HMGB1 Protein Does Not Mediate the Inflammatory Response in Spontaneous Spinal Cord Regeneration

A HINT FOR CNS REGENERATION*

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Background: HMGB1 in spontaneously regenerating spinal cord does not trigger the inflammation in contrast to those in injured mammalian cords.

Results: Gecko HMGB1 paralogs failed to interact with TLR2 and TLR4 but do with RAGE receptors to activate the signaling pathway.

Conclusion: HMGB1 is beneficial for spontaneous spinal cord regeneration by eliciting negligible inflammation and promoting oligodendrocyte migration.

Significance: HMGB1 displays distinct functions in regenerative vertebrates.

Uncontrolled, excessive inflammation contributes to the secondary tissue damage of traumatic spinal cord, and HMGB1 is highlighted for initiation of a vicious self-propagating inflammatory circle by release from necrotic cells or immune cells. Several regenerative-competent vertebrates have evolved to circumvent the second damages during the spontaneous spinal cord regeneration with an unknown HMGB1 regulatory mechanism. By genomic surveys, we have revealed that two paralogs of HMGB1 are broadly retained from fish in the phylogeny. However, their spatial-temporal expression and effects, as shown in lowest amniote gecko, were tightly controlled in order that limited inflammation was produced in spontaneous regeneration. Two paralogs from gecko HMGB1 (gHMGB1) yielded distinct injury and infectious responses, with gHMGB1b significantly up-regulated in the injured cord. The intracellular gHMGB1b induced less release of inflammatory cytokines than gHMGB1a in macrophages, and the effects could be shifted by exchanging one amino acid in the inflammatory domain. Both intracellular proteins were able to mediate neuronal programmed apoptosis, which has been indicated to produce negligible inflammatory responses. In vivo studies demonstrated that the extracellular proteins could not trigger a cascade of the inflammatory cytokines in the injured spinal cord. Signal transduction analysis found that gHMGB1 proteins could not bind with cell surface receptors TLR2 and TLR4 to activate inflammatory signaling pathway. However, they were able to interact with the receptor for advanced glycation end products to potentiate oligodendrocyte migration by activation of both NFκB and Rac1/Cdc42 signaling. Our results reveal that HMGB1 does not mediate the inflammatory response in spontaneous spinal cord regeneration, but it promotes CNS regeneration.

Inflammatory responses elicited by spinal cord injury (SCI) contribute substantially to secondary tissue damage characterized by a series of biochemical events and cellular dysfunction, eventually leading to the formation of an ellipsoidal, loculated cystic cavity (1–3). Controlling the infiltration of immune cells and their excessive releases of the pro-inflammatory cytokines has become the important therapeutic strategies for alleviating functional decline of the SCI (4, 5). The “sterile” inflammatory responses following SCI are significantly associated with the signaling of endogenous damage-associated molecular pattern (DAMP) molecules at sites of tissue damage, which bind with pattern recognition receptors expressed in most cells (6, 7). Ligation of DAMPs with pattern recognition receptors, including most toll-like receptors (TLRs) and advanced glycation end products (RAGE), elicits inflammatory signaling, resulting in the releases of cytokines that augment inflammatory reaction (8, 9). Many DAMPs are nuclear or cytosolic proteins with defined intracellular functions and are released from cells after tissue injury (10). A key DAMP molecule is the nuclear protein high mobility group box 1 (HMGB1, amphoterin) that is passively released from necrotic cells or is actively secreted by neurons and many other cell types upon stimulation (11, 12). HMGB1 promotes immune cell recruitment and activation and as consequences the secretion of a spectrum of inflammatory cytokines, including TNF-α, IL-1, and IL-6 (13–15). In various

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4. The abbreviations used are: SCI, spinal cord injury; DAMP, endogenous damage-associated molecular pattern; TLR, toll-like receptors; RAGE, receptor for advanced glycation end product; gHMGB1, gecko HMGB1; mHMGB1, mouse HMGB1; Q-PCR, quantitative PCR; CREB, cAMP-response element-binding protein; TRITC, tetramethylrhodamine isothiocyanate.
CNS injury models, inhibition of HMGB1 has been shown to reduce the severity of the lesion and microglial activation (16, 17). As such, HMGB1 has been proposed to be a significant proinflammatory cytokine mediating progressive spinal cord injury (15, 18).

Structurally, HMGB1 proteins are characterized by the presence of two L-shaped DNA-binding elements (HMG box A and B), each made up of 80 amino acids arranged in three positively charged α-helices, and a C-terminal extremely negatively charged domain (19, 20). By binding to the minor groove of double-stranded DNA, the protein participates in nucleosome formation and regulation of gene transcription (21, 22). HMGB1 of immunocompetent cells, including monocytes, macrophages, neutrophils, and dendritic cells, can shuttle from the nucleus to the cytoplasm, where it is further packaged into secretory vesicles and released into extracellular milieu under stimulation of exogenous pathogenic products (23). Alternatively, the protein is released by somatic cells undergoing necrosis and elicits progression of inflammation (11, 24–27). The extracellular HMGB1 mediates inflammatory signaling or other cellular responses, including proliferation, migration, and neurite outgrowth, through its interaction with RAGE, TLR2, and TLR4, etc., resulting in activation of transcription factor NFκB and mitogen-activated protein kinases (MAPK) (28–31). The peptide corresponding to the first 20 amino acids of the B-box domain is able to mimic the inflammatory potential of HMGB1 in vivo (32, 33), and a substitution of one amino acid might affect the interaction of HMGB1 with TLR4, providing a rational design and development of therapeutics for use in sterile and infectious inflammation (34).

