HMGB1, a cytokine-like mediator connecting acute neuronal death and delayed neuroinflammation in the postischemic brain

(렛트의 대뇌허혈모델에서 새로운 사이토카인 HMGB1의 역할 연구)

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HMGB1, a cytokine-like mediator connecting acute neuronal death and delayed neuroinflammation in the postischemic brain

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HMGB1, a cytokine-like mediator connecting acute neuronal death and delayed neuroinflammation in the postischemic brain
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Abstract

Cerebral ischemic injury is resulted from an excitotoxicity-induced acute neuronal death occurred in ischemic core and also from following delayed damaging process progressed in penumbra. However, direct mediators connecting these two phases remain to be explored. Here I demonstrated that High mobility group box 1 (HMGB1) might play a mediator-like role in the postischemic brain. HMGB1 has originally been identified as ubiquitously expressed, abundant nonhistone DNA-binding protein. The intracellular functions of HMGB1 include stabilization of nucleosome formation and facilitation of gene transcription. Recently, HMGB1 has been identified as a cytokine-like mediator of delayed endotoxin lethality and acute lung injury.

In the present study, function of HMGB1 in postischemic brain was investigated using animal model of middle cerebral artery occlusion (MCAO) and primary cortical and microglia cultures. The expression of HMGB1 in the infarction area in the ipsilateral side gradually declined over 2 d following 1 h of MCAO to below the basal level. After 3 d of reperfusion, HMGB1 levels increased to above the basal level, but then slowly declined again over the next few days. In
contrast, the plasma HMGB1 levels rapidly increased from 1 d after 1 h of MCAO, continued to increase for 6 d, and then declined slowly. shRNA-mediated HMGB1 down-regulation in the postischemic brain suppressed infarct size, microglia activation and proinflammatory maker induction, indicating that HMGB1 plays a crucial role in the inflammatory process. The inflammatory cytokine-like function of extracellular HMGB1 was further verified in primary cortical cultures and microglia cultures. HMGB1 was found to accumulate in the media of NMDA-treated primary cortical cultures, and supernatants collected from those cultures were found to trigger microglia activation, the hallmark of brain inflammation. Treatment with recombinant HMGB1 also induced microglia activation, but HMGB1-depleted supernatant produced by anti-HMGB1 antibody treatment or by HMGB1 shRNA-expression did not, thus demonstrating essential roles of HMGB1 in microglia activation. Together these results indicated that HMGB1 functions as a novel proinflammatory cytokine-like factor that connects excitotoxicity-induced acute damaging process and delayed inflammatory process in the postischemic brain.
국문요약

High mobility group box 1 (HMGB1)은 DNA에 결합하여 유전자 발현을 조절하는 핵 내의 구조 단백질로 알려져 있다. 그러나 최근 HMGB1이 핵 내 구조단백질로써의 역할 뿐 아니라 급성 폐 손상이나 늙은 시기의 내독소에의한 치사와 같은 병리 과정에서 cytokine과 유사한 역할을 한다는 것이 보고되고 있다.

본 연구는 뇌졸중 동물 모델, 1차 배양한 신경세포와 microglia를 사용하여 허혈에의한 신경세포 손상과정에서 HMGB1의 기능을 조사하였다. 임시적 국소 허혈 동물 모델 (middle cerebral artery occlusion, MCAO)을 사용하여 1시간 허혈 후 HMGB1이 시간적 공간적으로 조절되는 양상을 관찰하였다. 정상 쥐의 뇌에서는 모든 종류의 신경세포, astrocyte, microglia, oligodendrocyte를 포함한 글리아세포 그리고 혈관 내피세포 등 거의 모든 세포의 핵에 HMGB1이 존재한다. MCAO 동물 모델의 경우, 초기 급성 손상 시 흉부리성에 의한 신경세포 피사로 HMGB1이 조직 내로 유출되고 그 결과 혈청에서의 HMGB1이 급격히 증가되는 것이 관찰되었다. 이후 HMGB1은 활성화된 microglia, 특히 세포질에서 현저하게 증가하였다. HMGB1의 신경조직에서의 발현은 허혈 재관류이후 이틀까지는 줄어들어 있지만 3일 이후부터는 2배 이상의 발현증가를 보인다. shRNA를 이용하여 HMGB1 발현을 억제한 결과 허혈에 의한 경색의 크기가 현저히 줄어들고, microglia의 활성이 감소하였으며, iNOS, COX-2, IL-1, TNF와 같은 신경염증반응에 관련된 유전자발현이 억제되었다. 이것은
HMGB1이 허혈 후 뇌조직 손상을 유도하는데 관여하고 있으며 특히 신경 염증반응 과정에서 HMGB1이 중요한 역할을 하고 있다는 것을 시사한다.

허혈 뇌조직에서 홍분 독성에 의한 세포 파괴의 결과 세포 밖으로 유출된 HMGB1이 이후 진행되는 염증반응을 유도하는지 확인하기 위하여 일차 배양된 대뇌피질의 신경세포에 NMDA를 처리하였다. 신경세포가 파괴를 일으키는 과정에서 세포내의 HMGB1이 배양액으로 유출되는 것을 확인하였고, 이 배양액을 1차 배양 microglia 세포에 처리하면 microglia가 활성화되어 NO의 분비가 증가하고, 신경염증반응에 관련된 iNOS, COX-2, IL-1b, TNFa의 유전자 발현이 증가한다. 또한 제조합 HMGB1 단백질을 1차 배양한 microglia의 배양액에 처리한 경우에도 microglia의 활성화된다. 이때, HMGB1의 항체를 같이 처리하거나, HMGB1 shRNA를 발현시킨 신경세포에 NMDA를 처리한 배양액을 처리한 microglia는 활성이 감소된다. 이것은 배양액 중에 들어있는 HMGB1이 microglia의 활성에 중요한 역할을 했음을 시사한다.

