Genetic Ablation of Toll-Like Receptor 2 Reduces Secondary Brain Injury Caused by Cortical Contusion in Mice

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Abstract. Previous studies have shown that Toll-like receptor 2 (TLR2) was up-regulated after traumatic brain injury (TBI), but the potential contribution of TLR2 to TBI still remains unclear. The present study investigated the role of TLR2 in modulating TBI-induced secondary brain injury in mice. Wild-type TLR2(+/-) and TLR2(-/-)-deficient mice were subjected to a moderately severe weight-drop impact head injury. Brain samples were extracted at 24 hours after trauma. We measured TLR2 by western blot; motor function by Grip test; brain edema by wet/dry method; cortical apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method; and IL-1β, TNF-α and IL-6 by enzyme-linked immunosorbent assay (ELISA). We found the absence of TLR2 function in mice resulted in ameliorating brain injury as shown by the reduced severity of neurological deficit, apoptosis, and brain edema at 24 hours after TBI, which was associated with the decreased expression of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), compared with their wild-type counterparts after TBI. In combination, these results suggest that TLR2 might play an important aggravating role in the pathogenesis of TBI-induced secondary brain injury, possibly by regulating inflammatory cytokines in the cortex.

Key words: Traumatic brain injury; Cerebral inflammation; Toll-like receptor 2

Introduction

Although traumatic brain injury (TBI) represents a significant public health problem worldwide, there are currently no available therapeutic strategies that improve clinical outcome measures [1]. Increased levels of inflammatory molecules within the injured brain, including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), are believed to contribute to cerebral damage, cell death, and blood-brain barrier (BBB) dysfunction after TBI [2]. Most of the pro-inflammatory cytokines were downstream molecules in the toll-like receptor 2 (TLR2) signaling pathway, which may mediate the inflammatory damage after TBI [3]. Previous studies have demonstrated the post-TBI up-regulation of TLR2 in the brain [4, 5], which represents a possible target that can interrupt inflammatory mechanisms underlying the secondary brain injury following TBI. However until now, the possible role of TLR 2 in secondary brain damage after TBI has not been investigated.

There are now 10 mammalian toll-like receptors (TLRs) that have been identified by sequence analysis. Among them, TLR2, which is widely expressed in the brain, can detect endogenous agonists, such as the degradation products of macromolecules, heat shock proteins 60 and 70, products of proteolytic cascades, intracellular components of ruptured cells, and products of genes that are activated by inflammation [6]. Furthermore, TLR2 has been shown to play an important role in initiating the cerebral inflammation related to stroke, Alzheimer’s disease, Huntington’s disease, and Parkinson’s disease [7]. TBI is associated with a cerebral inflammatory response characterized by microglial and astrocytic activation, as well as the release of inflammatory mediators. In particular, it has been suggested that pro-inflammatory
cytokines such as IL-1β, IL-6, and TNF-α play an important role in early events mediating blood-brain barrier (BBB) breakdown and the subsequent development of cerebral edema [8]. Thus, therapeutic strategies that target the post-traumatic inflammatory cascade hold enormous potential for improving outcomes after TBI.

The aim of the current study was to determine whether genetic ablation of TLR2 could attenuate the TBI-induced secondary brain damage and over-expression of pro-inflammatory cytokines in the pericontusional area. We hypothesized that TLR2 knockout could protect neurons, reduce cerebral edema, and promote behavioral recovery after TBI, probably by modulating cerebral inflammation.

Materials and Methods

Animals. TLR2-deficient ICR mice were purchased from the Model Animal Research Center of Nanjing University, China. Homozygous TLR2 (+/+), and TLR2 (−/−) mice were generated from inbred heterozygous TLR2 (+/−) mice. Genotypes of TLR2 (+/+), and TLR2 (−/−) were confirmed by polymerase chain reaction (PCR) amplification of genomic DNA isolated from the blood. The mice were housed in temperature- and humidity-controlled animal quarters with a 12-h light/dark cycle. All procedures were approved by the Institutional Animal Care Committee and were in accordance with the guidelines of the National Institutes of Health on the care and use of animals.

Experiment protocol. A mouse model of TBI was employed as described elsewhere [9], with minor modification [10]. Male mice weighing 25 to 35 g were anesthetized with sodium pentobarbital (50 mg/kg ip). A round, flat, 6-mm diameter polytetrafluoroethylene impounder was centered between the ears and eyes using a stereotactic apparatus, and a 100 g weight was released, falling 12 cm along a stainless steel string, striking the impounder. Brain injury-induced apnea was then treated for 3 min with 100% oxygen administration and chest compression to stimulate respiration. This model is generally associated with 20% mortality within the first 5 minutes post-injury, and no delayed mortality was observed thereafter. The experimental groups consisted of sham TLR2 (+/+), sham TLR2 (−/−), injured TLR2 (+/+), and injured TLR2 (−/−) (n=12 per group). The mice in the sham and injured groups were subjected to identical anesthetic-alone or experimental TBI, respectively.

