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Scavenger receptor SRA attenuates TLR4-induced microglia activation in intracerebral hemorrhage

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ABSTRACT

Scavenger receptor A (SRA) has been shown to participate in the pattern recognition of pathogen infection. However, its role in intracerebral hemorrhage has not been well defined. In this study, we detected SRA and TLR4 expression and inflammatory response of microglia treated with erythrocyte lysate in vitro, and observed the cerebral water content and neurological deficit of ICH mice in vivo. We found that SRA deficiency leads to greater sensitivity to erythrocyte lysate-induced inflammatory response. SRA down-regulated inflammatory response expression in microglia by suppressing TLR4-induced activation. Collectively, we have identified the molecular linkage between SRA and the TLR4 signaling pathways in ICH. And our results reveal that SRA has important clinical implications for TLR-targeted immunotherapeutical strategy in ICH.

1. Introduction

Intracerebral hemorrhage (ICH) is one of the most devastating stroke subtypes with grave prognosis and has the highest mortality (Paciaroni and Agnelli, 2014; Specogna et al., 2014; Lei et al., 2013). Despite outstanding progress in ischemic stroke treatment technology in the past ten years, little progress has been achieved in ICH treatment (Trapani and Retta, 2015; Calnan et al., 2013; Howlett et al., 2013). Therefore, development of novel treatment strategies of ICH is warranted for successful clinical application.

Multiple studies have implicated this immune response in the pathogenesis of secondary injury after ICH (Lu et al., 2014; Xu et al., 2013; Ma et al., 2014). Toll-like receptors (TLRs) recognize pathogen and damage-associated molecular patterns and have a key role in innate immunity (Beutler, 2009; Leavy, 2012; Lavelle et al., 2010). In ICH, TLR4 has recently shown to be upregulated in a rat model, and TLR4 signaling indicates a target for therapeutic intervention (Sansing et al., 2011; Kokkinopoulos et al., 2005; Uronen-Hansson et al., 2004).

SRA is a prototypic member of a family of structurally diverse transmembrane receptors collectively termed as scavenger receptors (Yi et al., 2011). Several studies have shown that SRA deficiency resulted in impaired protection against pathogen infection (Yi et al., 2009; Greene et al., 2007; Hollifield et al., 2007). Emerging evidence also implicates SRA as a suppressor in an inflammatory response (Drummond et al., 2013; Yu et al., 2011; Ozment et al., 2012). However, the functional significance of SRA in ICH triggered by TLR4 signaling has not been identified. In this study, we explored the contribution of SRA to immune responses augmented by TLR4 in ICH.

2. Materials and methods

2.1. Mice

C57BL/6 mice (male, 8–10 weeks, 20–24 g) were obtained from the Animal Center of the FujianMedical University (Fuzhou, China). SRA knockout mice (SRA −/−, 8–10 weeks, 20–24 g) were obtained from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in individual cages with free access to sterile acidified water and irradiated food in a specific pathogen-free facility at the FujianMedical University. Experiments were conducted in accordance with animal care guidelines approved by the Animal Ethics Committee of the Fujian Medical University.

2.2. Primary cell cultures

Cortical neuronal cultures were prepared from whole cerebral cortices of C57BL/6 mouse embryos (E16) (both wild type and SRA −/− mice). After removal of the meninges, the tissue was digested by 0.005% trypsin/0.002% EDTA (10 min, 37 °C), mechanically dissociated, and centrifuged at 1000 × *g* for 5 min. The cell pellet was resuspended in neurobasal medium (Gibco) containing B27 serum free supplement (Gibco) and 500 Nm L-glutamine. 2×10^5 cells/well were seeded on sterile poly-L-lysine (Sigma-Aldrich) coated glass cover slips in a 24-well

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plate and incubated at 37 °C and 5% CO_2. After 1 h, a culture medium was changed completely. Purity of neuronal cultures was >95% as confirmed by random staining with neuronal and glia markers. 5 days after plating, neurons had developed a dense network of extensions. For primary microglial cells, cerebral hemispheres of 1-day old postnatal mice were digested with 0.1% trypsin. The cells were seeded into a six-well plate coated with poly-L-lysine and fed with Dulbecco's Modified Eagle Media (DMEM; Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Hyclone, Logan, UT, USA). Culture media were refreshed twice per week for 2 weeks. Microglia were detached by gentle shaking and filtered through a nylon mesh to remove astrocytes.

After centrifugation at 1000 × *g* for 10 min, the cells were resuspended in a fresh DMEM supplemented with 10% FBS and plated at a final density of 5×10^5 cells/mL on a poly-L-lysine coated 6-well culture plate. The following day, cells were subjected to the experiments. The cell purity was determined by immunohistochemical staining using a microglia specific antibody CD11b. The microglia cultures used were >95% pure.

2.3. Cell treatment

Microglia (1×10^5) was stimulated with 10 μL erythrocyte lysate or 10 μL BSA (bovine serum albumin). After 3 days, the supernatants were removed and further analyzed for cytokine production with ELISA. Neuron was cultured in a 96-well plate with 1×10^4 cells per well. For the toxicity experiments, neuron was serum starved for 4 h and then treated with a mixture of microglia conditioned medium. For MTT assay, cells were treated for 48 h.

