Delayed Treatment With Minocycline Ameliorates Neurologic Impairment Through Activated Microglia Expressing a High-Mobility Group Box1–Inhibiting Mechanism

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Background and Purpose—Minocycline, a semisynthetic tetracycline antibiotic, has been reported to ameliorate brain injury and inhibit microglial activation after focal cerebral ischemia. However, the cerebroprotective mechanism of minocycline remains unclear. In the present study, we investigated that mechanism of minocycline in a murine model of 4-hour middle cerebral artery (MCA) occlusion.

Methods—One day after 4-hour MCA occlusion, minocycline was administered intraperitoneally for 14 days. Neurologic scores were measured 1, 7, and 14 days after cerebral ischemia. Motor coordination was evaluated at 14 days by the rota-rod test at 10 rpm. Activated microglia and high-mobility group box1 (HMGB1), a cytokine-like mediator, were also evaluated by immunostaining and Western blotting. In addition, terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling immunostaining was carried out 14 days after cerebral ischemia.

Results—Repeated treatment with minocycline (1, 5, and 10 mg/kg) for 14 days improved neurologic score, motor coordination on the rota-rod test, and survival in a dose-dependent manner. Minocycline decreased the expression of Iba1, a marker of activated microglia, as assessed by both immunostaining and Western blotting. Moreover, minocycline decreased the activation of microglia expressing HMGB1 within the brain and also decreased both brain and plasma HMGB1 levels. Additionally, minocycline significantly decreased the number of terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling–positive cells and prevented ischemic brain atrophy 14 days after cerebral ischemia.

Conclusions—Our results suggest that minocycline inhibits activated microglia expressing HMGB1 and decreases neurologic impairment induced by cerebral ischemia. Minocycline will have a palliative action and open new therapeutic possibilities for treatment of postischemic injury via an HMGB1-inhibiting mechanism. (Stroke. 2008;39:951-958.)

Key Words: cerebral ischemia ■ high-mobility group box1 ■ minocycline ■ microglia ■ neuroprotection

Minocycline is a semisynthetic second-generation tetracycline with anti-inflammatory,1–3 glutamate antagonist,3,4 and antiapoptotic5–8 actions that have been demonstrated in many models of brain injury2,3,9–11 and that are completely separate and distinct from its antimicrobial action. These actions of minocycline, along with its superior human safety and blood-brain barrier penetration, make it an ideal candidate agent for clinical trials in stroke and other neurologic diseases.12 The neuroprotective effect of minocycline is thought to be associated with its ability to inhibit microglial activation induced by cerebral ischemia and inflammatory responses.

Microglial activation occurs very early in the injury process, and activated microglia exhibit morphological, immunologic, molecular, and functional changes in response to a given injury. In addition, microglial activation occurs in association with various types of brain injury,13 including damage associated with neurologic disease states.14,15 Although viewed as initiators of tissue repair and regeneration, microglia are predominantly known to initiate inflammation and induce apoptosis in the ischemic core owing to their release of and activation by cytotoxic products, such as reactive oxygen and nitrogen species, proinflammatory cytokines, and proteases.16–18 Several studies have attempted to use candidate drugs, including minocycline, to demonstrate that inhibition of microglial activation can afford protection against neuronal injury.19–21 Recently, high-mobility group box1 (HMGB1), a nonhistone DNA-binding protein, has been reported to be massively
released into the extracellular space immediately after an ischemic insult and to induce neuroinflammation and microglial activation in the postischemic brain. Moreover, anti-HMGB1 antibody–treated animals have demonstrated inhibition of inflammation and of microglial activation induced by cerebral ischemia. HMGB1 is widely expressed in various tissues, including the brain. As a chromosomal protein, HMGB1 has been implicated in diverse intracellular functions, including the stabilization of nucleosomal structure and the facilitation of gene transcription. HMGB1 is also actively secreted by macrophages and monocytes or released by necrotic cells into the extracellular milieu, induces expression of several genes related to inflammation, and leads to apoptosis. Furthermore, recent evidence has identified HMGB1 as a cytokine-like mediator of delayed endotoxin lethality. In the brain, HMGB1 has recently been reported to be released after cytokine stimulation and to be involved in the inflammatory process after it was administered intracerebroventricularly. Therefore, it is important to inhibit the released HMGB1 during treatment of postischemic injury.

