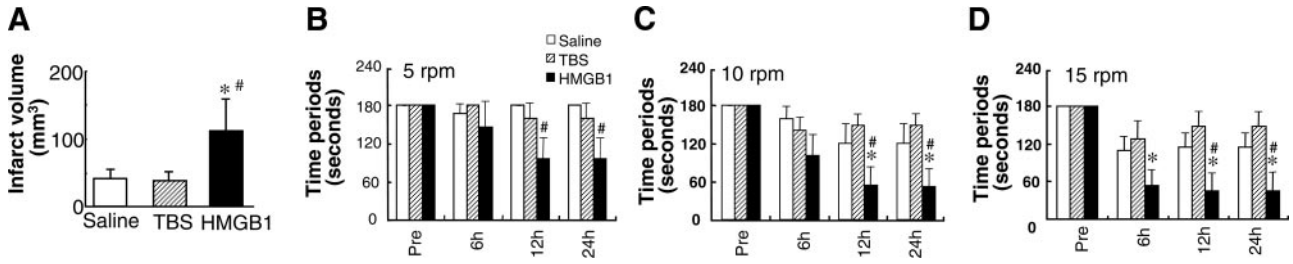

Figure 3. The effects of intracerebroventricular injection of HMGB1 on brain infarction induced by MCA occlusion in rats. *A)* The effect of i.c.v. injection of HMGB1 in Tris-buffered saline (TBS), TBS, or saline on brain infarction induced by MCA occlusion for 1.5 h. The results are the means ± SEM of 5 animals. $*P < 0.05$ compared with the TBS group. $\#P < 0.05$ compared with the saline group. *B–D)* Neurological deficits in rats treated with i.c.v. injection of HMGB1, TBS or saline were examined using the rota-rod test as described in Fig. 2. In the rota-rod test, trials were performed at 3 different speeds and the time intervals running on the rod were determined for each rat after reperfusion. The results are the means ± SEM of 4 animals. $*P < 0.05$ compared with the TBS group. $\#P < 0.05$ compared with the saline group.

immunoreactive nuclei disappeared from the striatum and were less prominent in the cerebral cortex 12 h after reperfusion (Fig. 5*A*). These changes in the distribution of HMGB1 immunoreactivity after ischemia strongly suggested that HMGB1 was translocated from nuclear stores to the extracellular space. Conversely, the immunoreactivity in the nerve fibers in the internal capsule appeared to be enhanced in the ischemic hemisphere as compared with the contralateral side (Fig. 5*C*). The paraventricular nucleus and anterior hypothalamic area on the ischemic side were strongly immunoreactive with diffuse and moderate staining in the surrounding areas, whereas staining was weak on the nonischemic side (Fig. 5*B*). There were also strongly immunoreactive neurons scattered in the reticular thalamic nucleus on the ischemic side (Fig. 5*C*). The wall of the internal carotid artery was strongly immunoreactive on the ipsilateral hemisphere (Fig. 5*B*), while no such immunoreactivity was observed on the contralateral side. The treatment with anti-HMGB1 mAb substantially diminished the changes observed in the ischemic hemisphere in control rats (Fig. 5*A–C*). These results indicated that neutralization of extracellular HMGB1 by the administration of a specific Ab inhibited the disappearance of HMGB1 immunoreactivity from nuclei and the up-regulation of HMGB1 in specific neurons in the ischemic brain hemisphere.

Analysis on the action mechanism of anti-HMGB1 mAb

Leakage of Evans blue dye during 3 h after the reperfusion was remarkable in the ipsilateral hemisphere and hypothalamus of control rats, whereas Evans blue leakage in the contralateral hemisphere was much lower (**Fig. 6*A***). There was no detectable leakage of Evans blue in the hemispheres of sham-operated rats (Fig. 6*A*). Treatment with anti-HMGB1 mAb signifi-

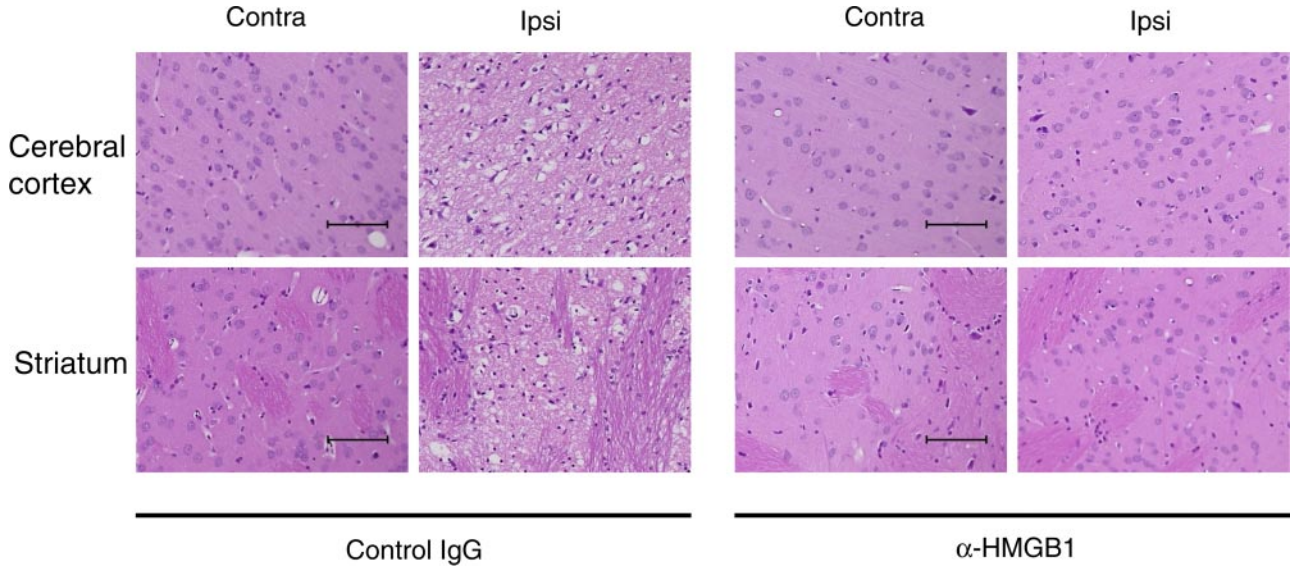

Figure 4. Histological examination of brain sections from MCA-occluded rats. The right MCA was occluded for 2 h. After reperfusion, the rats were treated with anti-HMGB1 mAb or control mAb. The tissues were fixed by infusing formalin transcardially 12 h after reperfusion. Brain sections of the cerebral cortex and striatum from the ipsilateral (Ipsi) and contralateral (Contra) sides were stained with hematoxylin-eosin. Scale bars represent 100 μm.