이와 같은 연구결과들에 근거할 때 일시적 국소 허혈 후 초기 홍분독성에 의한 신경세포의 파괴과정에서 과량의 HMGB1이 세포 밖으로 유출되며, 이 HMGB1은 주변 microglia 세포를 활성화시켜 신경염증을 유도하며, 이를 통해 지연성 신경세포손상에 관여할 것이라고 여겨진다.
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Introduction

Brain damage following cerebral ischemia/reperfusion is induced by various postischemic events, which constitutes the acute and delayed injury processes. Cerebral ischemia is a complex pathophysiological condition caused by an insufficient blood supply to the brain that in most cases is due to hemorrhage or occlusion of a cerebral artery by an embolus or local thrombosis. During an ischemic brain episode, deprivation of oxygen and glucose will induce multiple degenerative cellular reactions, including the release of excitatory amino acids, the formation of oxygen free radicals, Ca\(^{2+}\) overload, the activation of Ca\(^{2+}\)-dependent proteases and the initiation of genomic responses that ultimately result in neuronal dysfunction, excitotoxicity, Zn\(^{+2}\) toxicity, peri-infarct depolarization, inflammation and apoptosis (Dirnagl et al., 1999). Excitotoxicity and Zn\(^{+2}\) toxicity result in acute and massive neuronal death in the ischemic core, wherein the energy metabolism starts to fail, and significant reductions in mRNA and protein synthesis occur due to severe shortage of blood flow (Dirnagl et al., 1999; Lipton, 1999). This acute neuronal damage is followed by a second round of neuronal injury, called delayed neuronal death, in the
neighboring areas of the ischemic core. Of the many pathophysiological events that may contribute to this delayed injury, the cell-mediated processes that are associated with postischemic inflammation and apoptosis have been extensively studied (Kirino et al., 1984; Kirino et al., 2000; Lipton et al., 1999; Graham et al., 2001 Graham and Chen, 2001).

Several lines of evidence suggest that there is an alteration of gene expression in the course of neuronal injury in postischemic brain (Kirino et al., 1984; Kirino et al., 2000; Lipton et al., 1999; Graham et al., 2001). To identify genes whose expression was changed in rat brain following transient MCAO, especially with a hope to find those playing an important role in delayed neuronal death and recovery processes, cDNA microarray was utilized. A large number of genes were lately induced at 12 h and 1 d after MCAO, which was followed by the second surge in the number of down-regulated genes (Kim et. al., 2004). A list of genes up-
regulated in a delayed manner that could play a crucial role in delayed damage or recovery processes in postischemic brain was obtained (Kim et al., 2004).

High mobility group protein-1 (HMGB1) was one of the genes which showed delayed and sustained induction in postischemic brain. In striatum, expression of HMGB1 was increased 1.9-fold at 3 h and then continued to increase. Peak induction was detected at 4 d after 1 h of MCAO (2.3-fold). However, in cortex HMGB1 expression was reduced to 0.7-fold at 6 h and then expression was recovered reaching 2.5-fold induction after 1 h of MCAO (Kim et al. 2004).

HMGB1 has originally been identified as a ubiquitously expressed, gene abundant nonhistone DNA-binding protein that binds to the narrow minor groove of AT sequence rich-B form DNA (Bianchi et al. 1998). HMGB1 is a small protein (25-kD) of 215 amino acids and a highly dipolar structure that contains two DNA binding domains, HMG box A and box B, which are followed by an acidic COOH terminus composed of 30 glutamic and aspartic residues (Landsman and Bustin, 1993). HMGB1 is a highly conserved protein with >95% amino acid identity between rodents and humans (Masher et al. 1996). HMGB1 is
widely expressed in various tissues including the brain, excepting those that have eliminated their nucleus (such as erythrocytes and cornifying epithelial cell in the skin and proximal gut). The intracellular functions of HMGB1 include stabilization of nucleosome formation and facilitation of gene transcription. HMGB1 modulates the activities of nuclear targets, like, p53, NF-κB, steroid hormone receptors, glucocorticoid receptor, homeobox containing proteins and TBP (Huttunen et al., 2004). Moreover, HMGB1 facilitates V(D)J recombination through interaction with the RAG1 protein, and some types of transposition reactions (Muller et al., 2004). HMGB1 might also participate in transcriptional regulation at the chromatin level: HMGB1 was found to bind the nucleosomal linker DNA and enhance nucleosomal sliding in vitro (Banoldi et al., 2002). HMGB1 knockout mice failed to survive more than 24-48 h postpartum, probably due to hypoglycemia (Calogero et al., 1999).

Recent evidence identifies HMGB1 as a cytokine-like mediator of delayed endotoxin lethality and acute lung injury (Wang et al., 1999; Abraham et al., 2000). HMGB1 is actively secreted by activated macrophages and monocytes or released from necrotic cells into the extracellular milieu, where it triggers inflammation by activating
macrophages and pituicytes to release various proinflammatory cytokines (Wang et al., 1999; Abraham et al., 2000; Scaffidi et al., 2002; Bonaldi et al., 2003; Yang et al., 2004). In addition, HMGB1 triggers inflammation by stimulating neutrophils and smooth muscle cell chemotaxis (Abraham et al., 2000; Wang et al., 1999; Andersson et al., 2000 Degryse et al., 2001). Recombinant HMGB1 was found to induce acute inflammation in animal models of lung injury and endotoxemia (Wang et al., 1999; Abraham et al., 2000). And, anti-HMGB1 antibody attenuated endotoxin-induced lethality even when passive immunization was delayed until after the early cytokine response (Wang et al., 1999). In addition, high serum levels of HMGB1 in patients with sepsis or hemorrhagic shock have been reported to be associated with increased mortality and disease severity (Wang et al., 1999; Ombrellino et al., 1999)

In the brain, HMGB1 was identified to be a neurite outgrowth-promoting factor, and named amphoterin (Parkkinen et al., 1993). HMGB1 localized to the advancing plasma membrane of the filopodia at the leading edge in motile cells (Merenmies J et al. 1991). Recently, HMGB1 has been reported to be induced in the brain under pathogenic conditions, for example, in traumatic brain injury and Alzheimer's
disease (Takata et al., 2004). HMGB1 was found to be released after cytokine stimulation, and to participate in the inflammatory process when administered intracerebroventricularly (ICV) (Wang et al., 1999; Agnello et al., 2002). In addition, an ICV injection of HMGB1 was found to induce fever and hypothalamic IL-1 expression in rats (O'Connor et al., 2003), and very recently, it was reported that HMGB1 inhibited microglia Aβ clearance and enhanced Aβ neurotoxicity (Takata et al., 2003; Takata et al., 2004).

To investigate the functions of HMGB1 in the postischemic brain, its expression and localization after middle cerebral artery occlusion was examined in rat brain. Function of HMGB1 in the postischemic brain was investigated using shRNA-mediated knock-down of HMGB1. Successful application of shRNA to mammalian cells is dependent on efficient siRNA delivery. In this study, I used cationic polymer l-arginine-grafted-PAMAM dendrimer (PAMAM-Arg), as a novel nonviral gene delivery vector, which is composed of a backbone of PAMAM dendrimer. It has been reported that PAMAM-Arg/DNA complexes showed particularly high transfection efficiencies and low cytotoxicity in primary cortical cells (Kim et al. 2006), which have
been known to be extremely difficult to deliver genes.