Two rounds of genome duplication early in vertebrate evolution, followed by a single round of genome duplication in a common ancestor of sample teleosts, resulted in the occurrence of HMGB1 paralogs in basal vertebrate fishes (35). Recently, HMGB1 paralogs have been identified in amphibian and mammalian species, including the commonly used model organisms Xenopus (GenBank™ accession numbers U21933 and BC054148), mouse (GenBank™ accession numbers XP_889413 and NP_034569), and rat (GenBank™ accession numbers XP_003753270 and NP_037095). During evolution, the duplicated genes have usually undergone degeneration, neofunctionalization, or subfunctionalization, which might be associated with differential or complementary regulation of physiological functions (36, 37). A comparative study of the two paralogs of HMGB1 is therefore indispensable to clarify each physiological role and the associated molecular signaling, especially in their mediation of inflammatory responses spotlighted by clinical intervention. The neglected nuance might be beneficial for refining our knowledge on these proteins, thereby improving therapeutical development. The reptile is the lowest amniote located at a significantly evolutionary position bridging lower vertebrates and mammals. Similar to fishes and amphibians, several species in the taxa are capable of regenerating complex body structures, including significant portions of their central nervous system in tailed adulthood (38–41). However, the mechanisms for these animals to circumvent secondary tissue damage by limiting the vicious self-propagating cycle of inflammation are still poorly understood. Given the evidence that mammalian HMGB1 mediates CNS inflammation (18, 42), we speculate that the regenerative animals might share distinct HMGB1 regulatory mechanisms by limiting excessive inflammatory responses to facilitate spinal cord regeneration, i.e. either by differentially spatial-temporal regulation of two paralogs following injury or by alternative molecular signaling. To address this question, we investigated the distinct roles of two paralogs of Gekko japonicus HMGB1 (gHMGB1) following tail amputation. We have revealed that the two paralogs of HMGB1 are broadly retained from fish onward during evolution. gHMGB1 paralogs displayed differential responses to the challenges of infectious stimulation and spinal cord injury. The expression of gHMGB1 has been selectively switched in the traumatic spinal cord and has not mediated inflammatory responses, but it facilitates the functional recovery by promoting migration of oligodendrocytes through specific interaction with RAGE.

**EXPERIMENTAL PROCEDURES**

**Animals**—Adult G. japonicus were used as described by Wang et al. (43). Briefly, adult animals were fed mealworms ad libitum and housed in an air-conditioned room with a controlled temperature (22–25 °C) and saturated humidity. Anesthesia was induced by cooling the animals on ice prior to tail amputation. Amputation was performed at the sixth caudal vertebra, identified based on the special tissue structure present at that position (41), by placing a slipknot of nylon thread and pulling gently until the tail was detached, thus mimicking the autotomy undergone for natural defense.

For lipopolysaccharide (LPS) treatment, LPS (Sigma) was dissolved in normal saline and injected intraperitoneally (7.5 mg/kg body weight) according to the protocol of Hasegawa et al. (44). Control experiments were performed by injection of an equivalent amount of normal saline.

All experiments were conducted in accordance with guidelines of the National Institutes of Health (Guide for the Care and Use of Laboratory Animals, 1985), and the Guidelines for the Use of Animals in Neuroscience Research by the Society for Neuroscience. Experiments were approved according to the Animal Care and Use Committee of Nantong University and the Jiangsu Province Animal Care Ethics Committee. All geckos (n = 15) were anesthetized on ice prior to sacrifice.

**Surveys of HMGB1 Paralogs in Vertebrates**—A cDNA library of the brain and spinal cord from G. japonicus was constructed according to the methods described previously (45). In a large scale sequencing of the cDNA library, more than 5000 clones were analyzed for coding probability with the DNATools program (46). Sequences of HMGB1 paralogs were found based on BLASTP alignment against genome databases deposited at the National Center for Biotechnology Information (47). Phylogenetic tree was constructed using the PHYML implementation of Maximum-Likelihood, with the GTR (CD5 sequences) and JTT (protein sequences) substitution model (48).

**Preparation of Recombinant Proteins in Eukaryotic Cells and Immunization of Rabbit**—Recombinant constructs of gHMGB1 paralogs, gHMGB1a and gHMGB1b, and their mutants, gHMGB1ma and gHMGB1mb, in the pRAG2a expression vector were transfected into 293T cells using Lipo-
HMGB1 in Spinal Cord Regeneration

fectant 2000 transfection reagent (Invitrogen) according to the manufacturer’s instructions. After a 48-h culture in a 37°C humidified incubator with 8% CO₂, cells were removed by centrifugation at 10,000 rpm for 20 min. Both the cell supernatants and homogenate were filtered through a 0.45-μm filter prior to purification by Ni²⁺-nitrilotriacetic acid chromatography kit (Qiagen). The process for production of the recombinant proteins referred to the manufacturer’s instructions. Purity of the recombinant proteins was subsequently determined by separating each protein in a 12% SDS-PAGE and staining with Coomassie Brilliant Blue R-250.

Rabbits were immunized subcutaneously with 100 μg of recombinant HMGB1 mixed in 0.5 ml of Gurber adjuvant (Bio-tech, Poway, CA). Three booster injections were given at 2-week intervals using the same antigen dose. After the final booster dose, rabbits were bled, and sera were separated and stored at −20°C. Antibody titer was determined using an ELISA, and antibody reactivity was confirmed in a Western blot analysis. Mouse HMGB1 (mHMGB1) recombinant protein and polyclonal antibody were also prepared in parallel as positive controls.

Quantitative Real Time-PCR (Q-PCR)—Total RNA was prepared with TRIzol (Invitrogen) from different tissues, including the brain, spinal cord, kidney, liver, ovary, testis, lung, and spleen of adult geckos. Total RNAs were also extracted from 0.5-cm spinal cord segments of 20 geckos amputated from the sixth caudal vertebra at 1 and 3 days and 1 and 2 weeks, respectively.

For Q-PCR examination of gHMGB1 temporal expression, the first-strand cDNA was synthesized using Omniscript reverse transcription kit (Qiagen) in a 20-μl reaction system containing 2 μg of total RNA, 0.2 units/μl Moloney murine leukemia virus reverse transcriptase, 0.5 mM dNTP mix, 1 μM oligo(dT) primer. The cDNA was diluted 1:5 before use in Q-PCR assays. The sequence-specific primers and TaqMan probe were designed and synthesized by Invitrogen. The primer pair and probe for gHMGB1a are as follows: forward primer 5’-GAG TGA GGA GGC TGC GTA TG-3’, reverse primer 5’-CCC TTT GCC CAT ACT GAT ATG TG-3’, and TaqMan probe 5’-CCT CAC AGT CAG CTT GAG AG-3’. The process for production of the recombinant proteins referred to the manufacturer’s instructions. Purity of the recombinant proteins was subsequently determined by separating each protein in a 12% SDS-PAGE and staining with Coomassie Brilliant Blue R-250.