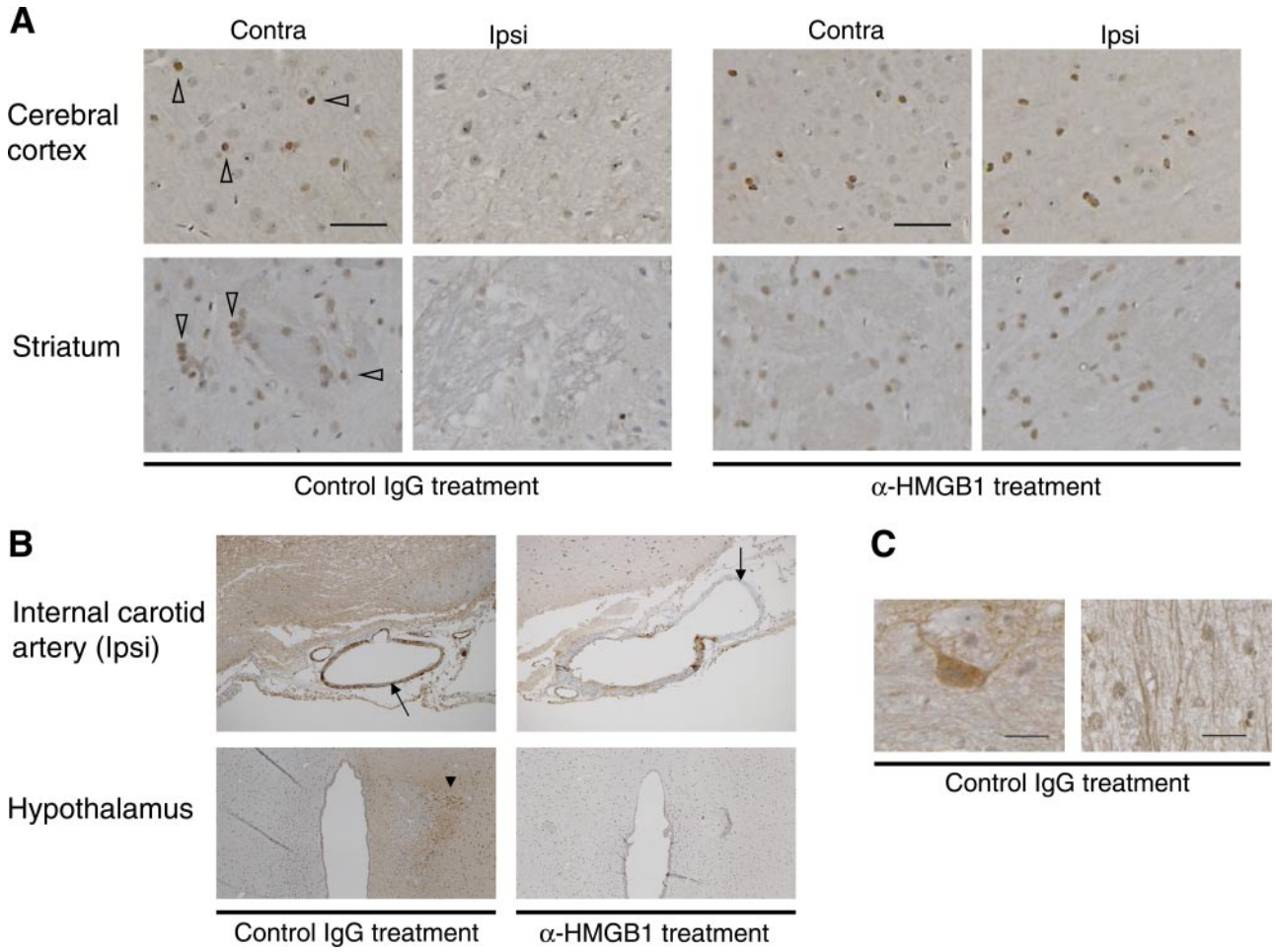

Figure 5. Immunohistochemical localization of HMGB1 in the brain sections from MCA-occluded rats. *A)* The right MCA was occluded for 2 h. After reperfusion, the rats were treated with anti-HMGB1 mAb or control mAb. The tissues were fixed by infusing formalin transcardially 12 h after reperfusion. Brain sections of the cerebral cortex and striatum from the ipsilateral (Ipsi) and contralateral (Contra) sides were stained with anti-HMGB1 mAb labeled with horseradish peroxidase. The reaction was developed by addition of diaminobenzidine and hydrogen peroxide. Counterstaining was performed using hematoxylin. The immunoreactive nuclei were indicated by arrow heads in the contralaterl side of the control rat brain. Scale bars represent 50 μm. *B)* Immunohistochemical staining of internal carotid artery and paraventricular nucleus using anti-HMGB1 mAb labeled with horseradish peroxidase. Arrows indicate the ipsilateral internal carotid artery from control (left) and anti-HMGB1 mAb-treated (right) rats. Arrowhead indicates the strong immunoreactive paraventricular nucleus on the ipsilateral side of a control rat (left). *C)* Large immunoreactive neuron in the reticular thalamic nucleus (left) and immunoreactive fibers in the internal capsule (right) of the ipsilateral hemisphere of a control rat. Scale bars represent 20 μm.

cantly reduced the extravasation of Evans blue dye in the ischemic hemisphere including the hypothalamus (Fig. 6*A*, *B*).

In coronal sections, high leakage of Evans blue dye was observed in the striatum and parietal and temporal cortex, which was consistent with the infarct areas determined 1 day after reperfusion (Figs. 2*A*, 6*A*). Treatment with anti-HMGB1 mAb appeared to inhibit the vascular leakage evenly throughout all brain regions (Fig. 6*A*, *B*).

The MMP activities determined by gelatin zymography clearly showed that pro-MMP-9 band was increased significantly in the ischemic hemisphere of control rats, whereas it was increased only minimally in anti-HMGB1 mAb-treated rats (Fig. 6*C*). MMP-2 activity was detected constitutively in both hemispheres and was not affected by ischemic insult (Fig. 6*C*).

Doppler flowmetry measurements showed that blood flow on the ipsilateral side of the temporal cortex in MCA-occluded rats immediately after occlusion was decreased to 24% of the preoccluded level (**Fig. 7**). By the end of the 2-h occlusion period, blood flow gradually recovered to 40% of the preoccluded level. Reperfusion slightly accelerated the recovery of blood flow. In the contralateral side, a change in blood flow was minimal. Treatment with anti-HMGB1 mAb (200 μg) did not affect the change in blood flow induced by MCA occlusion in either side of the cerebral cortex (Fig. 7).