HMGB1 was found to be massively released during the excitotoxicity-induced acute damaging process in the postischemic brain, and to trigger inflammatory processes like microgliosis activation, thus indicating that HMGB1 links excitotoxicity-induced acute damage and the subsequent inflammatory processes in the postischemic brain.
Materials and Methods

1. Surgical procedures for MCA occlusion.

All experiments were carried out in accordance with the animal research guidelines issued by the Inha University School of Medicine. Male Sprague-Dawley rats (250-300 g) were anesthetized with 5% isoflurane in a mixture of 30% oxygen and 70% nitrous oxide, and MCA was occluded for 1 h using the suture occlusion method previously described (Kim et al., 2004). Left femoral artery was cannulated for monitoring arterial blood pressure (Blood pressure analyzer; Digi Med, Louisville, KY) and for blood sampling to analyze pH, PaO₂, PaCO₂, and glucose concentration (I-STAT; Sensor Devises, Waukesha, WI). Regional cerebral blood flow (rCBF) was monitored using a laser Doppler flowmeter (Periflux System 5000; Perimed, Jarfalla, Sweden). A thermoregulated heating pad and an overhead-heating lamp were used to maintain rectal temperature of 37±0.5°C. In sham-operated rats, an incision was made over the MCA, but the artery was not occluded.

2. Intracranial injection.
Rats were anesthetized with an intramuscular injection (1-1.5 ml/kg body weight) of a mixture of ketamine and xylazine hydrochloride (3:1), and placed on a stereotaxic apparatus (Narishige Scientific Instrument Lab., Tokyo, Japan). The skull was leveled between bregma and lambda. Plasmid (2ug)/PAMAM-Arg complex were prepared in 5% glucose and 10 µl of the complexes were injected stereotaxically into the striatum (coordinates in mm with reference to bregma: AP 0; L 5.0; V 4.0) using a 26-G Hamilton microsyringe (80330, Hamilton Company, Reno, NV). 2-mm thick coronal brain slices, which localizes between 8 and 10 mm from the frontal pole, were taken from the brain using a brain matrix (RBM-40000, ASI Instruments, USA), and brain regions affected by shRNA-expressing plasmid were dissected. The tissue samples were kept at -80°C until required.

3. Preparation of mixed neuron-glia cultures.

Mixed cortical cells, including neurons and astrocytes, were prepared from embryonic day 15.5 (E15.5) mouse cortices and cultured as described by Kim et al. (2004). Dissociated cortical cells were plated at a density of five hemispheres per 24-well plate (approximately 4 x 10^5 cells per well), which is poly-D-lysine (100 µg/mL) and laminin (100
µg/mL)-coated. Cultures were maintained without antibiotics in MEM with 5% FBS and 5% horse serum. At day 6 *in vitro* (DIV6), when astrocytes had reached confluence underneath neurons, cytosine arabinofuranoside (ara-C) was added to a final concentration of 10 µM, and culture was maintained for 2 d to halt microglial growth. Fetal bovine serum (FBS) and glutamine were not supplemented from day 6, and the medium was changed twice a week after day 8. Cultures were used at DIV 13-15.

4. Primary microglial culture.

Primary microglial cultures were prepared as described previously. In brief, cells dissociated from the cerebral hemispheres of 1- to 3-day-old postnatal rat brains (Sprague-Dawley strain) were seeded into 75-cm² flasks at a density of 1.2×10⁶ cells/ml in minimal essential medium (MEM; Sigma Chemical, St. Louis, MO) containing 10% FBS (Hyclone, Logan, Utah). Culture media were changed after 24 h and then twice a week. After 2 weeks, microglia were then detached from the flasks by mild shaking and filtered through a nylon mesh to remove astrocytes. After centrifugation (1000×g) for 5 min, the cells were resuspended in a fresh DMEM supplemented with 10% FBS, and
plated at a final density of $4 \times 10^4$ cells/well on a 24 multi-well culture dish. The following day, cells were subjected to different treatments. The microglial cultures used were $> 96\%$ pure.

5. **NMDA and staurosporin treatment and cell supernatants preparation.**

Primary cortical cells were treated with serum-free MEM containing 30 µM NMDA (Sigma, St. Louis MO) and 10 µM staurosporin (Sigma, St. Louis MO) for 1 h. The medium was then removed and replaced with fresh MEM medium, and cells were cultured for 24 h. 400 µl of the culture medium was then collected and concentrated to 40 µl by using Centricon 10 (Millipore, Billerica, MA), and then either immunoblotted or used as culture medium for activating primary microglial cultures.

6. **NO measurement.**

Primary microglia ($4 \times 10^4$) plated on 24-well plates were treated with LPS (100 ng/ml) for 48 h. To measure the amount of NO produced by microglia, 100 µl of conditioned medium was mixed with an equal
volume of Griess reagent (0.5% sulfanilamide and 0.05% N-1-naphthylethylenediamine), and incubated for 10 min at room temperature. Absorbance of the mixture at 550 nm was measured using a microplate reader.

7. Generation of the HMGB1 shRNA transgene.

To generate shHMGB1-pU6, two 64-mer sense and anti-sense oligonucleotides containing 19-nt inverted repeat corresponding to the HMGB-1 coding region were synthesized (Fig. 3A). The inverted motif, which also contained the 7 nucleotide spacer and 5 Ts, was subcloned into the BamH1 and HindIII sites, immediately downstream of the U6 promoter of the pU6 plasmid (Ambion, Texas, USA). A mutant plasmid, MshHMGB1-U6, was constructed in the same way, except that 6 nt within the target region was substituted. The sequences inserted were as follows (only the sense sequences are shown): shHMGB1, 5'-GATCCCCGAAGCAACCGGATGCTTCTTTCAAGAG AAGAAGCATCCGGGTGCTTCTTTTTTGGAAA-3'; and MshHMGB1, 5'-GATCCCCGAAGCACTACTGCGCTTCTTTTTTGGAAA-3'.

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8. Transient transfections.

All plasmids, including shHMGB1-pU6 and MshHMGB1-pU6, were mixed with PAMAM-Arg at 1:3.5 charge ratio, and then administered to rat brain or transfected into primary cortical cultures. After 48 h, cell or tissues were subjected to various biochemical assays.

9. RNA preparation and RT-PCR.

Total RNA was prepared using TRIzol reagent (Gibco BRL, Gaithersburg, MD) according to manufacturer’s instructions, and 1 µg RNA samples were used for cDNA synthesis. First-strand cDNA synthesis was primed with random hexamers and conducted according to the manufacturer's specifications (RT-PCR kit; Roche, Mannheim, Germany). cDNA equivalent to 200 ng of total RNA was subjected PCR using the manufacturer's protocol (PCR core kit; Roche). The primer sequences of rat HMGB1, TNFα, IL-1β, iNOS, and COX-2 have been described previously (Kim et al., 2004; Yu et al., 2003).