2.4. MTT assay

Cell viability of neuron was assessed using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2 H-tetrazolium bromide (MTT, Sigma-Aldrich) assay. After 48 h, a MTT reagent was added to the wells, incubated for 4 h at 37 °C, 5% CO_2. After centrifugation, the supernatant was removed from each well. The colored formazan crystal produced from MTT was dissolved with 0.15 mL DMSO, then the optical density (OD) value A490 was measured by the multiscanner autoreader (Dynatech MR 5000; Dynatech Laboratories, Chantilly, VA, USA). The absorbance was measured at 570 nm. The mean of readings of triplicate wells was taken as one value. The OD value for the control cultures was considered as 100% viability and viability in other samples is expressed as a percentage of viability in the control cultures.

2.5. Western blot

Cells were washed with ice-cold PBS and then lysed in Triton X-100/glycerol buffer (50 mM Tris–HCl, 4 mM EDTA, 2 mM EGTA, 1 mM dithiothreitol, and 25 wt./vol.% sucrose, pH 8.0, supplemented with 1% Triton X-100 and protease inhibitor). After centrifugation at 5000 *g* for 15 min at 4 °C, the protein concentration was measured with a BCA protein assay kit (Pierce, 23227). Lysates were separated using SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline, pH 7.4, containing 0.05% Tween 20 (Sigma, P1379), and were incubated with primary anti-mouse antibodies and horseradish peroxidase-conjugated secondary anti-mouse antibodies (Jackson Immunoresearch Laboratories, 115-035-003) or anti-rabbit antibodies (Jackson Immunoresearch Laboratories, 111-035-003) according to the manufacturer's instructions. The protein of interest was visualized using Supersignal® West Dura Duration substrate reagent (Thermo, 34080).

2.6. ELISA

The production of TNF-α, IL-1β and IL-6 in the culture supernatants was measured by ELISA as specified by the manufacturer (R&D systems). Microglia plated in 24-well plates were cultured with anaerobic for 6 h. The culture supernatants were aspirated and stored at −70 °C until assayed by ELISA. The concentrations of TNF-α, IL-1β and IL-6 were determined using a standard curve obtained with TNF-α, IL-1β and IL-6 proteins.

2.7. ICH model

Briefly, mice were anesthetized with an intraperitoneal injection of 400 mg/kg chloral hydrate and fixed on a mouse stereotaxic frame (Stoelting). A 20-μL volume of autologous non-anticoagulated blood was collected from the tail vein of the mouse and then injected into the caudate nucleus at 2 μL/min under stereotactic guidance at the following coordinates relative to bregma: 0.8 mm anterior, 2 mm left lateral, and 3.5 mm deep during a period of 10 min. The needle was held in place for 10 min after injection, and the microsyringe was pulled out after the blood had coagulated. The craniotomy was then sealed with bone wax, and the scalp was closed with sutures. Body temperature was maintained at 37 °C throughout the procedure, and the mice were given free access to food and water after they woke up. The mice that died because of anesthesia were excluded.

2.8. Administration of Ad-TLR4 RNAi or Ad-scramble RNAi

To study the effects of TLR4, mice received an intracerebral ventricular injection of Ad-TLR4 RNAi or Ad-scramble RNAi (2 μL of a 10 mg/mL solution prepared in 0.9% NaCl) 10 min after ICH and sacrificed 48 h after ICH.

2.9. Histochemical evaluation of microglia

Three days after ICH, the animals were deeply anesthetized with pentobarbital and transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). After the mice were perfused and fixed, the perihematomal region of cerebral tissues were collected, fixed in 4% paraformaldehyde for 24 h, dehydrated in 30% sucrose solution for 48 h, embedded, frozen, and cut into 25-μm sections using a Leica CM1900 cryostat. The perihematomal region was treated with 3% H_2O_2 in 0.01 M phosphate-buffered saline (PBS) and preincubated in 5% normal goat serum. The samples were then incubated in a primary antibody solution containing rat anti-CD11b antibody (Serotec, Fullerton, CA, USA, 1:200) overnight at 4 °C. After washing, the samples were incubated in a secondary IgG antibody (1:200) for 1 h at room temperature (RT). Finally, the sections were incubated in horseradish peroxidase (HRP)–streptavidin (1:200) for 1 h at RT, and the color reaction was conventionally developed with diaminobenzidine (DAB) and H_2O_2.

2.10. Evaluation of neurological scores

The neurological scores were determined by Neurological Severity Scores, a composite of motor, sensory, reflex, and balance tests. Neurological function was graded on a scale of 1 to 18; a score of 1 point is awarded for the inability to perform the test or for the lack of a tested reflex. The higher the score, the more severe the injury (normal score: 2–3; maximal deficit score: 18).

2.11. Brain water content measurement

Brain water content was measured in mouse cerebral tissues after ICH. Briefly, mice were randomly sampled from each group and anesthetized by intraperitoneal injection with chloral hydrate ($n = 5$). Next, the cerebral tissues were removed, and the surface water on the cerebral tissues was blotted with filter paper. Brain samples were immediately weighed on an electric analytic balance to obtain the wet weight and then dried at 100 °C for 24 h to obtain the dry weight.