Inhibition of microglia activation by anti-HMGB1 mAb

Activated microglia detected by *Griffonia simplicifolia* (IB_4)-lectin were observed in the cerebral cortex, stria-

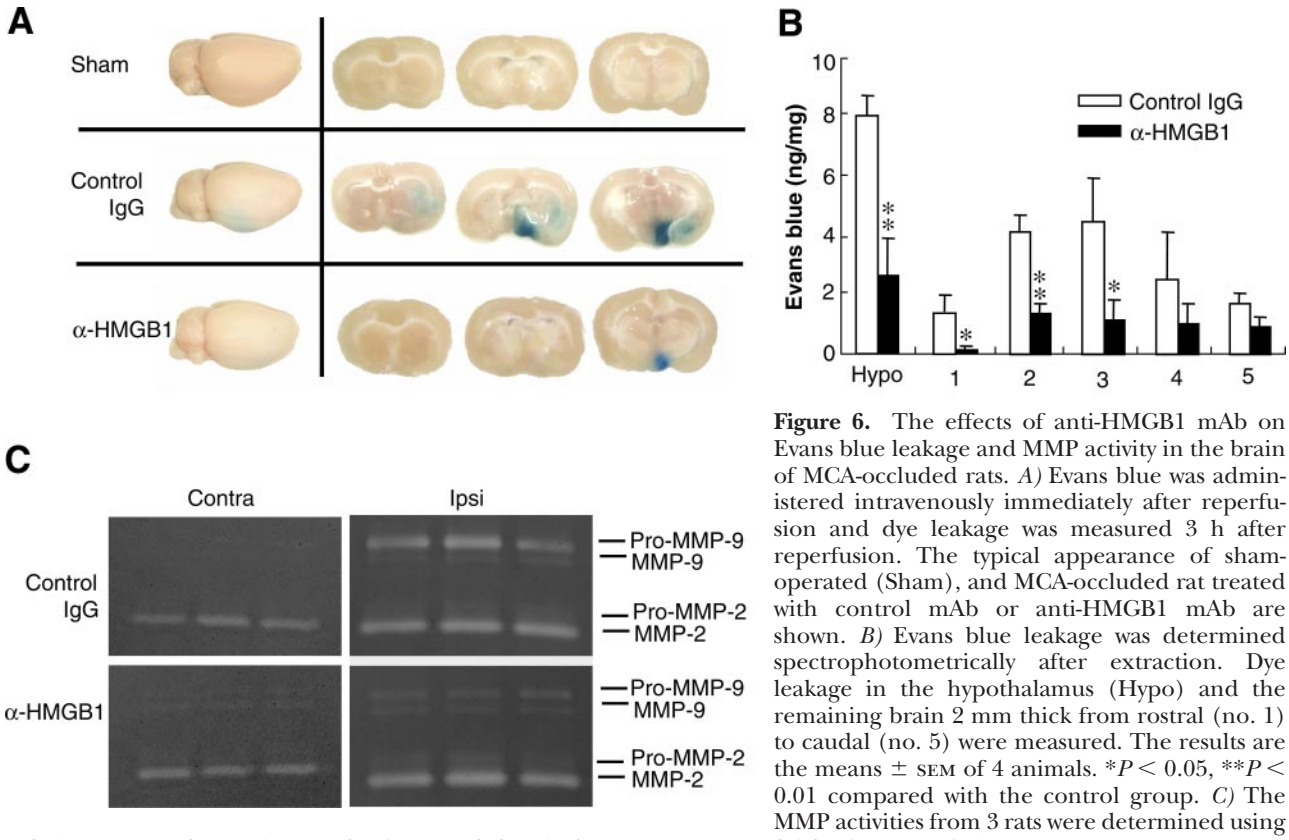

Figure 6. The effects of anti-HMGB1 mAb on Evans blue leakage and MMP activity in the brain of MCA-occluded rats. *A)* Evans blue was administered intravenously immediately after reperfusion and dye leakage was measured 3 h after reperfusion. The typical appearance of sham-operated (Sham), and MCA-occluded rat treated with control mAb or anti-HMGB1 mAb are shown. *B)* Evans blue leakage was determined spectrophotometrically after extraction. Dye leakage in the hypothalamus (Hypo) and the remaining brain 2 mm thick from rostral (no. 1) to caudal (no. 5) were measured. The results are the means ± SEM of 4 animals. $*P < 0.05$, $**P < 0.01$ compared with the control group. *C)* The MMP activities from 3 rats were determined using gelatin zymography. Brain samples from each hemisphere were prepared 6 h after reperfusion.

tum, and hypothalamus in the ischemic side of the control rat brain (**Fig. 8*A–D***); however, there were few such microglia in the ischemic side of the brain in anti-HMGB1 mAb-treated rats (Fig. 8*E*). However, no difference was seen between control and anti-HMGB1 mAb-treated rats with respect to the number of neutrophil infiltrates in the ischemic side of the striatum and remaining brain tissue 12 h after reperfusion (Fig. 8*F*).

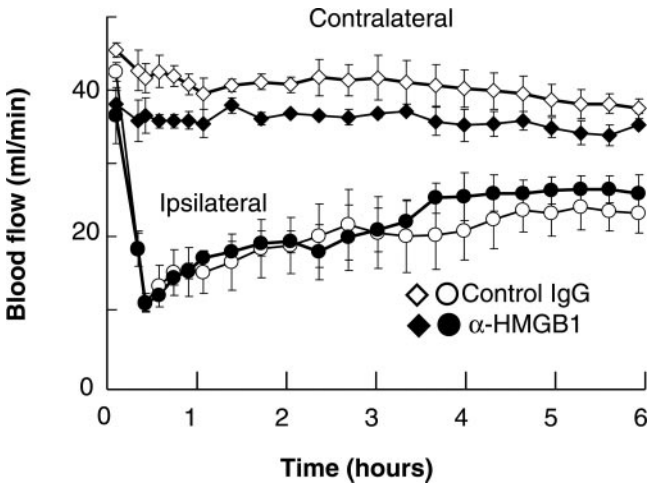

Figure 7. The effect of anti-HMGB1 mAb on blood flow in the brain of MCA-occluded rats. Changes in cerebral blood flow were determined bilaterally using Doppler flowmetry during the MCA-occlusion (0–2 h) and reperfusion (2–6 h) periods. The results are the means ± SEM of 5 animals.

There were few neutrophils in the contralateral side of the brain in the control and anti-HMGB1 mAb-treated groups.

Anti-HMGB1 mAb inhibits the expression of TNF-α and iNOS

The expression of mRNA coding for inflammation-related molecules, including inflammatory cytokines, was determined by RT-PCR. MCA occlusion significantly up-regulated the expression of TNF-α and iNOS in the cerebral cortex and striatum of the ischemic hemisphere as compared with the nonischemic side (**Fig. 9**). Treatment with anti-HMGB1 mAb almost completely inhibited the expression of TNF-α and iNOS in the cerebral cortex and striatum of the ischemic hemisphere (Fig. 9). In contrast, the ischemia-induced changes in the expression of HIF-1α, IL-18, COX-2, MMP-2, and MMP-9 were marginal and anti-HMGB1 mAb had no apparent effect on the expression of these mRNAs. The quantification of iNOS and TNF-α expression using real-time PCR confirmed marked increases in the expression of iNOS and TNF-α in the ischemic cerebral cortex and striatum compared with the contralateral side in the control rats whereas the increases were not significant in the anti-HMGB1-treated rats (**Fig. 10**). The treatment with anti-HMGB1 mAb significantly inhibited the TNF-α expression in the ischemic cerebral cortex and striatum (Fig. 10).