10. Northern analysis.

Northern analysis was performed as described previously (Kim et al., 2004). Each lane was loaded with 50 µg of total RNA. After transferred
to a nylon membrane (Roche, Mannheim, Germany), RNA blots were hybridized with 64 base pair DNA probe corresponding to HMGB1 shRNA. Probes were labeled with P\textsuperscript{32}-dCTP using a random prime labeling system (Amersham, CA, USA), and then purified using a robeQuant\textsuperscript{TM} G-50 micro column (Amersham, CA, USA). 1×10\textsuperscript{7} cpm of labeled probe was applied to each membrane in hybridization buffer (ULTRAhyb\textsuperscript{TM}, Ambion, Texas), and incubated at 42°C for 18 h. Membranes were washed and then exposed to film.

11. TTC staining.

Rats were decapitated after 2 d of reperfusion and whole brains were dissected coronally into 2-mm brain slices using a metallic brain matrix (RBM-40000, ASI, Springville, UT). Slices were immediately stained by immersion in 1% 2,3,5-triphenyl tetrazolium chloride (TTC) at 37°C for 15 min and then in 4% paraformaldehyde for preservation.

12. Lectin histochemistry.

Vibratome sections were incubated with horseradish peroxidase (HRP)-conjugated *Griffonia simplicifolia* isoelectin-B\textsubscript{4} (GSA I-B\textsubscript{4})
(Sigma, St. Louis, MO, USA) at 10 µg/ml in PBS at room temperature for 3 h. After three washes in PBS isolectin binding sites were visualized using HRP/3,3\text{-}diaminobenzidine (DAB).

13. Immunocytochemistry.

Brain sections and cells were prepared as previously described (Kim et al., 2004). Primary antibodies were diluted as follows: 1:50 for anti-HMGB1 (BD Bioscience, Franklin Lakes, NJ), and 1:100 for anti-NeuN (Chemicon, Temecula, CA) and anti-GFAP (Jackson ImmunoResearch Lab, West Grove, PA) antibodies. For double immunostaining, Rhodamine-labeled anti-rabbit IgG (Jackson ImmunoResearch Lab, West Grove, PA) was used as the secondary antibody for anti-HMGB1, and FITC-labeled anti-mouse IgG for anti-GFAP and anti-Neu N antibodies.

14. 5-Bromo-4-Chloro-3-indolyl-β-D-Galactopyranoside (X-Gal) histoc- hemistry.

Animals were perfused transcardially with 4% paraformaldehyde and brain slices (50 µm thick) were prepared. After three washes in 0.01%
sodium deoxycholate, 0.02% Nonidet P-40, 2 mM MgCl₂, 0.1 M NaHPO₄ (pH 7.3), X-gal staining was performed over 12-16 h at 37°C in X-gal buffer [2 mM MgCl₂/5 mM K₃Fe(CN)₆/5 mM K₄Fe(CN)₆/0.1 mM NaHPO₄, pH 7.3/0.01% sodium deoxycholate/0.02% Nonidet P-40] containing 1 mg/ml of X-gal.

**15. Immunoblotting.**

Brain homogenates were immunoblotted as previously described (Kim et al., 2004). Rats were deeply anesthetized with an overdose of sodium pentobarbital (100 mg/kg, i.p.), and brain slices were prepared as described above. Blood samples (1 ml) were drawn by cardiac puncture, after 15 min of centrifugation at 1,500×g, serum was collected and stored at -80°C in sterile polypropylene tubes until required for assay. Tissue and serum samples were incubated in 50 mM HEPES, pH 7.4/0.1% Nonidet P-40/150 mM NaCl supplemented with a protease inhibitor mixture (BD Biosciences, Franklin Lakes, NJ) for 15 min on ice, and then centrifuged at 6,000×g at 4°C. Protein concentrations were determined using Bradford's method (Bio-Rad, Hercules, CA). Lysates were incubated for 10 min in the presence of SDS sample buffer containing 2-mercaptoethanol, centrifuged at
16,000×g for 5 min at 4°C, and supernatants were loaded onto SDS-PAGE. Primary antibodies were diluted as follows: 1:1000 for anti-HMGB1 (BD Bioscience) and 1:500 for p38 MAPK and phospho-p38 MAPK and detected by using a Chemiluminescence kit (BD Bioscience, Franklin Lakes, NJ) using donkey anti-rabbit horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL).

16. HMGB1 Measurements.

Serum samples or cell-conditioned media were fractionated by SDS/PAGE, and HMGB1 levels were determined by immunoblotting using a standard curve for recombinant HMGB1 as a reference (Sigma, St. Louis MO). Briefly serum samples or cell-conditioned media (100-200 µl) were ultrafiltered using a Centricon 10 (Millipore, Billerica, MA), fractionated by SDS/PAGE, and transferred to PVDF membranes (Bio-Rad, Hercules, CA). The primary antibodies for anti-HMGB1 (BD Bioscience, Franklin Lakes, NJ) and anti-histone (Chemicon, Temecula, CA) were diluted 1:1000 and 1:500, respectively, Detection was performed using a Chemiluminescence kit (BD Bioscience, Franklin Lakes, NJ).
Results

1. Analysis of the gene expression patterns induced by transient MCAO

To provide evidence that transcriptional control is important in the pathophysiology of postischemic brain, genomic responses in the brain were examined at 3 h, 6 h, 12 h, 1 d, 2 d, or 4 d after transient middle cerebral artery occlusion (MCAO) by using a microarray harboring 5000 rat cDNAs. These analyses led us to present a list of 85 genes that were up-regulated greater than 2.3-fold between 12 h and 4 d after MCAO, which included 56 novel genes whose expression has not been previously implicated in ischemic pathophysiology (Fig. 1A) HMGB1 was identified as one of the novel genes. In striatum, expression of HMGB1 was increased 1.9- fold at 3 h and than continued to increase. Peak induction was detected at 4 d after 1 h of MCAO (2.3-fold). However, in cortex HMGB1 expression was reduced to 0.7-fold at 6 h and then expression was recovered reaching 2.5-fold induction after 1 h of MCAO (Fig. 1B).
Fig. 1. HMGB1 was identified as a gene showing delayed and sustained induct in the postischemic brain. mRNA samples were prepared at 3, 6 and 12 h, and 1, 2 or 4 d after 1 h of MCAO. Six-millimeter thick coronal brain slices, 6 12 mm apart, starting the frontal pole of the brain, were prepared using a brain matrix device, from which the cortex and striatum were dissected. Brain samples obtained from three independently manipulated animals were pooled to minimize experimental variation (A). HMGB1 expression up-regulated at 4 d after MCAO (B)
2. HMGB1 increases following transient focal cerebral ischemia

