Please cite this article in press as: Fan H et al. Involvement of endoplasmic reticulum stress in the necroptosis of microglia/macrophages after spinal cord injury. Neuroscience (2015), http://dx.doi.org/10.1016/j.neuroscience.2015.10.049

Neuroscience xxx (2015) xxx–xxx

INVOLVEMENT OF ENDOPLASMIC RETICULUM STRESS IN THE NECROPTOSIS OF MICROGLIA/MACROPHAGES AFTER SPINAL CORD INJURY

H. FAN,[a] H.-B. TANG,[b] J. KANG,[a] L. SHAN,[c] H. SONG,[d] K. ZHU,[e] J. WANG,[a] G. JU[a]* AND Y.-Z. WANG[a]*

[a] *Department of Neurobiology and Collaborative Innovation Center for Brain Science, Institute of Neurosciences, Fourth Military Medical University, 169 Chang Le Xi Road, Xi'an 710032, China*

[b] *Department of Clinical Laboratory Medicine, Xijing Hospital, Fourth Military Medical University, 127 Chang Le Xi Road, Xi'an 710003, China*

[c] *Department of Orthopedics, Tangdu Hospital, Fourth Military Medical University, Xin Si Road, Xi'an, Shaanxi 710038, China*

[d] *Department of Occupational and Environmental Health, School of Public Health, Fourth Military Medical University, Xi'an 710032, China*

[e] *Zhejiang University China Brain Bank, Department of Pathology and Pathophysiology, Department of Neuroscience, 866 Yu-Hang-Tang Road, Zhejiang University Zi-Jin-Gang Campus, Hangzhou, Zhejiang 310058, China*

Abstract—Microglia/macrophages play a crucial role in inflammation after spinal cord injury (SCI). Although extensive studies have been performed on the mechanisms of microglia/macrophage activation and recruitment, how microglia/macrophages are eliminated remains unclear. In the present study, we observed a high-level expression of mixed lineage kinase domain-like protein (MLKL), a key molecule in the execution of necroptosis, in microglia/macrophages after SCI in mice. *In vivo* PI-labeling and Necrostatin-1 treatment confirmed the necroptosis of microglia/macrophages. Interestingly, our electronic microscopic (EM) study revealed that MLKL localized not only at the membrane but also on the endoplasmic reticulum (ER) of necroptotic microglia/macrophages. Furthermore, receptor-interacting protein 3 (RIP3), another necrosome component, was also found on the ER of necroptotic microglia/macrophages. And Glucose-regulated protein 78 (GRP78), an ER stress sensor, was up-regulated in MLKL-positive microglia/macrophages after SCI, suggesting a possible link between necroptosis and ER stress. *In vitro*, oxygen–glucose deprivation (OGD) stress induced ER stress and necroptosis in microglia. Inhibiting ER stress by 4-phenylbutyrate (4-PBA) significantly blocked the OGD-induced necroptosis of microglia. In the end, our data showed that, GRP78 and phosphorylated MLKL were co-expressed by the microglia/macrophages in the injured human spinal cord. Taken together, these results suggested that microglia/macrophages undergo an ER-stress involved necroptosis after SCI, implying that ER stress and necroptosis could be manipulated for modulating inflammation post-SCI.

Key words: spinal cord injury, microglia/macrophages, necroptosis, ER stress.

INTRODUCTION

Spinal contusion is the major type of spinal cord injury (SCI) in the clinic, which often results in permanent loss of motor and sensory functions. Although several clinical trials have been tested, it still lacks effective treatment. A unique pathological change following SCI is the secondary injury which is characterized by gradual enlargement of lesion area and the presence of chronic inflammation (Allison and Ditor, 2015). The spinal microglia, together with recruited macrophages which exhibited an almost identical phenotype with activated microglia, plays a key role in the initiation and development of inflammation (David and Kroner, 2011). After SCI, the function of microglia/-macrophages has been thought to be mainly destructive, possibly due to the dominance of their M1 sub-population (Hu et al., 2015). Many studies have been carried out to elucidate the mechanisms of their proliferation, migration and M1/M2 phenotype conversion, with the aim of biasing the post-SCI inflammation toward better repair (Mabon et al., 2000; Zai and Wrathall, 2005; Kroner et al., 2014). However, how microglia/macrophages are eliminated from the injured spinal cord remains unclear.