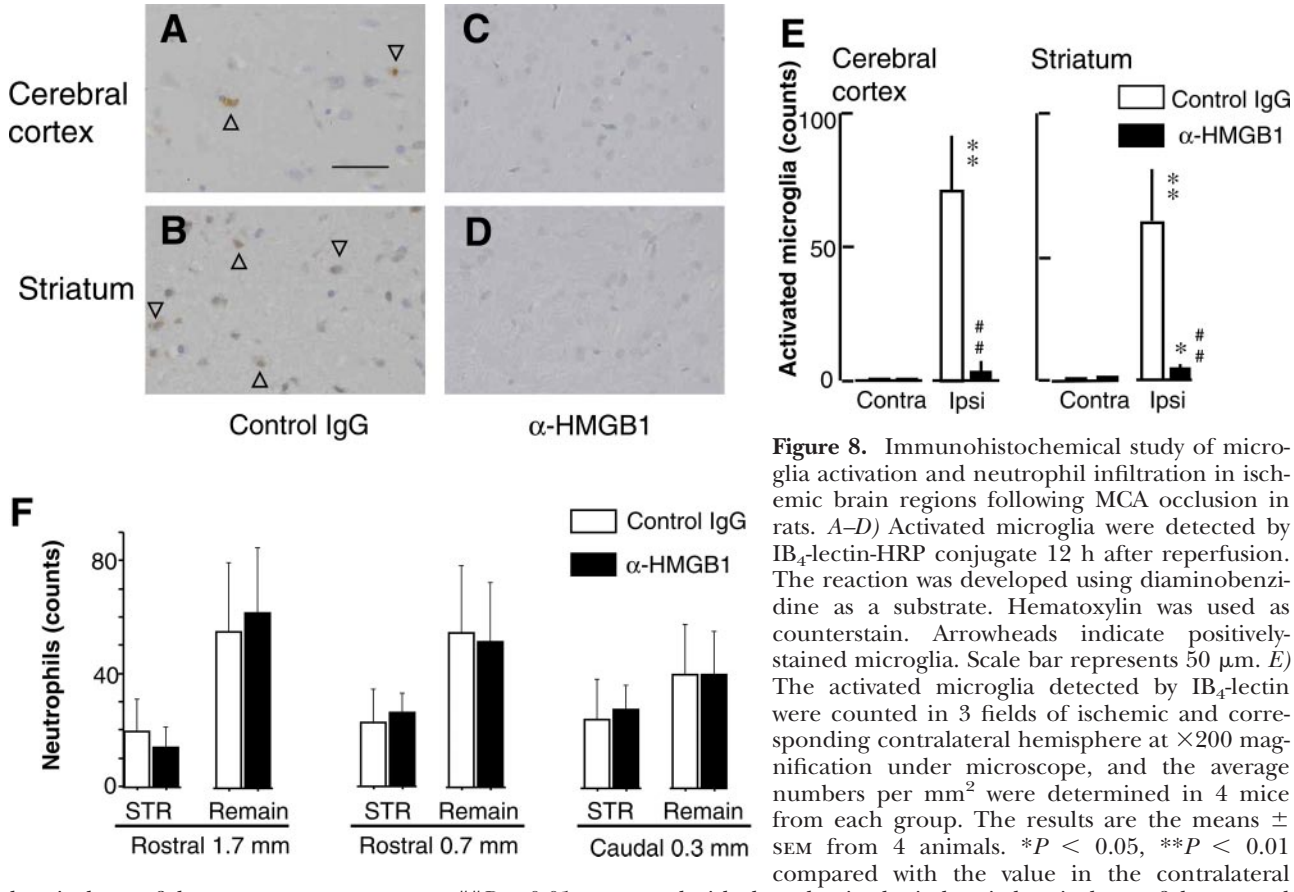

Figure 8. Immunohistochemical study of microglia activation and neutrophil infiltration in ischemic brain regions following MCA occlusion in rats. *A–D)* Activated microglia were detected by IB_4-lectin-HRP conjugate 12 h after reperfusion. The reaction was developed using diaminobenzidine as a substrate. Hematoxylin was used as counterstain. Arrowheads indicate positively-stained microglia. Scale bar represents 50 μm. *E)* The activated microglia detected by IB_4-lectin were counted in 3 fields of ischemic and corresponding contralateral hemisphere at ×200 magnification under microscope, and the average numbers per mm^2 were determined in 4 mice from each group. The results are the means ± SEM from 4 animals. $*P < 0.05$, $**P < 0.01$ compared with the value in the contralateral hemisphere of the same treatment group. $\#\#P < 0.01$ compared with the value in the ischemic hemisphere of the control group. *F)* The number of neutrophil infiltrates in the ischemic side of the brain 12 h after reperfusion was determined by subtracting the number of CD68-positive cells from the total number of myeloperoxidase-positive cells in 3 coronal sections (1.7 mm and 0.7 mm rostral and 0.3 mm caudal to bregma). The results are the means ± SEM per section from 7 animals.

DISCUSSION

In the present study, we demonstrated that i.v. treatment with a neutralizing anti-HMGB1 mAb produced a remarkable therapeutic effect on brain infarction induced by MCA occlusion/reperfusion in rats. Treatment with anti-HMGB1 mAb dramatically reduced the infarct size, and this effect was associated with a pronounced improvement in neurological deficits. Furthermore, the ischemic status before mAb treatment was confirmed by the appearance of hemiparesis, and the body temperature in control and anti-HMGB1-treated rats did not differ. Treatment with anti-HMGB1 mAb strongly inhibited the ischemia-induced increase in microvascular protein leakage that was likely associated with inhibition of MMP activity. It has been reported that induction and activation of MMP-9 (18, 24) contributes to disruption of the blood-brain barrier, leading to aggravation of brain infarction. Therefore, HMGB1 may play a key role in the development of brain injury under hypoxic and ischemic conditions by disrupting microvascular structure and neuron-matrix interactions. In addition, a recent study (16) showed that HMGB1 inhibits glial glutamate transport by GLAST in mouse gliosomes and suggested that HMGB1 can increase extracellular glutamate levels *in vivo*. Thus, it is possible that anti-HMGB1 mAb also prevented the elevation of glutamate levels that leads to neuronal excitotoxicity.