2.1 Plasma HMGB1 rapidly increases following transient focal cerebral ischemia

To investigate the role of HMGB1 in the brain following cerebral ischemia, we examined HMGB1 expression after 1 h of middle cerebral artery occlusion (MCAO). The expression of HMGB1 in the infarction area in the ipsilateral side (Fig. 2A, right panel) gradually declined over 2 d following MCAO/reperfusion to below the basal level (Fig. 2B). After 3 d of reperfusion, HMGB1 levels increased to above the basal level, but then slowly declined again over the next few days (Fig. 2B). In contrast, the plasma HMGB1 levels rapidly increased from 1d after 1 h of MCAO/reperfusion, continued to increase for 6 d (Fig. 2C), and then declined slowly.
Fig. 2. Plasma HMGB1 immediately increases following MCAO
Levels of HMGB1 in ischemic hemispheres (right panel in Fig 1A) were
determined by RT-PCR at various times after 1 h of MCAO/reperfusion
(B), and serum HMGB1 levels were examined by immunoblotting (C).
Data are presented as averages±SEM (n=3). * p < 0.05.
2.2 Localization of HMGB1 change following transient focal cerebral ischemia

Brain sections were prepared from sham-operated control and 1 d, 2 d, and 4 d after reperfusion, and immunostained with anti-HMGB1 antibody. The expression of HMGB1 in the core in the ipsilateral striatum (Fig. 3A) drastically declined over 1 d following MCAO/reperfusion to below the basal level (Fig. 3B). After 2 d of reperfusion, HMGB1 levels increase gradually and HMGB1 immunoreactivity was induced in reactive glia–like cells in the infarction core. At 4 d after MCAO, an increment in the size and in the number of HMGB1-positive glia–like cells was detected (Fig. 3B). In contrast the striatum immediate the down regulation and subsequent up-regulation of HMGB1 expression was induced slowly in cortex (Fig. 4B). The HMGB1 was decreased in the parietal cortex in the core at 1 and 2 d. At 4 d after reperfusion, the number of HMGB1 immunoreactive cells was markedly increased in the infarct core region in the cerebral cortex.

Double immunofluorescent staining showed that the majority of HMGB1 positive cells, which were stained with Rhodamine-conjugated secondary antibody, were microglia as confirmed by staining with FITC-conjugated isolectin-B4 (Fig 5). Furthermore, the HMGB1 immunoreact
Fig. 3. Immunohistochemical localization of HMGB1 in the ischemic striatum. Brain sections were prepared from sham-operated and 1, 2, and 4 d after reperfusion, and immunostained with anti-HMGB1 antibody (B). The prominent HMGB1 was decreased in the parietal cortex in the core at 1 d. At 2 d after reperfusion, HMGB1 immunoreactivity was induced in reactive glia–like cells in the core. At 4 d after MCAO, an increment in the size and in the number of HMGB1-positive cells was detected.
Fig. 4. Immunohistochemical localization of HMGB1 in the ischemic cortex. Brain sections were prepared from sham-operated and 1, 2, and 4 d after reperfusion, and immunostained with anti-HMGB1 antibody (B). The HMGB1 was decreased in the parietal cortex in the core at 1 and 2 d. At 4 d after reperfusion, HMGB1 immunoreactivity was markedly increased in the core.
Fig. 5. Double immunofluorescent staining

The majority of HMGB1 were localized in microglial cells in the ischemic striatum. The HMGB1 was localized in nucleus in rat brain. At 2 d after reperfusion, HMGB1 immunoreactivity was markedly increased in cytoplasm of activated microglial cells. HMGB1 positive cells, stained with Rhodamine-conjugated secondary antibody, were microglia and astrocytes as confirmed by staining with FITC-conjugated isolectin-IB4 and GFAP.
ivity was co-localized with isolectin-B4 in the cytoplasm of activated microglia.

3. shRNA-mediated suppression of HMGB1 expression following transient focal cerebral ischemia

3.1 Down-regulation of gene expression using a shRNA-expressing plasmid in primary cortical culture

Primary cortical cultures were derived from the cortex of E15.5 rat embryos, and consisted mainly of neurons and glial cells. These cells have been used in various experiments regarding neurogenesis, neurophysiology, and neuropathology studies. A plasmid expressing shRNA of the HMGB1 gene was constructed. The hairpin-forming 64 bp insert in the plasmid harbors the inverted repeat of 19-bp corresponding to the HMGB1 coding region, which is separated by a 7 bp spacer (shHMGB1-pU6) (Fig. 6A). A mutant shHMGB1-expressing plasmid containing the substitution of 6 nucleotides within the 19-bp region was also constructed (MshHMGB1-pU6). When shHMGB1-pU6 was co-transfected into primary neuronal cells with pEGFP-N1 plasmid, which was used as a transfection control, the marked HMGB1 suppression was detected in the GFP-positive cells. Over 75% of GFP-positive cells were HMGB1-negative, indicating that shHMGB1-pU6
transfection had suppressed HMGB1 expression (Fig 6B-C and D). In contrast, no suppression of HMGB1 was detected in the GFP-positive cells co-transfected with MshHMGB1, although its transfection efficiency as evidenced by GFP-positive cell numbers was comparable to shHMGB1-pU6. The results of this study provide a basis for future research upon the use of RNAi in primary neuronal cells.