Northern Blotting—Northern blotting was performed as described by Wang et al. (39). A total of 4 μg of RNA extracted from brain, spinal cord, kidney, liver, ovary, and testis of adult geckos was electrophoresed and blotted onto a nylon membrane (Osmonics Inc.). The blots were hybridized at high stringency with digoxigenin-labeled gHMGB1a or gHMGB1b riboprobe (1 μg/ml in DIG Easy Hyb) for 15 h at 58°C and washed twice in 2× SSC with 0.1% SDS at 25°C for 5 min each and twice in 0.1× SSC with 0.1% SDS at 65°C for 20 min each. They were then incubated in a blocking solution, pH 7.5, containing 100 mM maleic acid, 150 mM NaCl, and 1% blocking reagent (Roche Applied Science) followed by incubation in the blocking solution plus anti-digoxigenin-alkaline phosphatase (Roche Applied Science) diluted 1:10,000, for 1 and 2 h, respectively, at room temperature. After washing with 100 mM maleic acid buffer, pH 7.5, containing 150 mM NaCl and 0.3% Tween 20, and then 100 mM Tris-HCl buffer, pH 9.5, containing 100 mM NaCl, the hybridized bands were visualized by CDP-Star (Roche Applied Science) and recorded by exposure to x-ray film.

Plasmid Construction, Cell Culture, and Treatment—Open reading frame of gHMGB1a or gHMGB1b was constructed into pEGFP-N1 vector. gHMGB1a mutation (gHMGB1ma, K90E) and gHMGB1b mutation (gHMGB1mb, E90K) were made by PCR-based site-directed mutagenesis (QuikChange™, Stratagene) using primers 5’-CAA AAA AGA AGT AGG ACC CAA ATG CAC-3’ and 5’-GTG CAT TTG GCT CCT CGA ACT TCT TTG-3’ for gHMGB1ma and primers 5’-AAG TGG TCA GGC CAT CCC AAT GCA CCC AAG-3’ and 5’-CTT GGC TGG TAC GGT TTC CCA GCT CAA-3’ for gHMGB1mb.

Murine macrophage RAW 264.7 cells, human neuroblastoma cell line SH-SY5Y (Chinese Academy of Sciences, Shanghai Institutes for Biological Sciences Cell Resource Center), or gecko oligodendrocyte cell line Gsn3 (49) were grown in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% (v/v) fetal bovine serum in a 37 or 30°C humidified incubator with 5% CO₂. Cells were treated with 1, 5, and 10 μg/ml recombinant proteins or commercial human HMGB1 (hHMGB1, Sigma), respectively. Alternatively, RAW 264.7 or SH-SY5Y cells were transfected with pEGFP-gHMGB1a, pEGFP-gHMGB1b, pEGFP-gHMGB1ma, or pEGFP-gHMGB1mb plasmids by FuGENE HD transfection reagent (Roche Applied Science) following the manufacturer’s instructions. Cells were also counterstained with the Hoechst 33342 (1 μg/ml) for 10 min at 37°C or stained with annexin V-PE for 10 min at 20–25°C and mounted on slide glasses with mounting medium. Images were captured on a Nikon Diaphot microscope.

Western Blot—Protein was extracted from cells with a buffer containing 1% SDS, 100 mM Tris-HCl, 1 mM PMSF, and 0.1 mM...
were determined by RT-PCR using mouse and subsequent selection by flow cytometry (BD FACSAria).

72 °C before storing the samples at 4 °C.

72 °C for 30 s. A final extension of 10 min was performed at

ice-cold cell lysis buffer containing 50 mM Tris-HCl, pH 7.4, 2 mg/ml protease inhibitors from Roche Applied Science).

200 μl of rhotekin-RBD beads and 20 μl of PAK-PBD beads and rotated for 60 min at 4 °C. The beads were washed three times with lysis buffer and heated for 5 min at 100 °C in SDS-PAGE sample buffer, and then analyzed for total cells, anti-Rac1 antibody (1:500, Cytoskeleton), or anti-Cdc42 antibody (1:500, Cytoskeleton).

β-mercaptoethanol, following treatment with 1 μg/ml recombinant proteins or commercial hHMGB1 for 24 and 48 h. Alternatively, the cells were transfected with pEGFP-hHMGB1a, pEGFP-hHMGB1b, pEGFP-hHMGB1ma, or pEGFP-hHMGB1mb plasmids for 24 and 48 h and then were collected by flow cytometry (BD Biosciences, FACSAria). Protein concentration of each specimen was determined by the Bradford method to maintain the same loads. Protein extracts were heat-denatured at 95 °C for 5 min, electrophoretically separated on 10% SDS-PAGE, and transferred to PVDF membranes. The membranes were subjected to the reaction with a 1:1000 dilution of primary antibodies in TBS buffer at 4 °C overnight, followed by a reaction with secondary antibody conjugated with goat anti-rabbit HRP dilution 1:1000 (Santa Cruz Biotechnology) at room temperature for 2 h. After the membrane was washed, the HRP activity was detected using an ECL kit. The image was scanned with a GS800 Densitometer Scanner (Bio-Rad), and the data were analyzed using PDQuest 7.2.0 software (Bio-Rad). β-Actin (1:5000) was used as an internal control.

bodies used in Western blot are as follows: gHMGB1a and gHMGB1b; cleaved caspase 3 (Asp-175, Cell Signaling); RAGE, TLR2, TLR4, p-CREB, and CREB (Santa Cruz Biotechnology); JNK, p-JNK, p65NF-κB, p-ERK1/2, ERK1/2, p-p38, p38, and β-actin (Abcam).