In this study, we found that activated microglia expressing HMGB1 were increased in the striatum and ischemic core after cerebral ischemia and that HMGB1 was massively released during the cerebral ischemia-induced injury process. We studied whether minocycline could ameliorate ischemic injury as assessed by neurologic impairment scores, the numbers of terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL)–positive cells, and ischemic brain atrophy induced by middle cerebral artery (MCA) occlusion. In addition, we investigated whether delayed treatment with minocycline could decrease activated microglia expressing HMGB1 and the expression level of HMGB1 in the brain and plasma.

Materials and Methods

Animals

Male ddY mice (25–35 g; Kiwa Experimental Animal Laboratory, Wakayama, Japan) were kept under a 12-hour light/dark cycle (lights on from 7 AM to 7 PM) in an air-conditioned (23°C) room with food (CE-2; Clea Japan, Tokyo, Japan) and water available ad libitum. All procedures regarding animal care and use were performed in compliance with the regulations established by the experimental animal care and use committee of Fukuoka University.

Focal Cerebral Ischemia

Focal cerebral ischemia was induced according to the method described in our previous study. Mice were anesthetized with 2% halothane and maintained thereafter with 1% halothane (Flossen; Takeda Chemical Industries, Osaka, Japan). After a midline neck incision, the left common and external carotid arteries were isolated and ligated. A nylon monofilament (8-0 Ethilon; Johnson & Johnson, Tokyo, Japan) coated with silicone resin (Xantopren; Heleus Dental Material, Osaka, Japan) was introduced through a small incision into the common carotid artery and advanced to a position 9 mm distal from the carotid bifurcation for occlusion of the MCA. After vessel occlusion, the mice were reanesthetized with halothane, and reperfusion was established by withdrawal of the filament.

Triphenyltetrazolium Chloride Staining

Twenty-four hours after MCA occlusion, the animals were humanely killed by decapitation. The brains were removed and sectioned coronally into four 2-mm slices according to a mouse brain matrix. Slices were immediately stained with 2% 2,3,5-triphenyltetrazolium chloride (Sigma, St. Louis, Mo). The border between the infarcted and noninfarcted tissue was outlined with use of an image analysis system (NIH Image, version 1.63): the area of infarction was measured and the infarction volume was calculated.

Neurologic Score

Neurologic score was divided into 5 groups: 0 = normal motor function, 1 = flexion of the torso and of the contralateral forelimb on lifting of the animal by the tail, 2 = circling to the ipsilateral side but normal posture at rest, 3 = circling to the ipsilateral side, 4 = rolling to the ipsilateral side, and 5 = leaning to the ipsilateral side at rest (no spontaneous motor activity). Neurologic score was measured 4 hours and 1, 7, and 14 days after cerebral ischemia.

Rota-Rod Test in MCA-Occluded Mice

Motor coordination was measured by the rota-rod test as described previously. Mice were placed on a 3-cm-diameter rotating rod (Neuroscience Inc, Tokyo, Japan) with a nonskid surface, and the latency to fall was measured for up to 2 minutes. The rotating speed was 10 rpm.

Analysis of Ischemic Brain Atrophy

Mice (n = 3 in each group) were humanely killed by decapitation after perfusion with saline and 4% paraformaldehyde at 1, 7, 14, and 28 days after MCA occlusion. To quantify the degree of brain atrophy, we compared the ipsilateral hemisphere with the contralateral side in the striatum in each sample. Atrophy (%) was calculated as ipsilateral area/contralateral area × 100.

Microglia Immunohistochemistry

Mice (n = 3 in each group) were humanely killed by decapitation after perfusion with saline and 4% paraformaldehyde at 1, 3, 7, 14, and 28 days after MCA occlusion. The brains were removed of fat and water with use of an autodigesting unit (RH-12; Sakura Seiko Co, Tokyo, Japan) and then embedded in paraffin. Subsequently, 5-μm-thick sections were mounted on slides and dried at 37°C for 1 day. Sections (5 μm thick) were processed for immunohistochemistry with the Dako LSAB kit (Dako Inc, Carpinteria, Calif). After deparaffinization and rehydration, these sections were rinsed twice for 1 minute with phosphate-buffered saline (pH 7.4). After treatment with 3% H2O2, nonspecific binding was blocked with phosphate-buffered saline containing a carrier protein and 15 mmol/L NaNO3 for 5 minutes at room temperature. The sections were incubated in a 1:200 dilution of rabbit polyclonal anti-Iba1 (a macrophage/microglia-specific protein) primary antibody (Wako Pure Chemical Industries Ltd, Osaka, Japan) overnight at 4°C. The sections were then incubated with biotinylated anti-rabbit secondary antibody for 10 minutes at room temperature. After being rinsed twice for 1 minute with phosphate-buffered saline, the sections were incubated in streptavidin solution for 10 minutes at room temperature. Finally, they were treated with stable 3,3-diaminobenzidine tetrahydrochloride as a peroxidase substrate.