Necrosis and apoptosis are the two major types of cell death after SCI (Beattie et al., 2002). Extensive studies have revealed the cellular and molecular mechanisms of apoptosis after SCI (Yong et al., 1998). However, necrosis has long been thought to be uncontrollable, and the mechanism of necrosis after SCI remains largely unexplored. Recent studies have identified a type of programed necrosis (necroptosis) and uncovered its

*Corresponding authors. Tel: +86-29-84774565; fax: +86-29-83246270 (G. Ju). Tel: +86-29-84774562; fax: +86-29-83246270 (Y.Z. Wang).
E-mail addresses: jugong@fmmu.edu.cn (G. Ju), yazhouw@fmmu.edu.cn (Y.-Z. Wang).

Abbreviations: 4-PBA, 4-phenylbutyrate; CC3, cleaved Caspase-3; CXCR4, chemokine (C-X-C motif) receptor 4; EM, electronic microscopic; ER, endoplasmic reticulum; HMGB1, high-mobility group box protein 1; MLKL, mixed lineage kinase domain-like protein; Nec-1, Necrostatin-1; NGS, normal goat serum; OGD, oxygen–glucose deprivation; pMLKL, phosphorylated MLKL; RIP1, receptor-interacting protein 1; RIP3, receptor-interacting protein 3; SCI, spinal cord injury; EDTA, ethylenediaminetetraacetic acid; GRP78, Glucose-regulated protein 78; DMEM, Dulbecco's modified Eagle medium; PI, Propidium Iodide; PBS, phosphate-buffered saline.

http://dx.doi.org/10.1016/j.neuroscience.2015.10.049
0306-4522/

underlying mechanisms which involves an intracellular signaling cascade transduced by receptor-interacting protein 1/3 (RIP1/3) and mixed lineage kinase domain-like protein (MLKL) (Sun et al., 2012), thus offering new molecular tools for re-examining necrosis after SCI.

Endoplasmic reticulum stress (ER stress) is a cellular response to multiple injury conditions, which usually showed accumulation of unfolded proteins in the cytoplasm (Hoozemans and Scheper, 2012). Many studies have demonstrated that severe ER stress can activate the intracellular signaling that finally leads to apoptosis or autophagy (Gorman et al., 2012; Liu et al., 2015).Whether ER stress is also involved in the activation of necroptosis has been poorly studied.

In the present study, we investigated the necroptosis of microglia/macrophages after SCI, and the involvement of ER stress in the necroptosis of microglia/macrophages.

EXPERIMENTAL PROCEDURES

Human samples

Snap-frozen normal human spinal cord tissues were obtained from the human brain bank of the school of medicine at Zhejiang University. Biopsy of injured spinal cord tissues were performed with informed consent obtained from each patient prior to surgery and experiments involving human spinal tissues were approved by the Institutional Review Board of Tangdu Hospital, Fourth Military Medical University.

Spinal cord contusion and *in vivo* treatment

Male C57BL/6 mice were purchased from the Laboratory Animal Center of Fourth Military Medical University and all the protocols of animal experiments were approved by the Animal Care and Use Committee of the Fourth Military Medical University. Mice (6–8 w) were anesthetized with 1% sodium pentobarbital (80 mg/kg), followed by bilateral laminectomy of vertebrae T8–T9. Spinal cord lateral crushing was made at the T8 vertebra by using a forceps with a tip-gap set at 0.2 mm for 15 s. Manual bladder expression was performed once a day after surgery. Sham SCI was made by performing a dorsal laminectomy without crushing the spinal cord.