RhoA, Rac1, or Cdc42 activation was determined using the rhotekin-RBD that specifically binds activated Rho and the PBD-PAK that has a high affinity for both GTP-Rac and GTP-Cdc42 (RhoA/Rac1/Cdc42 Activation Assay Combo Biochem kit, Cytoskeleton, Denver). In brief, the cells were lysed with ice-cold cell lysis buffer containing 50 mM Tris-HCl, pH 7.4, 2 mM MgCl2, 1% Nonidet P-40, 10% glycerol, 100 mM NaCl, and protease inhibitor mixture (Roche Diagnostics) and centrifuged for 5 min at 14,000 x g. The equivalent protein amounts of lysate (500 μg of total cell protein) were performed using pulldown assay with 50 μg of rhotekin-RBD beads and 20 μg of PAK-PBD beads and rotated for 60 min at 4 °C. The beads were washed three times with lysis buffer and heated for 5 min at 100 °C in SDS-PAGE sample buffer, and then analyzed for bound RhoA, Rac1, and Cdc42 molecules by Western blotting using anti-RhoA antibody (1:500, Cytoskeleton), anti-Rac1 antibody (1:500, Cytoskeleton), or anti-Cdc42 antibody (1:500, Cytoskeleton).

Assays for TNF-α and IL-1α—For examination of TNF-α and IL-1α transcriptional expression, murine macrophage RAW 264.7 cells following transfection with plasmids at 1, 2, or 3 days and subsequent selection by flow cytometry (BD FACSAria) were determined by RT-PCR using mouse TNF-α- and IL-1α-specific primers (forward 5′-CCA CCA TCA AGG ACT CAA AT-3′ and reverse 5′-GGT GCC CAA ATC AGC GGT T-3′ for TNF-α and forward 5′-GCA CCT TAC ACC TAC CAG-3′ and reverse 5′-TGA ATA GAC TCA AGA AAT-3′ for IL-1α). Mouse GAPDH amplified from the same samples using gene-specific primers (forward 5′-TGA AGT CGC AGG AGA CAA CC-3′ and reverse 5′-GGT GGA GCC AAA AGG GTC A-3′) served as housekeeping controls. PCR parameters were 26 (TNF-α) or 38 (IL-1α) cycles of denaturation at 95 °C for 30 s, primer annealing at 50 °C for 30 s, and primer extension at 72 °C for 30 s. A final extension of 10 min was performed at 72 °C before storing the samples at 4 °C.

Two-site sandwich ELISA was used to quantify TNF-α and IL-1α in the extracts of murine macrophage RAW 264.7 cells cultured with 1 μg/ml recombinant proteins for 24 h or segments of 0.5 cm of proximal spinal cord injected with 5 μl of 300 μg/ml recombinant proteins at 24, 48, or 72 h, respectively, according to the manufacturer’s instructions (Duo-Set; R&D Systems, Minneapolis, MN).

Tissue Immunohistochemistry—The vertebral segments were harvested, post-fixed, and sectioned. Sections were allowed to incubate with polyclonal rabbit anti-gecko HMGB1a, rabbit anti-gecko HMGB1b antibody (1:100 dilution), polyclonal rat anti-bovine cd11b antibody (1:200 dilution, Millipore), or polyclonal mouse anti-human NF200 antibody (1:200 dilution, Abcam) at 4 °C for 36 h. The sections were further reacted with the FITC-labeled secondary antibody goat anti-mouse IgG (1:400 dilution, Invitrogen) or the TRITC-labeled secondary antibody donkey anti-rabbit IgG (1:400 dilution, Invitrogen) at 4 °C overnight, followed by observation under a confocal laser scanning microscope (Leica, Heidelberg, Germany).

Immunoprecipitation—RAW 264.7 or SH-SY5Y cells were washed twice with cold phosphate-buffered saline and then extracted with lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitors from Roche Applied Science). Whole cell extracts were centrifuged at 14,000 rpm for 20 min to remove the debris. The proteins in the supernatant were measured using a Protein Assay Kit II (Bio-Rad). For immunoprecipitation analysis, 500 μg of total cell lysates was precleared with protein A plus G-Sepharose before incubation with specific antibodies, followed by addition of protein A plus G-Sepharose. The precipitated proteins were resolved in 2× SDS-PAGE sample buffer and separated by electrophoresis on 10–12% SDS-PAGE. After transferring onto a polyvinylidene difluoride membrane (Millipore Corp.), they were incubated with anti-His-tag antibody and then with horseradish peroxidase-conjugated secondary antibody (Cell Biolabs, Inc.).

Cell Migration Assay—Migration of oligodendrocyte Gsn3 was studied using 6.5-mm Transwell chambers with 8-μm pores (Corning Costar) as described previously (50). 100 μl of oligodendrocytes (2 × 105 cells/ml) resuspended in DMEM/F-12 were transferred to the top chambers of each transwell and allowed to migrate at 30 °C in 5% CO2 for 36 h, and 600 μl of DMEM/F-12 including 1 μg/ml recombinant proteins or antibodies (1:50) was injected into the lower chambers. The upper surface of each membrane was cleaned with a cotton swab at the indicated time point. Cells adhering to the bottom surface of each membrane were stained with 0.1% crystal violet, imaged, and counted using a DMR inverted microscope (Leica Microsystems). Assays were done three times using triplicate wells.

RESULTS

HMGB1 Paralogs Are Broadly Retained from Fish Onward in the Phylogeny—To identify whether HMGB1 paralogs exist in the reptiles, in which some species are capable of spontaneously
Regenerating spinal cord, we constructed brain and spinal cord cDNA library from *G. japonicus*. Two cDNA clones (GenBank™ accession numbers HM239648 and HM239649) were thus obtained by 5′-rapid amplification of cDNA ends, and each encoded protein consisted of two highly conserved HMG boxes and a longest acidic tail, characteristic of the HMGB1 family (51, 52). Therefore, the two clones were designated as *gHMGB1a* and *gHMGB1b*, respectively. Our results indicated that reptiles, together with teleosts and amphibians with the ability to regenerate their adult spinal cord, retained HMGB1 paralogs.

A question then arises as to whether chicken and mammals, which have been shown to be a failure in regenerating their adult spinal cord, still keep two paralogs of HMGB1. It is interesting to note that mammalian HMGB1 has two seemingly diametrically opposed functions in promoting regeneration of many tissues and progression of inflammation (53). To address the issues, candidates of the HMGB1 paralogs in human, mouse, rat, and chicken genomic databases were searched using BLASTP alignment. Results have demonstrated that mouse and rat evidently preserve HMGB1 two paralogs with GenBank™ accession numbers as follows: mouse HMGB1-like XP_889413 and mouse HMGB1 NP_034569; mouse HMGB1-like XP_003753270 and rat HMGB1 NP_037095; rat HMGB1-like (XP_003753270); mouse HMGB1 (NP_034569); mouse HMGB1-like (XP_889413); and human HMGB1 (NP_002119).