Microglia/HMGB1 Double Staining

After deparaffinization and rehydration, the sections were incubated in a 1:200 dilution of rabbit polyclonal anti-Iba1 primary antibody and a 1:200 dilution of goat polyclonal anti-HMGB1 primary antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) overnight at 4°C. The sections were then incubated with a 1:200 dilution of donkey anti-goat IgG–fluorescein isothiocyanate secondary antibody (Santa Cruz Biotechnology) for 1 hour. Afterward, the sections were
also incubated with a 1:200 dilution of goat anti-rabbit IgG–fluorescein isothiocyanate secondary antibody (Santa Cruz Biotechnology) for 1 hour. The coverslips were mounted, and then the slides were analyzed by fluorescence microscopy.

TUNEL Staining
After deparaffinization and rehydration, the sections were assayed for TUNEL by direct binding of fluorescein-conjugated dUTP (green fluorochrome) with anti-mouse NeuN (Chemicon International, Temecula, Calif) providing the red counterstain and with the fluorescein isothiocyanate–apoptosis detection system (Promega, Tokyo, Japan). The coverslips were mounted, and then the slides were analyzed by fluorescence microscopy.

Immunoblotting
The expression of Iba1 and HMGB1 protein was evaluated by Western blotting after sample extraction and sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis. One hour after each treatment, tissue samples (striatum) were homogenized at 4°C for 1 minute in lysis buffer [20 mmol/L Tris (pH 7.4), 1 mmol/L EDTA, 1 mmol/L EGTA, and 0.1% Triton X-100] with a protease inhibitor cocktail. The tissue extract was centrifuged at 15 000 rpm at 4°C for 30 minutes. The supernatant was treated in the same way as the tissue extract.

SDS sample buffer [125 mmol/L Tris (pH 6.8), 2% SDS, 20% glycerol, 0.0001% bromophenol blue, and 10% β-mercaptoethanol] was added to aliquots of tissue extracts containing 15 µg total protein. Samples were heated at 95°C for 5 minutes. Protein (15 µg) was separated by SDS–polyacrylamide gel electrophoresis (20% gel). Blotting was performed at 2 mA/cm² by semidyne-type blotting (Bio-Rad, Tokyo, Japan). The blots were blocked with 5% nonfat dry milk in Tris-buffered saline in 0.1% Tween 20 (TBS-T) at 4°C and incubated with anti-Iba1 polyclonal antibodies (1:200) in TBS-T and anti-HMGB1 polyclonal antibodies (1:200) in TBS-T, followed by goat anti-rabbit IgG (H and L) alkaline phosphatase conjugate (1:1000) in TBS-T and bovine anti-goat IgG (heavy chain and light chain [H and L]) alkaline phosphatase conjugate (1:1000) in TBS-T. The blots were visualized with the use of alkaline phosphatase color reagents. The signal intensity of the blots was measured by an image analysis system (NIH Image, version 1.63).

HMGB1 Measurements
Plasma samples were fractionated by SDS–polyacrylamide gel electrophoresis, and HMGB1 levels were determined by immunoblotting with respect to a standard curve, with recombinant HMGB1 as a reference (Sigma-Aldrich, Tokyo, Japan).

Drug Preparation and Administration
Minocycline hydrochloride (Sigma-Aldrich) was dissolved in distilled water, and 1 day after 4-hour MCA occlusion, minocycline (1, 5, and 10 mg/kg IP) was administrated daily for 14 days.

Statistical Analysis
Results are expressed as mean±SEM. Student’s t test was used to evaluate differences between 2 groups. Multiple comparisons were...
evaluated by Tukey’s test after 1-way ANOVA. P<0.05 was considered statistically significant.

**Results**

**Effect of Minocycline on Neurologic Impairment in MCA-Occluded Mice**

MCA-occluded mice had significantly impaired neurologic function. Infarct volume in the vehicle- and minocycline-treated groups was measured 24 hours after cerebral ischemia by triphenyltetrazolium chloride staining (vehicle-treated group, 87.8±4.9 mm³; minocycline-treated group, 91.6±7.3 mm³). No differences in the level of cerebralischemia were found between these 2 groups. Repeated treatment with minocycline for 14 days improved the neurologic score in a dose-dependent manner. Minocycline (5 and 10 mg/kg) significantly improved the score 14 days after cerebral ischemia, compared with the vehicle-treated group (F3,19=8.147, P<0.01; 5 mg/kg minocycline, P<0.05; 10 mg/kg minocycline, P<0.01 compared with vehicle, Figure 1A).