3.2 shRNA-mediated suppression of HMGB1 expression in rat brain

To investigate the functional significance of the rapid accumulation of HMGB1 in the extracellular space of the postischemic brain, we knocked-down HMGB1 mRNA using a plasmid expressing the shRNA of the HMGB1 gene. The shRNA transcript was detected at day 1, persisted for more than 6 d, and showed high shRNA expression between days 3 and 5 (Fig. 7B). This shRNA was found to suppress endogenous HMGB1 expression to 56.3±8.9 % (n=3, p<0.05) of that of the control on day 4, and subsequently, HMGB1 expression recovered to back to its baseline level at 10 d after the injection (Fig. 7C).
Fig 6. HMGB1 shRNA transgene transfection in primary cortical cultures. (A) Schematic diagram of HMGB1 shRNA transgene showing the sense and antisense regions that target the HMGB1 gene. Fluorescence photographs prepared 48 h after co-transfection with pd2EGFP-N1 and shHMGB1-pU6 (B-D).
Fig. 7. shRNA-mediated silencing of HMGB1 gene expression in the normal brain. (A) β-gal staining was performed 2 d after pCN-Luci transfection using PAMAM-Arg as a gene carrier (left panel), and the boundary of the region expressing the exogenous gene was indicated (white box in right panel). sh HMGB1-pU6 or MshHMGB1-pU6 were injected into the indicated region, and biochemical assays were done on samples prepared from the indicated area. (B) Northern blotting for shHMGB1 transcripts in shHMGB1-pU6-transfected brains was performed at the indicated times. Each lane contained total RNA obtained from five animals, and blots were probed with 32P-labeled 64 nt sense oligonucleotide. (C) The levels of HMGB1 expression in shHMGB1-pU6- or MshHMGB1-pU6-administered animals were determined by RT-PCR at the indicated times after transfection. Data are presented as averages±SEM (n=4). * p < 0.05.
Fig. 8. shRNA-mediated silencing of HMGB1 induction in the postischemic brain. shHMGB1-pU6/PAMAM-Arg or MshHMGB1-pU6/PAMAM-Arg complexes were administered into the indicated region 24 h prior to 1 h of MCAO. (B) The serum levels of HMGB1 in shHMGB1-pU6-administered animals were determined by immunoblotting at the indicated times after 1 h of MCAO. (C) The levels of HMGB1 in the brain tissues of shHMGB1-pU6- or MshHMGB1-pU6-administered animals were determined by RT-PCR 24 h after 1 h of MCAO.
3.3 shRNA-mediated HMGB1 suppression has a neuroprotective effect in the postischemic brain

shHMGB1-pU6/PAMAM-Arg complex was administered 24 h prior to the 1 h of MCAO into the striatum, which encompasses infarction core later (Fig. 8A). Serum HMGB1 levels were found to be significantly reduced (Fig. 8B), and the suppressive effect obtained by a single administration of shHMGB1-pU6 plasmid was sustained for 10 days (Fig. 8B), which agrees well with the observed duration of shRNA expression (Fig. 7). The administration of shHMGB1-pU6 plasmid also repressed delayed HMGB1 induction (Fig. 8C) in the infarct core, but this was not detected in animals administered MshHMGB1-pU6 (Fig. 8C). Triphenyl tetrazolium chloride (TTC) staining revealed that shHMGB1-pU6 administration at 24 h prior to MCAO significantly suppressed infarct volumes in 21 of 23 animals (Fig. 9A). Almost all
Fig. 9. Neuroprotection by the shRNA-mediated silencing of HMGB1 induction in the postischemic brain. A TTC-stained infarction area in a coronal brain section 2 d after 1 h of MCAO (A). Arrow indicates the administration point of shHMGB1-pU6/PAMAM-Arg complex 24 h prior to 1 h of MCAO. (A) Representative pictures showing suppressed infarct formation (I) (II) and no suppression (III). (B) Numbers of rescued animals are presented in the bar graph. Data are presented as averages ± SEM (n=3). * p < 0.05.
animals exhibited infarct suppression along the syringe path (I, Fig. 9A). In contrast, no infarction suppression (III, Fig. 9A) was observed in any animals administered MshHMGB1-pU6.

3.4 Anti-inflammatory effect of HMGB1 suppression in the postischemic brain

Microglia activation is a hallmark of brain inflammation. The numbers of activated or phagocytic microglia were notably increased 4 d after 1 h of MCAO (Fig. 10A, black box in the inset in Fig. 10A), but they were significantly reduced in animals pre-administered with shHMGB-pU6 and relatively few ramified microglia were observed in the infarction core (Fig. 10C). In contrast, numerous activated or phagocytic microglia were detected in animals administered MshHMGB-pU6 (Fig. 10D), indicating anti-inflammatory effect of HMGB1 suppression in the postischemic brain. In addition, the inductions of proinflammatory cytokines, TNF-α, IL-1β, cytochrome oxidase (COX)-2, and inducible nitric oxide (iNOS) were repressed in the injected area (red box in the inset in Fig. 10A) 24 h after MCAO/reperfusion (Fig. 11A), suggesting that HMGB1 plays a role in proinflammatory cytokine release during this stage. In addition, p38 MAPK activation, detected as its
Fig. 10. Suppression of inflammatory markers by HMGB1 shRNA-expressing plasmid in the postischemic brain.

Microglia activation 4 d after 1 h of MCAO was visualized by anti-GSA I-B4 immunostaining (A-D). shHMGB1-pU6 or MshHMGB1-pU6 were administered 24 h prior to MCAO. Activated microglia were detected in the ischemic core (black rectangle in the inset in A) (B). Activated microglia numbers were notably reduced in wild type shRNA-expressing brains (C) but not in mutant shRNA-expressing brains (D). The insets in A-D are high magnification micrographs. The scale bar represents 500 mm in A-D.
Fig. 11. Suppression of inflammatory markers by HMGB1 shRNA-expressing plasmid in the postischemic brain.

The expressions of proinflammatory cytokines in the presence of shHMGB1-pU6 or MshHMGB1-pU6 were examined 1 d after 1 h of MCAO (A). Activation of p38 MAPK was examined 24 h after 1 h of MCAO by immunoblotting using anti-phosphorylated p38 MAPK antibody (B).
phosphorylated form, was significantly suppressed in shHMGB-pU6-administered animals, but not in MshHMGB-pU6-administered animals (Fig. 11B). Together these results indicate that HMGB1 suppression has anti-inflammatory effect in the postischemic brain.

4. Massive HMGB1 release following excitotoxicity-induced neuronal cell death in primary cortical cultures

We next investigated the function of extracellular HMGB1, which accumulated during the acute damaging process in the postischemic brain, by using primary cortical cultures. Levels of HMGB1 in cell homogenates or in supernatants of primary cortical cultures were measured after NMDA-, glutamate-, or staurosporin treatment. In normal conditions, HMGB1 was mainly localized to the nuclei of neurons and astrocytes (Fig. 12A, B). However, in NMDA-treated cells, the diffuse translocation of HMGB1 to the cytoplasm was detected in some neurons, although the number of HMGB1-positive intact neurons decreased due to excitotoxicity-induced death (Fig. 12D). In contrast, HMGB1 was sustained in nuclei of astrocytes (Fig. 12E). In staurosporine-treated cells, HMGB1 was retained by nuclei of neurons (Fig. 11G) and of astrocytes (Fig. 11H), which had a shrunken appearance and pyknotic nuclei. Interestingly, HMGB1 was found to
have accumulated in culture medium after incubating primary cortical cells with NMDA for 24 h (Fig. 12A). Similar enrichment of HMGB1 in culture medium was observed for glutamate-treated cells. However, in marked contrast, HMGB1 levels in staurosporine-treated cells were increased in cell homogenates but not in media (Fig. 12B). In all cases, histone was not detected in culture medium and levels in cell homogenates were similar (Fig. 12).