Evaluation of neurological status. Two experimenters blinded to the mouse genotype evaluated the mice for neurological status by a grip test, as previously described, 24 hours after sham or injury [9]. The mice (n=12 per group) were each picked up by the tail and placed on a taut string that was 60 cm long and suspended 40 cm above a table between two metal bars. The grip score was measured as the length of time (in seconds) during which the mouse remained on the string in some manner (1-4 paws, tail, or paws and tail) with a cut-off at 30 sec.

Sacrifice and brain removal. At 24 hours after sham or injury, the mice were anesthetized with sodium pentobarbital (80 mg/kg IP) after being tested for neurological deficits. For terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) analysis, the mice (n=6 per group) were perfused via left ventricular puncture.

Figure 1. Diagram showing the site of impact of the impounder and the areas taken for assay.
with cold saline (48 ºC), followed by 4% neutral buffered formalin. The brains were removed and placed on a frozen plate. Tissue samples of cerebral cortex were rapidly taken from the fresh brain at the site of the lesion (Figure 1), stored overnight in 4% neutral buffered formalin, and then embedded in paraffin. For wet:dry weight ratio, western blot, enzyme linked immunosorbent assay (ELISA), and enzyme activity assay, the mice (n=6 per group) were exsanguinated by cardiac puncture. The brains were removed and tissue samples were taken as described above. A portion of the tissue was harvested to determine the wet:dry weight ratio, and the remainder was immediately stored in liquid nitrogen until analysis.

**Western blot analysis.** The frozen brain tissue was mechanically lysed in 20 mM Tris, pH 7.6, which contains 0.2% SDS, 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 0.11 IU/ml aprotinin (all purchased from Sigma-Aldrich, Inc., St. Luis, MO, USA). Lysates were centrifuged at 12,000 x g for 20 min at 4ºC. The protein concentration was estimated by the Bradford method using the Nanjing Jiancheng (NJJC) protein assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The samples (60 µg per lane) were separated by 8% SDS-PAGE and electro-transferred onto a polyvinylidene-difluoride (PVDF) membrane (Bio-Rad Lab, Hercules, CA, USA). The membrane was blocked with 5% skimmed milk for 2 hours at room temperature, incubated overnight at 4ºC with primary antibodies directed against the TLR2 protein in PBS+Tween 20 (PBST) at a dilution of 1:200, with β-actin (diluted 1:8000 in PBST, Sigma-Aldrich, Inc., St. Luis, MO, USA) used as a loading control. After the membrane was washed six times for 10 min each in PBST, it was incubated in the appropriate HRP-conjugated secondary antibody (diluted 1:400 in PBST) for 2 hours. The blotted protein bands were visualized by enhanced chemiluminescence (ECL) Western blot detection reagents (Amersham, Arlington Heights, IL, USA) and were exposed to X-ray film. Developed films were digitized using an Epson Perfection 2480 scanner (Seiko Corp, Nagano, Japan). Optical densities were obtained using Glyko Bandscan.
software (Glyko, Novato, CA, USA) and the TLR2 expression levels were normalized to β-actin.

**TUNEL staining and quantitation of apoptotic cells.** The formalin-fixed tissues were embedded in paraffin and sectioned at 4 mm thickness with a microtome. The sections were examined for apoptotic cells by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method. TUNEL: In situ cell death detection Kit POD (ISCDD, Boehringer Mannheim, Germany) was used. The procedures were performed according to the protocol of the kit and a previous study [11]. Briefly, sections were deparaffinized, rehydrated, and washed with distilled water (DW). The tissues were digested with 20g/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) at room temperature for 15 minutes. Endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide/methanol in PBS at 37°C for 30 minutes. The sections were then incubated with terminal deoxynucleotidyl transferase at 37°C for 60 minutes to add the dioxigenin-conjugated dUTP to the 3’-OH ends of fragmented DNA. Anti-digoxigenin antibody peroxidase was applied to the sections to detect the labeled nucleotides. The sections were stained with DAB and counterstained slightly with hematoxylin. The positive cells were identified, counted, and analyzed under the light microscope by an investigator blinded to the grouping. The extent of brain damage was evaluated by an apoptotic index, which was the average number of TUNEL-positive cells in each section counted in 10 microscopic fields (at ×200 magnifications).

**Measurement of brain edema.** Brain edema was determined using the wet: dry weight method [12]. Brain cortical samples ~20mg in size were harvested and immediately weighed to obtain the wet weight (WW). The samples were dried in an oven for 24 h at 110°C and weighed again to obtain the dry weight (DW). Water content was calculated as [(WW–DW)x100]/WW.