**Effect of Minocycline on Motor Coordination in MCA-Occluded Mice**

MCA-occluded mice had significantly impaired motor coordination. Repeated treatment with minocycline for 14 days improved motor coordination on the rota-rod test in a dose-dependent manner. Minocycline (5 and 10 mg/kg) significantly improved the score 14 days after cerebral ischemia compared with the vehicle-treated group (F4,23=14.715, P<0.01; vehicle, P<0.01 compared with sham; 10 mg/kg minocycline, P<0.01 compared with vehicle; Figure 1B).

**Effect of Minocycline on Survival After MCA Occlusion**

Repeated treatment with minocycline (5 and 10 mg/kg) for 14 days improved survival after cerebral ischemia in a dose-dependent manner (Figure 1C).

**Effect of Minocycline on Microglial Activation Induced by Cerebral Ischemia**

Repeated treatment with minocycline (10 mg/kg) for 14 days decreased expression of Iba1, a marker of activated microglia, in the striatum by both immunostaining (A) (scale bar=500 μm) and Western blotting (B). Values are expressed as mean±SEM. *P<0.05 vs sham-operated group; †P<0.05 vs 14-day vehicle-treated group (n=3; Tukey’s test). GAPDH indicates glyceraldehyde 3-phosphate dehydrogenase.

**Microglia Expressing HMGB1**

In MCA-occluded animals without minocycline treatment, microglia expressing HMGB1 increased in the striatum during days 1 to 14, but not at 28 days, after cerebral ischemia (Figure 3).

**Expression of TUNEL-Positive Cells and Analysis of Ischemic Brain Atrophy**

In MCA-occluded animals without minocycline treatment, a significant increase in the number of TUNEL-positive cells was observed in the striatum during days 7 to 14, but not at 28 days, after cerebral ischemia. One day after MCA occlusion, the ischemic hemisphere tended to be larger on the ipsilateral than the contralateral side. However, the ipsilateral side progressively decreased in size compared with the contralateral side during 7 to 14 days after cerebral ischemia (F4,20=18.527, P<0.01; 14 days, P<0.01 compared with sham; 28 days, P<0.01 compared with sham; Figure 4).

**Effect of Minocycline on HMGB1 Expression in the Brain and Plasma**

The HMGB1 expression level was significantly increased in the striatum 14 days after cerebral ischemia (F3,8=14.999, P<0.01; 14 days, P<0.05 compared with sham; Figure 5A). Similarly, plasma levels of HMGB1 also increased 1 to 14 days after cerebral ischemia and were greater at 7 days...
compared with day 1 ($F_{1,3} = 41.067$, $P < 0.01$; 1 day, $P < 0.01$; 7 days, $P < 0.01$; 14 days, $P < 0.01$ compared with sham; 7 days, $P < 0.05$ compared with 1 day; Figure 5B). Repeated treatment with minocycline (10 mg/kg) significantly decreased HMGB1 expression in both the striatum and plasma (striatum: 14 days, 10 mg/kg minocycline, $P < 0.05$ compared with vehicle; Figure 5A; plasma: 7 days, 10 mg/kg, minocycline, $P < 0.05$; 14 days, 10 mg/kg minocycline, $P < 0.05$ compared with vehicle; Figure 5B).

**Effect of Minocycline on Brain Injury Induced by Cerebral Ischemia**

Repeated treatment with minocycline (10 mg/kg) significantly decreased the numbers of TUNEL-positive cells and activated microglia expressing HMGB1 14 days after cerebral ischemia (Figure 6A). In addition, minocycline significantly improved atrophy of the striatum 14 days after cerebral ischemia (10 mg/kg minocycline, $P < 0.01$ compared with vehicle; Figure 6C).
Discussion

Repeated treatment with minocycline for 14 days improved the neurologic score, motor coordination on the rota-rod test, and survival in a dose-dependent manner. Minocycline decreased the expression of activated microglia in the striatum as assessed by immunostaining and significantly decreased Iba1 protein levels compared with the vehicle-treated group as assessed by Western blotting 14 days after cerebral ischemia. Moreover, minocycline decreased the numbers of activated microglia expressing HMGB1 and HMGB1 levels in both the striatum and plasma. In addition, minocycline significantly decreased the numbers of TUNEL-positive cells and inhibited ischemic brain atrophy 14 days after cerebral ischemia. These results suggest that minocycline inhibits activated microglia expressing HMGB1 and shows a cerebroprotective effect.

