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Role of Toll like receptor 4 signaling pathway in the secondary damage induced by experimental spinal cord injury

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ABSTRACT

Toll-like receptors (TLRs) are signaling receptors in the innate immune system that is specific immunologic response to systemic bacterial infection and injury. TLRs contribute to the initial induction of neuroinflammation in the CNS. In spinal cord injury (SCI) intricate immune cell interactions are triggered, typically consisting of a staggered multiphasic immune cell response, which can become deregulated. The present study aims to evaluate the role of TLR4 signaling pathway in the development of secondary damage in a mouse model of SCI using TLR4-deficient (TLR4-KO) mice such as *C57BL/10ScNJ* and *C3H/HeJ* mice. We evaluated behavioral changes, histological, immunohistochemistry and molecular assessment in TLR4-KO after SCI. SCI was performed on TLR4-KO and wild-type (WT) mice by the application of vascular clips (force of 24 g) to the dura via a four-level T5-T8 laminectomy. Mice were sacrificed at 24 h after SCI to evaluate the various parameters. SCI TLR4 KO mice developed severer hind limb motor dysfunction and neuronal death by histological evaluation, myeloid differentiation primary response 88 (Myd88) expression as well as an increase in nuclear factor NF-κB activity, tumor necrosis factor (TNF)-α and interleukin (IL)-1β levels, glial fibrillary acidic protein (GFAP), microglia marker (CD11β), inducible nitric oxide synthases (iNOS), poly-ADP-ribose polymerase (PARP) and nitrotyrosine expression compared to WT mice. Moreover, the absence of TLR4 also caused a decrease in phosphorylated interferon regulatory transcription factor (p-IRF3) and interferon (IFN-β) release. In addition, SCI TLR4 KO mice showed in spinal cord tissues a more pronounced up-regulation of Bax and a down-regulation of Bcl-2 compared to SCI WT mice. Finally, we clearly demonstrated that TLR4 is important for coordinating post-injury sequel and in regulating inflammation after SCI.

Introduction

Pathophysiology of spinal cord injury (SCI)

The pathophysiology of SCI is initiated by a '*primary injury*', which is the direct consequence of the mechanical injury of the spinal cord (SC) and it is obviously not preventable. In the few hours following injury, there is a progressive extension of cell death which largely induces tissue damage and expansion of the lesion for many days up to months after the initial injury. This phase is the so called "*secondary injury*". The contemporary management of SCI consists of supportive care and stabilization of the spine (Samadikuchaksaraei 2007; Genovese et al. 2009). Currently, there is no drug treatment that effectively improves outcome after SCI. Notably, animal studies suggest that early drug treatments may reduce SCI-associated damage could significantly improve the neurological outcome (Genovese et al. 2009). High-dose corticosteroids (GCs) increases blood flow to the injured spinal cord and decrease the amount of swelling (Nockels and Young 1992). GCs, given within the first 8 h after injury and continued for 24–48 h were regarded as part of the standard treatment regimen (Bracken et al. 1990, 1992, 1997, 1998).

Toll-like receptors (TLRs) and SCI

After primary SCI, resident microglia, activated macrophages and dendritic cells could work as antigen presenting cells through

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TLRs signaling at and around the injury site (Trivedi et al. 2006). TLRs are a family of receptors first discovered in Drosophila (Anderson et al. 1985). The TLRs, as key mediators of innate immunity, responding to diverse microbial products and injury-induced endogenous ligands (Akira 2006). The activation signal diverges, following either of two inflammatory cascades, the myeloid differentiation primary response 88 (MyD88) pathway to nuclear factor kappa-B (NF-κB) activation or the toll receptor-containing adaptor inducing interferon IFN-β (TRIF) pathway, also called MyD88-independent pathway. TLRs MyD88-dependent pathway activates NF-κB and subsequently results in the production of inflammatory cytokines (Akira and Takeda 2004), while MyD88-independent pathway is related to transcriptional activation of type I interferons (Toshchakov et al. 2002) and also activates late phase of NF-κB. NF-κB is essential for neurons survival against oxidative stress and ischemic degeneration but it also contributes to inflammation and apoptosis after CNS injury (Xu et al. 2002). Therefore, MyD88-dependent pathway is correlated with the development of SC secondary injury via inflammatory reaction. Our preliminary studies showed that the absence of TLR4 could also reduce the TRIF-mediated pathway by decreasing the expression of phosphorylated interferon regulatory transcription factor (pIRF-3) and consequently, reducing the release of IFN-β that could counteract the inflammation caused by MyD88-dependent pathway. How and when TLR4 signaling recruits MyD88 vs. TRIF is the subject of much research and however, the exact role of these pathways in the pathophysiology of SCI remain unclear. Common TLR4 ligands appear to utilize the same pathway or both pathways consistently. Based on these data, in this study we wanted to better understand whether TLR4 signaling could be important in regulating the pathophysiological sequel of SCI.