Differential Responses of *gHMGB1a* and *gHMGB1b* to the Spinal Cord Trauma and Inflammatory Challenge—Both Northern blotting and real-time PCR were conducted to elucidate a basic expression pattern of the two *gHMGB1* paralogs. As shown in Fig. 2, *A* and *B*, all samples revealed two transcripts, similar to the HMGB1 signals obtained in canine tissues of about 1.3 and 2.4 kb (54). The more sensitive real-time PCR technique showed that *gHMGB1a* was ubiquitously expressed in all tissues examined but with low abundance in the kidney and liver (Fig. 2*C*). For *gHMGB1b*, the ovary displayed strong transcripts of 1.3 and 2.4 kb, with weak expression in the other tissues (Fig. 2*B*).

To understand the potential roles of *gHMGB1* two paralogs, which might be involved in differential responses to the different stimuli, we established spinal cord trauma and lipopolysaccharide (LPS) injection models to determine the transcriptional expression changes of *gHMGB1a* and *gHMGB1b*. Real-time PCR detection showed that the abundance of *gHMGB1a* mRNA in the injury site of the spinal cord displayed no obvious changes at 1 and 3 days and 1 and 2 weeks after tail amputation (Fig. 2*D*). In contrast, *gHMGB1b* transcription in spinal cord significantly increased at 3 days after amputation and decreased to the control level at 1 week. At 2 weeks after amputation, the expression of *gHMGB1b* increased again (Fig. 2*E*).

LPS dissolved in normal saline was injected intraperitoneally (7.5 mg/kg body weight). Samples were analyzed for 1 day (*n* = 15), 2 days (*n* = 15), and 3 days (*n* = 15) after injection. Tissues, including brain, spinal cord, spleen, and lung, were selected for analysis. Lung was chosen for its association with high-level expression of HMGB1 after inflammation or acute injury (23, 55), and kidney and liver were excluded for the low abundance of HMGB1 (*Fig. 2* *A* and *B*). Real-time PCR revealed that *gHMGB1a* transcripts in brain decreased at 3 days after injection. However, its expression markedly increased at 2 and 3 days in spleen (Fig. 2*F*), an organ that contains quite a number of macrophages involved in the induction and maintenance of both innate and acquired immune defense (56). Comparatively, *gHMGB1b* transcripts demonstrated no obvious changes in all tissues examined (Fig. 2*G*).

These results indicated that *gHMGB1a* was probably involved in the response of external inflammatory stimuli, and *gHMGB1b* was probably involved in the regulation of spinal cord regeneration following injury.

*gHMGB1a* and *gHMGB1b* Colocalized with Neuron and Microglia—In CNS, HMGB1 displays a complex temporal and spatial distribution pattern and is implicated in facilitating neurite outgrowth and cell migration (57, 58). HMGB1 also serves to induce neuroinflammation after injury, such as lesions in the spinal cord and brain (16, 18, 59). To elucidate whether neuron and microglia, a type of glial cells that are the resident macrophages of the brain and spinal cord, have participated in the physiological processes through activation of one or both HMGB1 paralog signalings, we first succeeded in preparation of the recombinant proteins by eukaryotic expression in 293T cells and then of the polyclonal antibodies (Fig. 3, *A* and *B*). We detected the cellular localization of *gHMGB1a* and *gHMGB1b* after spinal cord injury at 3 days, using double immunofluorescent staining with combinations of antibodies, namely
gHMGB1a/NF200 or gHMGB1b/NF200 for neurons and gHMGB1a/CD11b or gHMGB1b/CD11b for microglia. The results indicated that gHMGB1a was ubiquitously distributed in the gray and white matter, displaying colocalization with both neurons and microglia (Fig. 3, C and D). In comparison, the injury-induced gHMGB1b expression was mostly distributed in the gray matter, with colocalization with both neurons and microglia (Fig. 3, C and D). The results demonstrated that both neurons and microglia of the gecko could produce two HMGB1 paralogs, although only gHMGB1b exhibited marked responses to spinal cord injury.

Intracellular gHMGB1a and gHMGB1b Induced Differential Inflammatory Responses—HMGB1 was found to induce the synthesis of various inflammatory cytokines, including TNF-α and IL-1β by macrophages (60, 61). To gain insight into the gHMGB1a and gHMGB1b functions on mediating the release of the inflammatory cytokines in microglial cells, we overexpressed the plasmids of pEGFP-gHMGB1a or pEGFP-gHMGB1b into murine macrophage RAW 264.7 cells for 24 hours.

**FIGURE 2.** Transcriptional expression of gHMGB1 paralogs in tissues and differential responses to injury and infectious challenges. A and B, Northern blotting analysis of gHMGB1a and gHMGB1b transcripts in different tissues. C, quantitative real-time PCR analysis of gHMGB1a transcripts in different gecko tissues. Gecko EF-1α was used for normalization. D and E, quantitative RT-PCR amplification of gHMGB1a and gHMGB1b in the spinal cord from L13 to the 6th caudal vertebra following tail amputation at 0 day (con), 1 and 3 days (d) and 1 and 2 weeks (w). F and G, quantitative RT-PCR amplification of gHMGB1a and gHMGB1b in different tissues after injection of LPS (7.5 mg/kg body weight) intraperitoneally. Quantities were normalized to endogenous EF-1α expression. Error bars represent the standard deviation (*, p < 0.01).