Minocycline confers neuroprotection in a number of experimental models of brain injury, including cerebral focal and global ischemia. Low doses of minocycline have been reported to inhibit caspase-1 and inducible nitric oxide synthetase and to decrease infarct size. However, treatment with minocycline 1 mg/kg IP did not prevent infarction

Figure 5. Effect of minocycline on HMGB1 in the brain and plasma. The HMGB1 expression level significantly increased in the striatum 14 days after cerebral ischemia. Similarly, the plasma level of HMGB1 also increased 14 days after cerebral ischemia. Repeated treatment with minocycline (10 mg/kg) significantly decreased HMGB1 expression in both the striatum and plasma. S indicates sham-operated group. Values are expressed as mean±SEM. *P<0.05, **P<0.01 vs sham-operated group; †P<0.05 vs 1 day after MCA occlusion; ††P<0.05 vs vehicle-treated group (n=3; Tukey’s test).

Figure 6. Effect of minocycline on brain injury induced by cerebral ischemia. Repeated treatment with minocycline (10 mg/kg) significantly decreased the numbers of TUNEL-positive cells and activated microglia expressing HMGB1 14 days after cerebral ischemia. Neun-positive cells (red), TUNEL-positive cells (green), and merged image (yellow) are shown (n=3). Scale bar=20 μm (A). In addition, minocycline significantly improved atrophy of the striatum 14 days after cerebral ischemia (n=5; B and C). Values are expressed as mean±SEM. ††P<0.01 vs vehicle-treated group (Student’s t test).
24 hours after MCA occlusion in our study. However, 10 mg/kg minocycline significantly prevented cerebral infarction caused by MCA occlusion, which suggests that this dose was effective in the brain, so we chose that dose for long-term treatment.

In the present study, we found that the number of activated microglia expressing HMGB1 increased in the striatum and massive release of HMGB1 occurred during the cerebral ischemia-induced injury process. Activated microglia expressing HMGB1 gradually increased in the striatum during 1 to 14 days, but not at 28 days, after cerebral ischemia. Similarly, the number of TUNEL-positive cells significantly increased in the striatum, and atrophy of the striatum was also observed at 14 days. Activated microglia expressing HMGB1 cause a potent inflammatory response and chronic neurodegeneration after cerebral ischemia, which suggests that microglial HMGB1 may be a parameter of activated microglia.

HMGB1 is usually released either passively by necrotic or damaged cells or actively by macrophages/monocytes in the early phase of ischemia, and then extracellularly released HMGB1 triggers microglia activation. HMGB1 connects the acute excitotoxicity-induced neuronal death to delayed injury processes, such as inflammation, in the postischemic brain. In this study, although elevated HMGB1 protein levels were not observed on day 1 (Figure 5A), they progressively increased in the striatum in comparison with those in the sham-operated group 7 to 14 days after cerebral ischemia; such increased values may have been derived from activated microglia. In addition, HMGB1 secreted into the blood reached a maximum value at 7 days but not in the ischemic early phase. Our previous studies and other reports have shown that macrophages/monocytes and necrotic cells increase for 1 to 3 days in the ischemic early phase. After that time, apoptotic cells gradually increase in the ischemic region, but apoptotic cells do not release significant quantities of HMGB1. Taken together, these results suggest that activated microglia themselves not only express HMGB1 but also might contribute to the development of tissue injury by releasing HMGB1 into the extracellular milieu. However, this hypothesis is tentative, and further study will be required to confirm or refute this scenario.

Minocycline has been shown to be neuroprotective in a variety of animal models of both chronic neurodegeneration and acute central nervous system injury. In addition, minocycline has also been known to inhibit inductive nitric oxide synthetase upregulation and to delay mortality in a transgenic mouse model of Huntington disease. Delayed treatment with minocycline significantly prevented an increase in the number of activated microglia expressing HMGB1 and ameliorated neurologic impairment, tissue injury, and survival 14 days after MCA occlusion. In addition, minocycline decreased HMGB1 protein levels in the striatum at 14 days and in the plasma at 7 to 14 days after cerebral ischemia. We propose that activated microglia expressing HMGB1 induce neurologic impairment and tissue injury and aggravate mortality in cerebral ischemic conditions and that minocycline moderates neurologic impairment by activated microglia via an HMGB1-inhibiting mechanism.

Conclusions
Repeated treatment with minocycline (10 mg/kg) for 14 days is effective in reducing the number of activated microglia expressing HMGB1, which leads to decreased neurologic impairment and tissue injury and increased survival under cerebral ischemic conditions in mice. Minocycline appears to have a palliative action and opens new therapeutic possibilities for treatment of postischemic injury via inhibition of activated microglia expressing HMGB1.

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Disclosures
None.

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