5. Activation of microglia by extracellular HMGB1

5.1 HMGB1 released into excitotoxin-treated cell supernatants is capable of triggering microglia activation

To examine whether HMGB1 released into excitotoxin-treated cell supernatants is capable of triggering microglia activation, primary microglia cultures were incubated with media collected from NMDA-treated primary cortical cells. To remove residual NMDA in supernatant, cells were cultured in fresh medium after being exposed to NMDA (30 µM) for 1 h. Supernatants obtained from NMDA-treated cortical cultures induced microglial activation, as demonstrated by NO secretion.
Fig. 12. Localization of HMGB1 in bath medium containing primary cortical cell cultures undergoing excitotoxicity-induced cell death

Primary cortical cultures were incubated in serum free-MEM containing 30 µM NMDA (D, E, F) or 10 µM staurosporin (G, H, I) for 1 h. After 3 h (D, E) or 12 h (G, H) of treatments, HMGB1 localizations in neurons and astrocytes were visualized by double immuno-staining with anti-HMGB1 and anti-NeuN antibodies (A, D, G), or with anti-HMGB1, anti-GFAP antibodies (B, E, H) and Hoechst22230 and propyridiumdiodied (C, F, I) respectively. The scale bar represents 20 µm in A,B,D,E,G and H 10 µm in C, F and I.
Fig. 13. Accumulation of HMGB1 in bath medium containing primary cortical cell cultures undergoing excitotoxicity-induced cell death

Primary cortical cultures were incubated in serum free-MEM containing 30 µM NMDA or 10 µM staurosporin for 1 h. After 1d of treatments, both culture media and cell homogenates were analyzed by immunoblotting with anti-HMGB1 or anti-histone antibody. Data are presented as averages±SEM (n=3). * p < 0.05.
(Fig. 14A). However, NMDA-treated supernatants complemented with anti-HMGB1 antibody showed no microglial activation (Fig. 16), indicating that HMGB1 in medium plays an important role in microglial activation. Regardless of the presence of anti-HMGB1 antibody, staurosporin-treated cell supernatant was incapable of activating microglia (Fig. 16). NMDA-induced release of HMGB1 into culture media was notably reduced for shHMGB1-pU6-transfected cells (Fig. 14B). In addition, supernatants from shHMGB1-pU6-expressing primary cortical cells were incapable of activating microglia (Fig. 14B), further supporting the notion that HMGB1 plays a crucial role in microglial activation. Microglial activation by excitotoxin-treated cell supernatant was further confirmed by the marked inductions of proinflammatory cytokines, e.g., iNOS, IFNγ, COX-2 and TNFα (Fig. 15). Together, these results demonstrated that extracellular HMGB1 released by excitotoxin-treated neurons plays a crucial role in microglial activation in vitro and in vivo.
Fig. 14. Microglial activation by extracellular HMGB1. shHMGB1-pU6 or MshHMGB1-pU6 were transiently transfected into primary cortical cells 24 h prior to NMDA treatment. The amounts of HMGB1 in NMDA-treated cells and in medium bathing were determined by western blot after 24 h of NMDA treatment (A). Primary microglia cultures were treated with media collected from NMDA-treated primary cortical cultures, transfected or not transfected with shRNA-expressing plasmid or mutant shRNA-expressing plasmid, and NO production was determined using the Griess method (B). The results of four independent experiments are presented as averages±SEM. * p < 0.05.
Fig. 15. Changes in the RNA levels of the pro-inflammatory cytokines by extracellular HMGB1

shHMGB1-pU6 or MshHMGB1-pU6 were transiently transfected into primary cortical cells 24 h prior to NMDA treatment. The amounts of HMGB1 in NMDA-treated cells were determined by RT-PCR after 24 h of NMDA treatment. Changes in the RNA levels of the pro-inflammatory cytokines, TNF-α, COX-2, and IL-1β, in the presence or absence of HMGB1 shRNA-expressing plasmid were followed by RT-PCR. LPS (100 ng/ml) treatment was used as a positive control. The results of four independent experiments are presented.
Fig. 16. Supression of microglial activation by anti-HMHB1 antibody. Primary microglial cultures (1x104 cells) were incubated with media collected from NMDA- or staurosporine-treated primary cortical cultures for 24 h in the presence or absence of anti-HMGB1 antibody, and NO production was determined using the Griess method. The results of four independent experiments are presented as averages±SEM. * $p < 0.05$. 
5.2 Recombinant HMGB1 is capable of triggering microglia activation

Purchased recombinant human HMGB1 (rHMGB1) (verified to be endotoxin-free by HPLC) dose-dependently induced microglial NO release and maximal effect was obtained at 500 ng/ml (Fig. 17A and B). Marked proinflammatory cytokine induction was also observed in rHMGB1-treated cells (Fig. 18).
Fig. 17. Induction of microglia activation by recombinant HMGB1
Primary microglia cultures were treated with the indicated concentrations of rHMGB1 for 48 h (A) or treated with 100 or 500 ng of rHMGB1 for indicated times (B), and NO production was determined by measuring nitrite in medium using the Griess method. The data of four independent experiments are presented as means±SEM * p < 0.05.
Fig. 18. Changes in the RNA levels of the pro-inflammatory markers by recombinant HMGB1 in primary microglia cultures.

Primary microglia cultures were treated with the indicated concentrations of rHMGB1 for 48 h treated with 100 or 500 ng of rHMGB1. Changes in the RNA levels of the pro-inflammatory markers, iNOS, TNF-α, COX-2, and IL-1β, in the presence of increasing amounts of rHMGB1 were followed by RT-PCR.
Discussion

The present study demonstrates that HMGB1 is a mediator that connects acute excitotoxicity-induced neuronal death to delayed damaging processes, like inflammation, in the postischemic brain. HMGB1 is massively released into the extracellular milieu during the acute excitotoxic damaging phase, and it triggers inflammation. Extracellular HMGB1 might function as a proinflammatory cytokine. It might activate microglia, a major inflammatory cell in the brain, stimulate the release of other cytokines and aggravate brain injury. Therefore, the shHMGB1-mediated protective effects shown in Fig. 8 and Fig. 9 might be derived from a reduction in the amount of HMGB1 released during the acute damaging process (Scaffidi et al., 2002; Degryse et al., 2001; Muller et al., 2001). These results are in accord with previous reports which found that HMGB1 is passively released by necrotic or damaged cells, and with a report that Hmgb1−/− necrotic cells have a greatly reduced ability to promote inflammation (Scaffidi et al., 2002).