**FIGURE 3.** Distribution of gHMGB1a and gHMGB1b in the spinal cord. A, preparation of gHMGB1a and gHMGB1b recombinant proteins by eukaryotic expression in 293T cells. B, gHMGB1a and gHMGB1b polyclonal antibodies react specifically with spinal cord tissue by Western blot analysis. C, colocalization of gHMGB1a and gHMGB1b with NF-positive cells (arrowhead). D, colocalization of gHMGB1a and gHMGB1b with CD11b-positive cells (arrowhead). Lane M indicates protein marker. Rectangle indicates region magnified below. Scale bars, 100 μm.
Results showed that gHMGB1a is localized in the cytoplasm and nucleus, whereas gHMGB1b is primarily localized in the nucleus of macrophages (Fig. 4B). The positive cells were selected by flow cytometry and subjected to transcriptional analysis (Fig. 4C). The expressions of TNF-α and IL-1α were significantly up-regulated after transfection with pEGFP-gHMGB1a for 48 h, and they were slightly up-regulated or unchanged after transfection with pEGFP-gHMGB1b (Fig. 4, D and E). Interestingly, there is only one amino acid difference in the first 20-residue fragment of the B-box between gHMGB1a and gHMGB1b (Fig. 4A), which has been found to affect the inflammatory activity (32). We subsequently exchanged the distinct amino acid residue by mutation (Lys → Glu), and we examined the transcription of proinflammatory cytokines by transfection of mutated gHMGB1a (gHMGB1ma, Lys → Glu) or mutated gHMGB1b (gHMGB1mb, Glu → Lys) in RAW 264.7 cells. The potential of mediating the release of TNF-α and IL-1α by intracellular gHMGB1a and gHMGB1b has accordingly been exchanged. gHMGB1mb was capable of up-regulating the transcripts of IL-1α, but the role of gHMGB1ma was reversed (Fig. 4, F and G). The results revealed that the intracellular gHMGB1a and gHMGB1b induced differential inflammatory responses, and gHMGB1a was the main inflammatory mediator.

Involvements of Intracellular gHMGB1 Two Paralogs in the Apoptosis of Neurons—HMGB1 was found to activate not only macrophages but also neurons (23, 42). A plethora of studies have revealed that HMGB1 plays double-edged roles in neural...
development and neurodegeneration (59). Therefore, we transfected the plasmids of gHMGB1a and gHMGB1b into PC12 and SH-SY5Y cell lines to examine their effects on neurites. Results indicated that the two paralogs were incapable of promoting neurites outgrowth and elongation (data not shown), quite distinct from mammalian HMGB1 (57, 58). However, apoptosis was induced by overexpression of gHMGB1a, gHMGB1b, or their mutants for 72 h (Fig. 5, A and B). Annexin-V staining also demonstrated a marked correlation with apoptotic transfected cells (Fig. 5, C–Q). These indicated that both intracellular gHMGB1a and gHMGB1b could induce the neuronal apoptosis.

**Extracellular gHMGB1 Two Paralogs Do Not Facilitate the Release of Inflammatory Cytokines**—Previous studies have indicated that immune cells and damaged or necrotic cells can release HMGB1 into the extracellular milieu, where it triggers inflammatory responses by acting on various immune cells (33). To examine whether the extracellular gHMGB1 paralogs of regenerative gecko, especially for the significantly up-regulated gHMGB1a and gHMGB1b in the traumatic spinal cord, enacted the analogical inflammation-mediating function, RAW 264.7 cells were cultured with 0, 1, 5, or 10 μg/ml recombinant proteins for 24 h. The transcriptional expression levels of *TNF-α* and IL-1α exhibited no changes in macrophages (Fig. 6, A and B). We further conducted the parallel experiments by applying commercial hHMGB1 or mHMGB1 recombinant protein prepared by the same protocol. Assays by two-site sandwich ELISA showed that the recombinant proteins of gHMGB1 two paralogs, as well as their mutants, did not elicit the synthesis and secretion of *TNF-α* and IL-1α (Fig. 6, C–F), whereas mHMGB1 recombinant protein and commercial hHMGB1 significantly triggered the production of the two inflammatory cytokines (Fig. 6, C–F).

Following injection of 5 μl of 300 μg/ml gHMGB1 or mHMGB1 recombinant proteins, as well as equivalent commercial hHMGB1 into the spinal cord proximal to the injured site immediately after tail amputation, we collected 0.5-cm segments at 0–3 days, respectively, and we evaluated the expression levels of inflammatory cytokines. A dose of 5 μl of saline or 5 μl of 200 μg/ml LPS was performed as control. ELISAs displayed that *TNF-α* and IL-1α could be directly initiated by spinal cord amputation, reaching a basic amount of inflammatory cytokines. Injection of gHMGB1 recombinant proteins was unable to potentiate the release of *TNF-α* and IL-1α compared with saline control (Fig. 6, G and H). But injection of LPS, mHMGB1 recombinant protein, or commercial hHMGB1 significantly elicited the up-regulation of *TNF-α* and IL-1α in *vivo* (Fig. 6, G and H). Our results demonstrated that extracellular gHMGB1 paralogs did not enact inflammatory functions in the spontaneously regenerating spinal cord.

**gHMGB1a and gHMGB1b Exhibit Interactions with RAGE but Not with TLR2 or TLR4 Receptor**—Studies have shown that HMGB1 initiates the multiple intracellular signaling by interaction with RAGE, TLR2, TLR4, or other family members, leading to the activation of NFκB (28, 28). To shed light on the relationship of gHMGB1a and gHMGB1b with TLR2, TLR4, and RAGE receptors, we cultured macrophage RAW 264.7 or SH-SY5Y cells with 1 μg/ml recombinant gHMGB1a, gHMGB1b, or 0.1 μM PBS, pH 7.4, respectively. The two cell lines expressed the three surface receptors. However, NFκB has not been activated by the gHMGB1 two paralogs (Fig. 7, A and B), in contrast to what has been done by mammalian HMGB1 (28, 29). We further carried out immunoprecipitation experiments using anti-TLR2, -TLR4, or -RAGE antibody, and then we detected the components of the TLR2-, TLR4-, and RAGE-associated complexes with anti-His antibody, to avoid cross-reacting with endogenous HMGB1 of the cultured cells. As shown in Fig. 7, C–E, gHMGB1a or gHMGB1b coprecipitated with RAGE, but not with TLR2 or TLR4 receptor. These results indicated that gHMGB1a or gHMGB1b in spontaneously regenerating spinal cord could not bind with cell surface recep-
tors TLR2 or TLR4 to initiate the production of inflammatory cytokines.