Recent studies in various models of systemic inflammation have indicated that HMGB1 has proinflammatory cytokine activity. HMGB1 has been identified as a late mediator of septic shock (8). Anti-HMGB1 mAb has been shown to decrease the lethality induced by LPS (8) and cecal ligation puncture (25) in mice, even though the mAb was administered after the induction of disease. There is mounting evidence that HMGB1 may be involved in other inflammatory diseases including acute lung injury (14), hepatic injury (15), and rheumatoid arthritis (12). In monocytes/macrophages, HMGB1 has also been reported to stimulate the production of IL-1α/β, TNF-α, IL-6, IL-8, and MIP-1α/β (26); induce iNOS (27); and stimulate monocyte chemotaxis (28). In the present study, we demonstrated that ischemia-induced up-regulation of iNOS and TNF-α was inhibited by the neutralization of HMGB1. The induction of iNOS (29) and TNF-α (30) following an ischemic insult was reported to occur mainly in microglia. Thus, it is likely that HMGB1 activates microglia in the brain, leading to the up-regulation of iNOS and TNF-α expression. In our study, the detection of activated microglia by IB_4-lectin in the ischemic

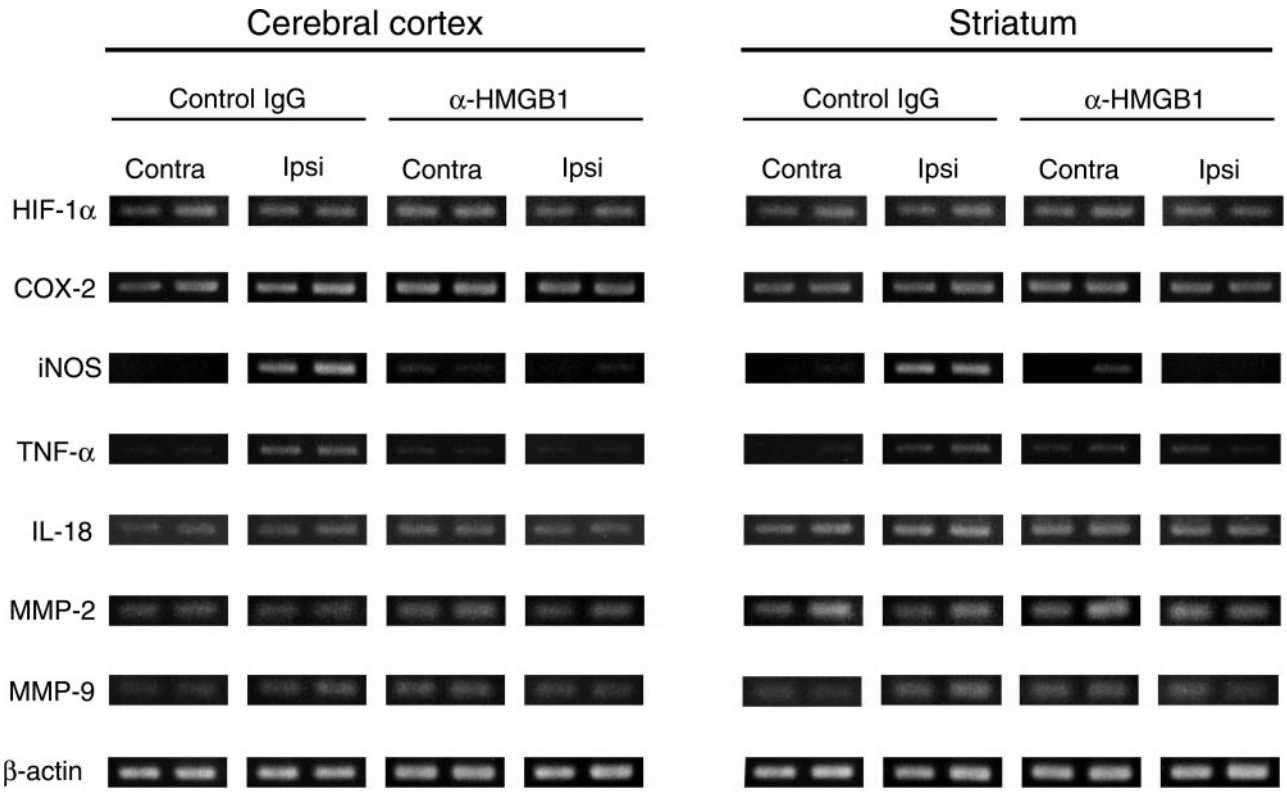

Figure 9. Expression of inflammation-related molecules in the brain of MCA-occluded rats. The right MCA was occluded for 2 h. Anti-HMGB1 mAb or control mAb was administered immediately after reperfusion. Six hours after reperfusion, the rats were decapitated under deep anesthesia with pentobarbital and both sides of the cerebral cortex and the striatum were dissected. Total RNA was extracted from each sample and RT-PCR was performed using the indicated primer pairs. Representative results from 3 rats are shown.

hemisphere supported this notion. Taken together, this evidence suggests that HMGB1 may play a key role in the development of brain injury under hypoxic and ischemic conditions by disrupting the structure of the microvasculature and by activating microglia to trigger the excess production of TNF-α and NO-derived radicals, which can lead to further development of the inflammatory response and exacerbation of neuronal injury. In fact, the induction of iNOS (31), TNF-α (32) and MMP-9 (18, 24) has been reported to be involved in the inflammatory response and disruption of the blood-brain barrier, leading to aggravation of brain infarction. The regulation of any one of these factors has been postulated to reduce ischemic injury (24, 31, 32). Therefore, it is reasonable that anti-HMGB1 mAb, which has the ability to substantially reduce the expression of all three proinflammatory factors, exerted profound therapeutic effects on brain infarction.

Recently, Kim *et al.* (33) reported that microinjection of short hairpin RNAi for HMGB1 into the striatum inhibited neuronal death in the transfected area after MCA occlusion in rats. They also showed that the RNAi inhibited the expression of TNF-α and iNOS, in addition to IL-1β and COX-2, in the restricted area 24 h after MCA occlusion. Although their intervention modulated the expression of HMGB1, it was not possible to determine whether intracellular or extracellular HMGB1 was a more important mediator of brain injury. Moreover, the effect of hairpin RNAi was limited in the restricted micromilieu and, therefore, the evaluation of neurological deficits in the treated rats could not be performed (33). However, our findings on the expression of TNF-α and iNOS were consistent with their results.

There are two main sources of extracellular HMGB1 (34, 35). The first is released from the nuclei of necrotic cells and the second is secreted from cells of the monocyte/macrophage lineage in an activation-dependent manner. The lack of HMGB1-immunoreactivity in the nuclei of cells in necrotic areas supported that HMGB1 may also be released from necrotic cells in the brain. Also, based on the observation of the diffuse immunoreactive staining for HMGB1 in the paraventricular and anterior hypothalamic areas, it seemed that HMGB1 might be released from surviving neurons without inducing necrosis. Under such conditions, HMGB1 may bind to extracellular matrix components, i.e., neurocan and phosphacan (36), which have been reported to have high affinity for HMGB1. Thus, it is possible that the release of HMGB1 from different types of cells into the extracellular environment occurred during the ischemic insult in the ipsilateral hemisphere. Interestingly, treatment with anti-HMGB1 mAb also inhibited the ischemia-induced changes in the distribution pattern of HMGB1 immunoreactivity, suggesting that HMGB1 may be involved in the control