Excitotoxicity is a leading cause of neuronal death following focal
cerebral ischemia. The activation of glutamate receptors, by the attendant failure of ion homeostasis and increase in intracellular Ca$^{2+}$ concentrations, is a major factor in the initiation of ischemic cell death (Dirnagl et al., 1999). In view of the fact that glutamate receptors are present in neurons but not in glial cells, HMGB1 released during acute excitotoxic insult is derived from neurons. Massive release of HMGB1 from damaged neurons would serve as a danger signal and evoke inflammatory reactions, which include activations of glial cells, of various immune cells in blood, and of endothelial cells. The mediator-like function of HMGB1 with respect to connecting the early and secondary damaging processes might also be relevant in other neuropathological conditions, such as, epilepsy or spinal cord injury, which also feature delayed damage progresses following acute and massive neuronal death (Rozengweig et al., 2004; Huttunen et al., 2002).

Although the mechanism by which HMGB1 exerts its proinflammatory cytokine-like effects in the CNS is unknown, the results of the present study indicate that HMGB1 directly triggers microglial activation probably by activating signaling cascades and also indirectly regulates the expression of classic proinflammatory cytokines. The present and
other reports show that the activations of several MAPKs and NF-kB are involved in the proinflammatory function HMGB1 (Huttunen et al., 2002; Sappington et al., 2002; Park et al., 2003; Fig. 11). They are downstream molecules of RAGE or TRL family members, which are important receptors in HMGB1 signaling (Yang et al., 2002). Although RAGE, TRL2, and TRL4 are present in microglia, interaction between and the relative contributions of different signaling pathway require further investigation. The inductions of proinflammatory cytokines by HMGB1 were notable \textit{in vivo} (Fig, 11) and \textit{in vitro} (Fig. 15). Thus HMGB1 functions synergistically with other cytokines, in the same manner as other cytokines, particularly with IFN\textsubscript{\gamma} during NO production in activated microglia. Moreover, synergistic effect could be achieved by both HMGB1 secretion upregulation by IFN\textsubscript{\gamma} and IFN\textsubscript{\gamma} secretion upregulation by HMGB1, as was described in NK cells (DeMarco et al., 2005).

Despite the fact that the level of HMGB1-induced NO release in microglia was relatively low compared to those induced by LPS- or IFN\textsubscript{\gamma} -treatment, the reduction in infarct size and the suppression of inflammatory processes observed in HMGB1 shRNA-expressing brain
was remarkable (Fig. 9). This may have been because extracellular HMGB1 *in vivo* acted on target cells other than microglia. In cultured macrophage, monocytes, or neutrophils, HMGB1 potently stimulates the release of various proinflammatory cytokines (Andersson et al., 2000; Park et al., 2003). In addition, the activation of human microvascular endothelial cells by HMGB1 has also been reported to result in the inductions of various adhesion molecules and proinflammatory cytokines, RAGE expression, and the activations of MAPKs and NF-kB (Fiuza, et al., 2003; Treutiger et al., 2004). Recently, HMGB1 was shown to induce dendritic cell maturation, as evidenced by the increased expressions of CD40, CD54, CD58, CD80 and CD80, and by the secretions of many proinflammatory cytokines, which result in immune reaction enhancement (Messner et al., 2005; Rovere-Querini et al., 2004). Therefore, HMGB1 might function pleiotropically, and its effects may be interconnected and affect various target cells *in vivo*, which may result in a profound effect on brain damage.

In view of the finding that serum HMGB1 levels were sustained after 6 days of reperfusion (Fig. 8B), it may be that HMGB1 is also actively secreted by inflammatory cells activated in a delayed manner in the
postischemic brain. In this regard, HMGB1 was induced in phagocytic microglia in the infarction core 3-4 days after MCAO/reperfusion (Fig. 3 and 4), which might be responsible for the observed delayed increase in HMGB1 in the ischemic brain hemisphere reperfusion (Fig. 3 and 4). HMGB1 is highly acetylated by nuclear acetyltransferase prior to secretion, whereas passively released HMGB1 is not acetylated (Bonaldi et al., 2003). Since it is highly possible that acetylated HMGB1 has biological properties and functions that differ from released or recombinant HMGB1, it may be that HMGB1 derived from microglia may exert different functions through distinct signaling molecules and pathways.

Recently, RNAi has emerged as a powerful genetic tool for silencing gene expression in multiple organisms, and it has been widely used to study gene function and to investigate the effects of various treatments. (Aoki H et. al., 2004; Cho YY et. al., 2004; Takei Y et. al., 2004; Jacque JM Y et. al., 2002; McCaffrey AP et. al., 2003). In this study, instead of using synthetic siRNA, pSUPER, a siRNA-expression vector system, was used for gene silencing. The plasmid vector used in this study (pSUPER) could drive the expression of shRNA in mammalian cells
In contrast to *C. elegans*, where RNAi effects are stable and long lasting, and are passed onto the offspring (Grishok & Mello, 2002), gene silencing by transfected siRNA duplexes in mammalian cells is transient. This is because mammalian cells lack the RNA-dependent RNA polymerases that amplify siRNAs in *C. elegans*. As a result, gene silencing is dependent on the number of siRNA molecules transfected into the cells and the duplexes become progressively diluted as cells divide. The persistence of siRNA activity in mammalian cells varies with the proliferative status of the cells. shRNA expressed within the cells by delivery of shRNA expression constructs has the potential to mediate longer-term RNAi than synthetic iRNA by virtue of their continual production. Therefore, the ability to use shRNA expression from vectors has greatly increased the range of applications in which RNAi can be applied.

The therapeutic applications of shRNA require the sustained intracellular production of shRNA in targeted tissues (Omi et al., 2003). Here we demonstrate that the stereotaxic injection of shRNA-expressing...
plasmids provided the stable expression of a silencing trigger for more than 7 d after a single administration (Fig. 7). Accumulating information regarding the impact of delayed neuronal death following transient global (Kirini et al., 1982, Abe et al., 1995) or focal ischemia (Wang et al., 2004) raises the possibility that delayed damaging process could be a potential therapeutic target. The sustained suppression of a targeted gene for more than 6 d in the ischemic brain by a single treatment suggests that shHMGB1-pU6 might be an effective means of controlling transient cerebral ischemia, until HMGB1 expression can be suppressed by other means.
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