**gHMGB1a and gHMGB1b Promote the Oligodendrocyte Migration by Activation of Rac1/Cdc42 Signaling**—In the CNS, extracellular HMGB1 is highly active in enhancing neurite outgrowth through interaction with RAGE to communicate with the GTPases Cdc42 and Rac (62–64). To verify the effects of gHMGB1 paralogs on the neurons, we cocultured SH-SY5Y cells with 1/2 H9262 g/ml exogenous recombinant gHMGB1a or gHMGB1b and then assayed the neurite length. Statistical analysis displayed no significant differences between control and treatment groups. Also, the paralogs did not mediate neuronal apoptosis, which could be induced by endogenous overexpression (Fig. 8, A and B). Daston and Ratner (65) have ever described that HMGB1 was the early marker of oligodendrocytes in the developing rat spinal cord, and Fages et al. (57) found that HMGB1 was involved in the migration of neuroblasto
toma cells. Given such defined functions, we therefore assayed the migration of oligodendrocyte GSN3 cells cocultured with 1/2 H9262 g/ml exogenous recombinant gHMGB1a or gHMGB1b. Transwell experiments demonstrated that both recombinant gHMGB1a and gHMGB1b proteins were able to potentiate the cell migration (Fig. 8, C–E and H), and anti-gHMGB1a or anti-gHMGB1b antibody (1:50 dilution) was able to block the effect, accordingly (Fig. 8, F–H).

RAGE engagement by HMGB1 ligand activates various signaling pathways in a cell-specific manner, eventually impinging on canonical NFκB and (or) CREB with consequent regulation of gene expression.
of migration. RAGE signaling-dependent regulation of cell motility also occurs independently of its effects on gene transcription via a Dia-1/Src/Ras/PI3K/RhoA/ROCK or a Dia-1/Rac1/Cdc42 pathway (66). To unveil the migration-associated signaling(s) activated by gHMGB1a/RAGE or gHMGB1b/RAGE interaction, oligodendrocyte Gsn3 cells were cocultured with 1 μg/ml exogenous recombinant gHMGB1a or gHMGB1b. Western blots revealed that oligodendrocytes expressed the RAGE receptor (Fig. 9A), rather than TLR2 and TLR4 receptors (data not shown). Transcriptional factors NFκB and its upstream regulators p-ERK1/2 have been shown to be activated, whereas CREB and other pathway molecules p-p38 and p-JNK have not (Fig. 9A). Rac1 and Cdc42, but not RhoA, were significantly activated to mediate oligodendrocyte migration (Fig. 9B). These results demonstrated that gHMGB1a and gHMGB1b could potentiate the oligodendrocyte migration by activation of both NFκB and Rac1/Cdc42 signaling, and gHMGB1b, the up-regulated protein in spontaneously regenerating spinal cord, was the critical mediator that was possibly implicated in the promoting spinal cord regeneration.

**DISCUSSION**

Many conflicting lines of evidence have resulted in confusion and considerable debate regarding the detrimental versus beneficial role of inflammation, particularly after CNS injury (67). On the one hand, acute inflammatory response after spinal cord injury has been well documented to mediate the progressive tissue damage (68, 69); on the other hand, some observations indicate that inflammation can also be beneficial for injured CNS tissue by promoting clearance of cell debris and secretion of neurotrophic factors (70, 71). Nevertheless, it is generally acceptable that uncontrolled inflammation drives the progression of the CNS tissue damages, whereas HMGB1 protein, a self-derived immune activator, facilitates the vicious self-propagating inflammatory circle contributing to the pathophysiology (4, 72). In the condition of uncontrolled inflammatory reaction, neutrophils, monocytes/macrophages, and lymphocytes were rapidly recruited and activated, contributing to the expansion of the lesion size and series of pathological changes by releasing a spectrum of inflammatory cytokines or generation of free radicals such as NO (69, 73–76). Extracellular HMGB1 acts as ligand and sensor for innate immunity to promote recruitments of inflammatory cells and to elicit release of inflammatory cytokines, thus initiating a vicious circle between injured neurons and uncontrolled inflammation (13, 72, 77). In traumatic spinal cord of rat, HMGB1 was significantly up-regulated from 6 h, acting on the roles of the release of TNF-α, IL-1β, and IL-6 (15, 18). Monocytes cultured with HMGB1 release TNF-α, IL-1, IL-6, IL-8, and macrophage inflammatory protein-1 but not anti-inflammatory cytokines, such as IL-10 and IL-12 (23, 25). As such, HMGB1 has become a "necrotic marker" participating in the proinflammatory cascade of sec-
ondary damage after spinal cord injury (18, 42). In contrast to the failures of adult spinal cord regeneration in chicken and mammals, several vertebrates, including fish, amphibians, and reptiles, are capable of spontaneous regeneration following cord transection or tail amputation (38, 78). Although changes in regenerative ability resulted from combinations of several factors, such as multipotent neural progenitors, extraneuronal milieu, and cellular and molecular changes acquired during

FIGURE 8. Effects of gHMGB1 paralog recombinant proteins on the neurons and oligodendrocytes. A, Western blot analysis of cleaved caspase3 in the SH-SY5Y cells cultured with 1 μg/ml exogenous recombinant gHMGB1a, gHMGB1b, or mutant proteins. B, immunofluorescence of SH-SY5Y cells detected by NF-200 antibody, and analysis of the neurite length. C–G, assays of oligodendrocytes Gsn3 migration by transwell experiments after cells cultured with PBS, pH 7.4 (C), recombinant gHMGB1a (D), or gHMGB1b proteins (E) for 36 h; anti-gHMGB1a antibody (F) and anti-gHMGB1b antibody (G) were able to accordingly block the effects. H, statistical analysis of migrated cell numbers in C–G. Error bars in H represent the standard deviation (*, p < 0.01). Scale bars, 25 μm in B and 50 μm in C–G.

FIGURE 9. Examination of HMGB1/RAGE signal pathway associated with oligodendrocyte migration. A, Western blot analysis of RAGE, p65NFkB, CREB, p-CREB, JNK, p-JNK, p-p38, ERK1/2, and p-ERK1/2 implicated in the pathway activation. B, determination of Rac1/Cdc42/RhoA in signaling activation. Error bars in A and B represent the standard deviation (*, p < 0.01). Con, control.
evolution, any valuable clues derived from the comparative studies might be beneficial for potential regeneration in mammals. Interestingly, injured spinal cords in regeneration-competent lower vertebrates do not lead to secondary tissue damage, which often occurs in chicken and mammals. HMGB1 has not been shown to be involved in the pathophysiological events, suggesting that there is close association between tightly regulated inflammatory responses by gHMGB1 paralogs and successful spinal cord regeneration.