Materials and methods

Animals

Adult male C57BL/10ScNJ mice (20–22 g; Charles River; Milan; Italy) and adult male C3H/HeJ mice (20–22 g; Jackson Laboratory, USA) with a targeted disruption of the Toll like receptor 4 gene (KO) and littermate wild-type controls (WT) were housed in a controlled environment and provided with standard rodent chow and water. A spontaneous mutation occurred in C3H/HeJ at the lipopolysaccharide (LPS) response making C3H/HeJ mice more resistant to endotoxin compared to C57BL/10ScNJ mice. The study was approved by the University of Messina Review Board for the care of animals. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purposes (D.M.116192) as well as with the EEC regulations (O.J. of E.C. L 358/1 12/18/1986).

Materials

All compounds were obtained from Sigma–Aldrich (Milan, Italy). All chemicals were of the highest commercial grade available. All stock solutions were prepared in non-pyrogenic saline (0.9% NaCl; Baxter, Italy, UK).

SCI

We used the clip compression model as previously described (Impellizzeri et al. 2012). Mice were anesthetized under intraperitoneal ketamine and xylazine (2.6/0.16 mg/kg of body weight, respectively). A longitudinal incision was made on the midline of the back, exposing the paravertebral muscles. These muscles were dissected away exposing T5-T8 vertebrae. The spinal cord was exposed via a four-level T5-T8 laminectomy and SCI was produced by extradural compression at T6-T7 level using an aneurysm clip with a closing force of 24 g. In all injured groups, the spinal cord was compressed for 1 min. Sham animals were only subjected to laminectomy. Following surgery, 1.0 cc of saline was administered subcutaneously in order to replace the blood volume lost during the surgery. During recovery from anesthesia, the mice were placed on a warm heating pad and covered with a warm towel. The mice were individually housed in a temperature-controlled room at 27 °C for a survival period of 20 days. Food and water were provided to the mice ad libitum. During this time period, the animals' bladders were manually voided twice a day until the mice were able to regain normal bladder function.

Experimental groups

Mice were randomly allocated into the following groups: (i) *SCI WT group*: mice were subjected to SCI as described above ($n = 30$), (ii) *SCI TLR-4 KO group*: TLR-4 KO *C57BL/10ScNJ* mice were subjected to SCI as well as the WT group ($n = 30$), (iii) *Sham WT group*: WT mice were subjected to the surgical procedures, as reported above, except that the aneurysm clip was not applied ($n = 30$), (iv) *Sham TLR-4 KO group*: TLR-4 KO *C57BL/10ScNJ* mice were subjected to the surgical procedures, as reported above, except that the aneurysm clip was not applied ($n = 30$). In addition, to better investigate whether the observed phenotype was really associated with the gene defect, we added another experimental group with a different strain of TLR 4 KO (C3H/HeJ mice); (v) *SCI TLR-4 KO group*: TLR-4 KO *C3H/HeJ* mice were subjected to SCI as described above ($n = 30$). The mice ($n = 30$ from each group) were killed at 24 h after SCI to evaluate the various parameters. In a separate set of experiments to investigate the motor score, additional animals (10 animals/each group) were observed until 10 days after SCI.

Light microscopy

Spinal cord tissues from perilesional zone were taken at 24 h following trauma. Tissue segments containing the lesion (1 cm on each side of the lesion) were paraffin embedded and cut into 5-μm-thick sections. The tissue segments were fixed for 24 h in paraformaldehyde solution (4% in phosphate-buffered saline (PBS) 0.1 M) at room temperature, dehydrated by graded ethanol and embedded in Paraplast (Sherwood Medical, Mahwah, NJ). Tissue sections were deparaffinized with xylene, stained with Hematoxylin/Eosin (H&E) and studied using light microscopy connected to an Imaging system (AxioVision, Zeiss, Milan, Italy).

The histopathological changes of the gray matter were scored on a 6-point scale as previous demonstrated (Impellizzeri et al. 2012): 0, no lesion observed, 1, gray matter contained 1–5 eosinophilic neurons; 2, gray matter contained 5–10 eosinophilic neurons; 3, gray matter contained more than 10 eosinophilic neurons; 4, small infarction (less than one third of the gray matter area); 5, moderate infarction; (one third to one half of the gray matter area); 6, large infarction (more than half of the gray matter area). The scores from all the sections from each spinal cord were averaged to give a final score for individual mice. All the histological studies were performed in a blinded fashion.

Grading of motor disturbance

The motor function of mice subjected to compression trauma was assessed once a day for 10 days after injury. Recovery from motor disturbance was graded using the Basso Mouse Scale (BMS) open-field score as previously described (Impellizzeri et al. 2012) since the BMS has been shown to be a valid locomotor rating scale for mice.