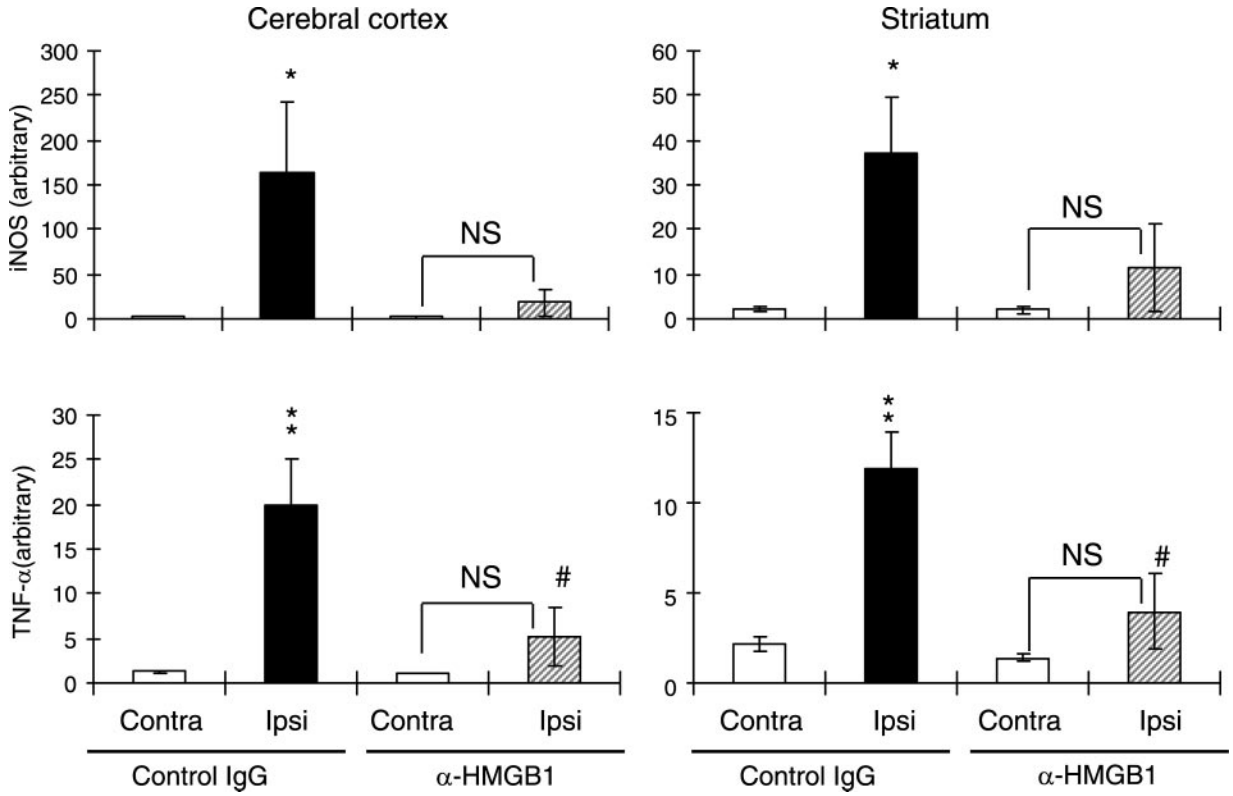

Figure 10. Real-time PCR. The expression of iNOS and TNF-α in the brain of MCA-occluded rats was quantified using real-time PCR by normalization with β-actin expression. The right MCA was occluded for 2 h. Anti-HMGB1 mAb or control mAb was administered immediately after reperfusion. Six hours after reperfusion, the rats were decapitated under deep anesthesia with pentobarbital and both sides of the cerebral cortex and the striatum were dissected. Total RNA was extracted from each sample, and the real-time PCR was performed using the indicated primer pairs. The expression of β-actin was used to normalize cDNA levels. The results are the means ± SEM from 4 animals. $*P < 0.05$, $**P < 0.01$ compared with the value in the contralateral hemisphere of the same treatment group. $\#P < 0.05$ compared with the value in the ischemic hemisphere of the control group. NS; not significant.

of its own translocation and redistribution in the ischemic brain.

HMGB1 has plural receptors, RAGE (37) and TLR-2/TLR-4 (38, 39). It is not clear at this moment which receptor signaling system is most important for ischemic brain injury. The number of infiltrating neutrophils into the ischemic hemisphere 12 h after reperfusion did not differ between the control and anti-HMGB1-treated groups, suggesting that infiltrated neutrophil in the early phase of brain ischemia did not play a significant role in the development of the inflammatory response.

The present results strongly suggested that HMGB1 plays a crucial role in inducing massive infarction by activating multiple injurious cascades after an ischemic insult due to arterial thrombus or embolus. It should be pointed out that anti-HMGB1 mAb had little effect on blood flow to the ischemic regions. Therefore, blocking the deleterious effects of HMGB1 on ischemic stroke in humans might be effective for rescuing neurons and dramatically reducing the resultant infarct size without restoring blood flow to the ischemic regions. In other words, neurons may be much more resistant to hypoxic and ischemic insults if the activity or release of HMGB1 is minimized. Anti-HMGB1 mAb, with the ability to neutralize HMGB1, prevented the development of brain infarction induced by MCA occlusion by inhibiting very early inflammatory responses and possibly by inhibiting neurotoxicity due to elevated glutamate levels. It is noteworthy that for the treatment of acute brain infarction, anti-HMGB1 mAb had higher efficacy among several types of drugs, including FK506 (40), MMP inhibitors (41, 42), radical scavengers (43), endothelin type A receptor antagonists (44), glutamate receptor antagonists (45), anti-α4 integrin Abs (46), proteasome inhibitors (47), and PPAR-γ inhibitors (48). The finding that i.c.v. injection of HMGB1 aggravated infarction was consistent with the effects of anti-HMGB1 mAb and implies the functional role of endogenous HMGB1. Concerning the accessibility of mAb to ischemic regions, it is likely that the mAb can be sufficiently delivered to the BBB-disrupted ischemic area spatially and temporally in the early phase of brain ischemia, resulting in the marked suppression of vascular permeability in the following phase. Therefore, anti-HMGB1 mAb appears to be a good therapeutic candidate for the treatment of ischemic stroke.