Two paralogs of intracellular gHMGB1 were able to trigger the apoptosis of neurons, and how these intracellular proteins activated the apoptotic signaling has not been determined. Bioinformatic analysis has even revealed that overexpressed HMGB1 can influence cell fate, such as apoptosis or proliferation, through regulation of cell cycle factors, including c-Myc, p53, pRb, Ras, etc. (79). The possibility of intracellular gHMGB1-mediated neuronal apoptosis may be caused by aberrant expression of these cell cycle factors. The apoptosis of neurons, although much less than those of oligodendrocytes, has been assumed as an important process that affects the development of neuronal tissue damage following acute spinal cord injury (80–82). But it has been shown to be required for the successful regeneration of the amputated tail, including spinal cord (83). In Xenopus tadpole, inhibition of caspase-dependent apoptosis resulted in a failure to induce proliferation in the growth zone, a mispatterning of axons in the regenerate, and the appearance of ectopic otoliths in the neural tube (83). The requirement of apoptosis may be non-cell autonomous for some cells during regeneration (83, 84). Distinct with necrosis, cells undergoing programmed cell death induce negligible inflammation in the surrounding tissue, due to the retention of HMGB1 within the apoptotic cells (13, 85). In regenerating spinal cord of gecko, the neuronal apoptosis mediated by up-regulation of gHMGB1b following injury would therefore produce negligible inflammatory response. In addition, intracellular gHMGB1b in immune cells of spinal cord, as shown in cultured macrophage RAW 264.7 cells, triggered less release of inflammatory cytokines. Taken together, gHMGB1b elicits limited inflammatory responses in the injured spinal cord.

One of the pathological outcomes of acute and chronic SCI is demyelination of axons at traumatic site due to oligodendrocyte death through necrosis and/or apoptosis (82). Primary demyelination occurs within the 1st day, and the subsequent progression, which is independent of injury intensity, is attributed to the second injury (86). The poorly understood inflammatory mechanisms have contributed to the increased loss of myelin after SCI (86). It is defined that both gHMGB1 paralogs have not militated against integrity of myelin by eliciting enormous inflammatory cytokines compared with those of mammals. Instead, they have been shown to potentiate migration of oligodendrocytes, a prerequisite for normal myelination in the vertebrate CNS. The cellular events might be involved in the replenishment of necrotic and/or apoptotic oligodendrocytes suffering from direct insults or ensheathment of regenerating axons.

HMGB1 could interact with multiple cell surface receptors, including RAGE, TLR2, TLR4, TLR9, and Mac-1, etc. (6, 29, 87). Among them, RAGE, TLR2, and TLR4 pathway signalings
have been strongly implicated in the pathogenesis of sterile injury by activation of NFκB (29, 33, 34). HMGB1/TLR4 signaling is mostly required for the activation of inflammatory cytokine release in macrophages, whereas HMGB1/TLR2 and HMGB1/RAGE are dispensable. Exposure of macrophages to HMGB1, for example, was able to induce TNF production by macrophages derived from TLR2 knock-out and RAGE knock-out in quantities comparable with wild-type macrophages, whereas TNF release was significantly inhibited in macrophages from TLR4 knock-out mice (34). Similar results could be observed for other HMGB1-induced cytokine mediators in these macrophages, demonstrating that the TLR4 knock-out macrophages also failed to produce MIP-2, IL-6, IL-1β, and IL-10, although these were produced normally by TLR2 and RAGE knock-out macrophages (34). Furthermore, HMGB1 activation of macrophages from double knock-out of TLR2 and RAGE led to significant stimulation of TNF release, but double knock-out of TLR4 and RAGE completely prevented TNF release in response to HMGB1 (34). So, it is understandable that two paralogs of gHMGB1 failed to initiate the release of inflammatory cytokines in macrophages due to undetectable interaction with TLR2 and TLR4. RAGE has been shown to be expressed on neurons, endothelial macrophages, oligodendrocytes, and immature dendritic cells (64, 88–92). RAGE signaling requires adaptor proteins, such as diaphanous-1, TIRAP, and MyD88. The structural properties of ligand, the cell type, and the number of RAGE molecules expressed on the cell surface may condition RAGE signaling (66). HMGB1/RAGE signaling has been documented to contribute to the outgrowth of the neurites (64, 88) and cell migration (57, 89). In this study, extracellular gHMGB1a and gHMGB1b failed to promote the neurite outgrowth, but it potentiated oligodendrocyte migration in a manner of activation of both NFκB and Rac1/Cdc42 signaling, suggesting a cell-specific HMGB1/RAGE activation mechanism.

Genome duplication is an evolutionary innovation to create functionally diverse proteins from a limited number of genes. These duplicated genes were assigned to execute specific functions to meet various physiological requirements, except those suffered from degeneration (37, 93). In the meantime, animals have evolved alternative switches to regulate these paralogs, and such cases were found in the regulation of Globin gene superfamily and other proteins (43, 94, 95). Both paralogs of gHMGB1 were found to be ubiquitously expressed and share similar binding patterns with cell surface receptors. However, traumatic spinal cord induces only gHMGB1b up-regulation, rather than gHMGB1a, which is induced in spleen and lungs by infectious signals and LPS, suggesting that established switch(es) might control spatial expression of the two paralogs. The exquisite regulatory strategy, by which gHMGB1b participates in spinal cord regeneration, could be readily explained by the need of repetitive repair of tail loss in complex natural environments.

In conclusion, as illustrated in Fig. 10, the spontaneously regenerating spinal cord switches on the up-regulation of gHMGB1b rather than gHMGB1a. The intracellular protein could not create a vicious circle of inflammation by negligible release of inflammatory cytokines in macrophages or by promoting neuronal apoptosis, nor could the extracellular protein activate inflammatory signaling by undetectable interaction with TRL2 and TLR4 receptors. Instead, gHMGB1b interacted with RAGE to facilitate oligodendrocytes migration by activation of both NFκB and Rac1/Cdc42 signaling. Such cellular events under tightly controlled inflammatory milieu might be implicated in the spinal cord regeneration.

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HMGB1 in Spinal Cord Regeneration

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