Necrostain-1 (Nec-1) administration

Nec-1 (7.8 mg/kg) was administrated (i.v.) twice a day for 5 days for examining its effect on the necroptosis after SCI.

Primary microglia and N9 cells culture

Microglial cells were isolated from the brain tissue of neonatal C57BL/6 mice. Briefly, the skin and skull were peeled away and the meningeal lining was then gently removed under a microscope. The cerebral cortical tissue was then minced into a fine slurry with scissors, followed by digestion with 0.125% trypsin/0.02% EDTA. Cells were suspended in 12 ml of Dulbecco's modified Eagle medium (DMEM) containing 10% FBS, 100 μg/ml penicillin–streptomycin and 2 mM L-glutamine, seeded onto poly-D-lysine coated T-75 flasks and maintained at 37 °C in an incubator with humidified 5% CO_2 atmosphere. After 9 days culture, when the cells were approximately 90% confluent, and purified by shaking at 260 rpm/min for 1 h. Immunostaining of calcium-binding adapter molecule 1 (Iba-1) was adopted to ensure that over 95% of the cells were Iba-1- positive. Microglial cells were then seeded in 6-well or 24-well plates at a density of 2×10^6 cells or 0.5×10^6 per well.

Mouse microglial N9 cells were maintained in DMEM supplemented with 5% FBS, 100 μg/ml penicillin–streptomycin and 2 mM L-glutamine. For experiments, cells were plated onto 6-well or 24-well plates at a density of 3×10^6 cells or 1×10^6 cells per well.

Oxygen–glucose deprivation (OGD) injury and Nec-1, 4-phenylbutyrate (4-PBA) treatment

OGD in N9 cells and primary microglia was performed by replacing the medium with glucose-free DMEM (Gibco, USA) and cultures were incubated in a hypoxic chamber (95% N_2 and 5% CO_2) at 37 °C for 12 h. After OGD treatment, cells were transferred back to normal condition with fresh culture medium for 4, 16, 24, 36 and 48 h for N9 cells and 36 h for microglia respectively. Control cells were maintained in regular DMEM under normoxic conditions. For Nec-1 and 4-PBA treatment, 20 μM Nec-1 and 4 mM 4-PBA were added into the culture medium when OGD was begun, until the end of the experiment. Immediately at the end of treatments, total cellular proteins were isolated for Western-blotting analysis, or cells were stained with Propidium Iodide (PI) for death detection.

PI staining

In vitro *PI labeling.* N9 cells and primary microglia were exposed to OGD for 12 h and subsequently placed back to normal culture medium for 36 h. PI (5 μM, Sigma) and Hoechst 33342 (5 μg/ml, Sigma) were added into the culture medium and incubated for 30 min at 37 °C. Cells were then washed three times with 0.01 M phosphate-buffered saline (PBS) and fixed with 4% (w/v) paraformaldehyde in phosphate buffer (PB) (4% PFA) for 10 min at room temperature and then imaged under an inverted fluorescence microscope (IX71, Olympus) equipped with an Olympus DP72 digital camera. For each of triplicate experiments, pictures were taken from eight random fields. All cells in the images were analyzed. Image Tool (University of Texas Health Sciences Center at San Antonio) was used for quantification.

In vivo PI staining. PI (10 mg/ml) was diluted in 0.9% NaCl. Twenty milligram per kilogram of PI in a total volume of not more than 100 μl was administered (i.p.) to mice 1 h before sacrifice as described (Ito et al., 1997; Oerlemans et al., 2012).

Please cite this article in press as: Fan H et al. Involvement of endoplasmic reticulum stress in the necroptosis of microglia/macrophages after spinal cord injury. Neuroscience (2015), http://dx.doi.org/10.1016/j.neuroscience.2015.10.049