The clear demonstration of the effectiveness of anti-HMGB1 mAb against ischemic stroke beyond diverse types of candidates, strongly indicated that this treat-

ment has opened a novel strategy for the treatment of ischemic stroke in humans. Monoclonal Abs constitute a new group of drugs, and these targeted therapies are expected to have profound potential for the treatment of cancer (49) and inflammatory diseases (50). To promote the clinical application, the development of human mAbs against HMGB1 and the evaluation of their efficacy in animal models are important objectives for future research. FJ

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REFERENCES

1. Danton, G. H., and Dietrich, W. D. (2004) The search for neuroprotective strategies in stroke. *Am. J. Neuroradiol.* **25,** 181–194
2. Slevin, M., Krupinski, J., Kumar, P., Gaffney, J., and Kumar, S. (2005) Gene activation and protein expression following ischaemic stroke: strategies towards neuroprotection. *J. Cell. Mol. Med.* **9,** 85–102
3. Lindsberg, P. J., and Kaste, M. (2003) Thrombolysis for acute stroke. *Curr. Opin. Neurol.* **16,** 73–80
4. Thomas, J. O., and Travers, A. A. (2001) HMG1 and 2, and related 'architectural' DNA-binding proteins. *Trends Biochem. Sci.* **26,** 167–174
5. Rauvala, H., and Pihlaskari, R. (1987) Isolation and some characteristics of an adhesive factor of brain that enhances neurite outgrowth in central neurons. *J. Biol. Chem.* **262,** 16625–16635
6. Merenmies, J., Pihlaskari, R., Laitinen, J., Wartiovaara, J., and Rauvala, H. (1991) 30-kDa Heparin-binding protein of brain (Amphoterin) involved in neurite outgrow. *J. Biol. Chem.* **266,** 16722–16729
7. Parkkinen, J., Raulo, E., Merenmies, J., Nolo, R., Kajander, E. O., Baumann, M., and Rauvala, H. (1993) Amphoterin, the 30-kDa protein in a family of HMG1-type polypeptides. *J. Biol. Chem.* **268,** 19726–19738
8. Wang, H., Bloom, O., Zhang, M., Vishnubhakat, J. M., Ombrellino, M., Che, J., Frazier, A., Yang, H., Ivanova, S., Borovikova, L., *et al.* (1999) HMG-1 as a late mediator of endotoxin lethality in mice. *Science* **285,** 248–251
9. Lotze, M. T., and Tracey, K. J. (2005) High-mobility group box 1 protein (HMGB1): nuclear weapon in the immune arsenal. *Nat. Rev. Immunol.* **5,** 331–342
10. Ulloa, L., and Tracey, K. J. (2005) The 'cytokine profile': a code for sepsis. *Trends Mol. Med.* **11,** 56–63
11. Abraham, E., Arcaroli, J., Carmody, A., Wang, H., and Tracey, K. J. (2000) HMG-1 as a mediator of acute lung inflammation. *J. Immunol.* **165,** 2950–2954
12. Kokkola, R., Li, J., Sundberg, E., Aveberger, A. C., Palmblad, K., Yang, H., Tracey, K. J., Andersson, U., and Harris, H. E. (2003) Successful treatment of collagen-induced arthritis in mice and rats by targeting extracellular high mobility group box chromosomal protein 1 activity. *Arthritis Rheum.* **48,** 2052–2058
13. Taniguchi, N., Kawahara, K., Yone, K., Hashiguchi, T., Yamakuchi, M., Goto, M., Inoue, K., Yamada, S., Ijiri, K., Matsunaga, S., *et al.* (2003) High mobility group box chromosomal protein 1 plays a role in the pathogenesis of rheumatoid arthritis as a novel cytokine. *Arthritis Rheum.* **48,** 971–981
14. Ueno, H., Matsuda, T., Hashimoto, S., Amaya, F., Kitamura, Y., Tanaka, M., Kobayashi, A., Maruyama, I., Yamada, S., Hasegawa, N., *et al.* (2004) Contributions of high mobility group box protein in experimental and clinical acute lung injury. *Am. J. Respir. Crit. Care Med.* **170,** 1310–1316
15. Tsung, A., Sahai, R., Tanaka, H., Nakao, A., Fink, M. P., Lotze, M. T., Yang, H., Li, J., Tracey, K. J., Geller, D. A., *et al.* (2005) The nuclear factor HMGB1 mediates hepatic injury after murine liver ischemia-reperfusion. *J. Exp. Med.* **201,** 1135–1143
16. Pedrazzi, M., Raiteri, L., Bonanno, G., Patrone, M., Ledda, S., Passalacqua, M., Milanese, M., Melloni, E., Raiteri, M., Pontremoli, S., *et al.* (2006) Stimulation of excitatory amino acid release from adult mouse brain glia subcellular particles by high mobility group box 1 protein. *J. Neurochem.* **99,** 827–838
17. Sumi, T., and Lo, E. H. (2002) Involvement of matrix metalloproteinase in thrombosis-associated hemorrhagic transformation after embolic focal ischemia in rats. *Stroke* **33,** 831–836
18. Aoki, T., Sumi, T., Wang, X., and Loh, E. H. (2002) Blood-brain barrier disruption and matrix metalloproteinase-9 expression during reperfusion injury. Mechanical versus embolic focal ischemia in spontaneously hypertensive rats. *Stroke* **33,** 2711–2717
19. Kauer, J., Zhao, Z., Klein, G. M., Lo, E. H., and Buchan, A. M. (2004) The neurotoxicity of tissue plasminogen activator? *J. Cereb. Blood Flow Metab.* **24,** 945–963
20. Zhao, B.-Q., Ikeda, Y., Ihara, H., Urano, T., Fan, W., Mikawa, S., Suzuki, Y., Kondo, K., Sato, K., Nagai, N., *et al.* (2004) Essential role of endogenous tissue plasminogen activator through matrix metalloproteinase 9 induction and expression on heparin-produced cerebral hemorrhage after cerebral ischemia in mice. *Blood* **103,** 2610–2616
21. Hiraga, N., Adachi, N., Liu, K., Nagaro, T., and Arai, T. (2007) Suppression of inflammatory cell recruitment by histamine receptor stimulation in ischemic rat brains. *Eur. J. Pharmacol.* **557,** 236–244
22. Bederson, J. B., Pitts, L. H., Tsuji, M., Nishimura, M. C., Davis, R. L., and Bartkowski, H. (1986) Rat middle cerebral artery occlusion: evaluation of the model and development of a neurologic examination. *Stroke* **17,** 472–476
23. Sado, Y., and Okigaki, T. (1996) A novel method for production of monoclonal antibodies. Evaluation and expectation of the rat lymph node method in cell and molecular biology. *Cell Biol. Int.* **20,** 7–14
24. Asahi, M., Wang, X., Mori, T., Sumii, T., Jung, J. C., Moskowitz, M. A., Fini, M. E., and Lo, E. H. (2001) Effects of matrix metalloproteinase-9 gene knock-out on the proteolysis of blood-brain barrier and white matter components after cerebral ischemia. *J. Neurosci.* **21,** 7724–7732
25. Yang, H., Ochani, M., Li, J., Qiang, X., Tanovic, M., Harris, H.E., Susarla, S.M., Ulloa, L., Wang. H., DiRaimo, R., *et al.* (2004) Reversing established sepsis with antagonists of endogenous high-mobility group box 1. *Proc. Natl. Acad. Sci. U. S. A.* **101,** 296–301
26. Andersson, U., Wang, H., Palmblad, K., Aveberger, A.C., Bloom, O., Erlandsson.-Harris, H., Janson, A., Kokkola, R., Zhang, M., Yang, H., *et al.* (2000) High mobility group 1 protein (HMG-1) stimulates proinflammatory cytokine synthesis in human monocytes. *J. Exp. Med.* **192,** 565–570
27. Ren, D., Sun, R., and Wang, S. (2006) Role of inducible nitric oxide synthase expressed by alveolar macrophages in high mobility group box 1-induced acute lung injury. *Inflamm. Res.* **55,** 207–215
28. Rouhiainen, A., Kuja-Panula, J., Wilkman, E., Pakkanen, J., Stenfors, J., Tuominen, R. K., Lepantalo, M., Carpen, O., Parkkinen, J., Rauvala, H., *et al.* (2004) Regulation of monocyte migration by amphoterin (HMGB1). *Blood* **104,** 1174–1182
29. Han, H. S., Karabiyikoglu, M., Giffard, R. G., and Yanari, M. A. (2002) Influence of mild hypothermia on inducible nitric oxide synthase expression and reactive nitrogen production in experimental stroke and inflammation. *J. Neurosci.* **22,** 3921–3928
30. Gregersen, R., Lambertsen, K., and Finsen, B. (2000) Microglia and macrophages are the major source of tumor necrosis factor in permanent middle cerebral artery occlusion in mice. *J. Cereb. Blood Flow Metab.* **20,** 53–65
31. Iadecola, C., Zhang, F., and Xu, X. (1995) Inhibition of inducible nitric oxide synthase ameliorates cerebral ischemic damage. *Am. J. Physiol.* **268,** R286–R292
32. Barone, F. C., Arvin, B., White, R. F., Miller, A., Webb, C. L., Willette, R. N., Lysko, P. G., and Feuerstein, G. Z. (1997) Tumor

necrosis factor-alpha. A mediator of focal ischemic brain injury. *Stroke* **28,** 1233–1244
33. Kim, J.B., Sig, Choi,J., Yu, Y.M., Nam, K., Piao, C.S., Kim, S.W., Lee, M.H., Han, P.L., Park, J.S., Lee, J.K., *et al.* (2006) HMGB1, a novel cytokine-like mediator linking acute neuronal death and delayed neuroinflammation in the postischemic brain. *J. Neurosci.* **26,** 6413–6421
34. Dumitriu, I. E., Baruah, P., Manfredi, A. A., Bianchi, M. E., and Rovere-Querini, P. (2005) HMGB1: guiding immunity from within. *Trends Immunol.* **26,** 381–387
35. Yang, H., Wang, H., Czura, C. J., and Tracey, K. J. (2005) The cytokine activity of HMGB1. *J. Leukoc. Biol.* **78,** 1–8
36. Milev, P., Chiba, A., Haring, M., Rauvala, H., Schachner, M., Ranscht, B., Margolis, R. K., and Margolis, R. U. (1998) High affinity binding and overlapping localization of neurocan and phosphacan/proteintyrosine phosphatase-zeta/beta with tenacin-R, amphoterin, and the heparin-binding growth-associated molecule. *J. Biol. Chem.* **273,** 6998–7005
37. Hori, O., Brett, J., Slattery, T., Cao, R., Zhang, J., Chen, J. X., Nagashima, M., Lundh, E. R., Vijay, S., Nitecki, D., *et al.* (1995) The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. *J. Biol. Chem.* **270,** 25752–25761
38. Park, J. S., Gamboni.-Robertson, F., He, Q., Svetkauskaite, D., Kim, J. Y., Strassheim, D., Sohn, J. W., Yamada, S., Maruyama, I., Banerjee, A., *et al.* (2002) High mobility group box 1 protein interacts with multiple Toll-like receptors. *Am. J. Physiol.* **290,** C917–C924
39. Yu, M., Wang, H., Ding, A., Golenbock, D. T., Latz, E., Czura, C. J., Fenton, M. J., Tracey, K. J., and Yang, H. (2006) HMGB1 signals through toll-like receptor (TLR) 4 and TLR2. *Shock* **26,** 174–179
40. Maeda, M., Furuichi, Y., Ueyama, N., Moriguchi, A., Satoh, N., Matsuoka, N., Goto, T., and Yanagihara, T. (2002) A combined treatment with tacrolimus (FK506) and recombinant tissue plasminogen activator for thrombotic focal cerebral ischemia in rats: increased neuroprotective efficacy and extended therapeutic time window. *J. Cereb. Blood Flow Metab.* **22,** 1205–1211
41. Gu, Z., Cui, J., Brown, S., Fridman, R., Mobashery, S., Strongin, A. Y., and Lipton, S. A. (2005) A highly specific inhibitor matrix metalloproteinase-9 rescues laminin from proteolysis and neurons from apoptosis in transient focal cerebral ischemia. *J. Neurosci.* **25,** 6401–6408
42. Yang, Y., Estrada, E. Y., Thompson, J. F., Liu, W., and Rosenberg, G. A. (2007) Matrix metalloproteinase-mediated disruption of tight junction proteins in cerebral vessels is reversed by synthetic matrix metalloproteinase inhibitor in focal ischemia in rat. *J. Cereb. Blood Flow Metab.* **27,** 697–709
43. Nishi, H., Watanabe, T., Sakurai, H., Yuki, S., and Ishibashi, A. (1989) Effect of MCI-186 on brain edema in rats. *Stroke* **20,** 1236–1240
44. Matsuo, Y., Mihara, Si., Ninomiya, M., and Fujimoto, M. (2001) Protective effect of endothelin type A receptor antagonist on brain edema and injury after transient middle cerebral artery occlusion in rats. *Stroke* **32,** 2143–2148
45. Roussel, S., Pinard, E., and Seylaz, J. (1992) Effect of MK-801 on focal brain infarction in normotensive and hypertensive rats. *Hypertension* **19,** 40–46
46. Becker, K., Kindrick, D., Relton, J., Harlan, J., and Winn, R. (2001) Antibody to the alpha4 integrin decreases infarct size in transient focal cerebral ischemia in rats. *Stroke* **32,** 206–211
47. Phillips, J. B., Williams, A. J., Adams, J., Elliott, P. J., and Tortella, F. C. (2000) Proteasome inhibitor PS519 reduces infarction and attenuates leukocyte infiltration in a rat model of focal cerebral ischemia. *Stroke* **31,** 1686–1693
48. Shimazu, T., Inoue, I., Araki, N., Asano, Y., Sawada, M., Furuya, D., Nagoya, H., and Greenberg, J. H. (2005) A peroxisome proliferator-activated receptor-gamma agonist reduces infarct size in transient but not in permanent ischemia. *Stroke* **36,** 353–359
49. Harris, M. (2002) Monoclonal antibodies as therapeutic agents for cancer. *Lancet Oncol.* **5,** 292–302
50. Singh, R., Robinson, D. B., and El-Gabalawy, H. S. (2005) Emerging biologic therapies in rheumatoid arthritis: cell targets and cytokines. *Curr. Opin. Rheumatol.* **17,** 274–279

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