**Enzyme-linked immunosorbent assay (ELISA).** The levels of inflammatory mediators were quantified using specific ELISA kits for rats according to the manufacturers’ instructions (TNF-α from Diaclone Research, Besançon, France; IL-1β, IL-6 from Biosource Europe SA, Nivelles, Belgium) and our previous study [13]. Values were expressed as ng/g protein.
Statistical Analysis. All data were presented as mean ± SD. SPSS 12.0 was used for statistical analysis of the data. The measurements were subjected to one-way ANOVA. Differences between experimental groups were determined by the Fisher’s LSD post-test. Statistical significance was inferred at P<0.05.

Results

Western blot results for confirming TLR2 in the injured brain. Figure 2 showed no expression of TLR2 in the TLR2 (-/-) mice. At 24 hours after TBI, the protein level of TLR2 was significantly increased in the TBI group of TLR2 (+/+) mice compared to the sham group of TLR2 (+/+) mice (P <0.01) (Figure 2).

Effect of TLR2 deletion on neurological status. The grip scores in both the sham-operated TLR2 (+/+) and TLR2 (-/-) mice were similar. At 24 hours after TBI, decreased grip score, reflecting impaired sensory motor function, was found in both injured TLR2 (+/+) and TLR2 (-/-) mice as compared with their respective sham-operated mice. However, the neurological deficit was significantly improved in TLR2 (-/-) mice compared to TLR2 (+/+) mice (Figure 3).

Effect of TLR2 deletion on apoptosis in the cortex. Similar apoptotic indices were found in the brain samples of both sham-operated TLR2 (+/+) and TLR2 (-/-) mice, representing a physiological change and in accordance with the results of the previous study [4]. At 24 hours after TBI, an increased apoptotic index was found in both injured TLR2 (+/+) and TLR2 (-/-) mice compared to their respective sham-operated mice. However, the pathological change was better in TLR2 (-/-) mice compared with TLR2 (+/+) mice (Figure 4).

Effect of TLR2 deletion on brain edema. Similar brain water content was detected in both sham-operated TLR2 (+/+) and TLR2 (-/-) mice. At 24 hours after TBI, brain water content significantly increased in both injured TLR2 (+/+) and TLR2 (-/-) mice compared with their respective sham-operated mice. However, the pathological change was more severe in TLR2 (+/+) mice than in TLR2 (-/-) mice (Figure 5).

Effect of TLR2 deletion on expression of inflammatory cytokines in the brain. The protein expression levels of TNF-α, IL-1β, and IL-6 in the brain samples of both sham-operated TLR2 (+/+) and TLR2 (-/-) mice were similar. At 24 hours after TBI, higher cortical protein expression levels of inflammatory cytokines were detected in both injured TLR2 (+/+) and TLR2 (-/-) mice compared with their respective sham-operated mice. Similarly, higher protein expression levels of these inflammatory cytokines were found in TLR2 (+/+) mice than in TLR2 (-/-) mice (Figure 6).
Discussion

This study revealed that mice lacking TLR2 function have significantly altered secondary brain injury characterized by increased neurological deficit, apoptotic cell death, and brain edema after TBI. Compared to those of TLR2(+/-) mice, the brain samples of TLR2(-/-) mice showed decreased TBI-induced expression of the inflammatory cytokines TNF-α, IL-1β, and IL-6. To our knowledge, the findings reported here suggest for the first time that TLR2 plays an important role in the pathogenesis of TBI-induced secondary brain injury, possibly by regulating levels of inflammatory cytokines and inducing cerebral inflammation.

The family of TLRs plays a key role in controlling the innate immunity that responds to a wide variety of pathogen-associated molecules [14]. A variety of TLRs have been identified in human cells and in the brains of some other species [15]. Several studies have suggested that TLR2 is critical for inflammation-induced injury in the central nervous system [15]. Thus, TLR2 is well-positioned in the central nervous system (CNS) and seems to possibly initiate inflammation following TBI. The TLR2-mediated intracellular signaling pathways converge to activate the transcription of a series of cytokine/chemokine genes that are involved in the initiation or regulation of the inflammatory response [16]. The functional importance of TLR2 in acute inflammation is based on its ability to regulate the promoters of a variety of genes whose products, such as IL-1β, TNF-α, IL-6, ICAM-1, and acute phase proteins, are critical to inflammatory processes [17]. In the present study, we found that the levels of TLR2 protein in the contused brain were increased following TBI, which concurs with the previous studies [4, 5]. In TLR2 knockout mice, the concentration of pro-inflammatory cytokines was suppressed and the parameters of secondary brain damage such as brain edema, cell apoptosis, and motor dysfunction were attenuated.

In the previous research regarding TBI and TLR2, Park et al [18] investigated the role of TLR2 in glial cell activation using a stab-wound injury (SWI) model with TLR2 knock-out mice. They found that TBI resulted in an 18- and 4-fold up-regulation of glial fibrillary acidic protein (GFAP) and CD11b mRNA, respectively, in the injury area. In TLR2 knockout mice, the induced expression of these genes was reduced by 70% and 40%, respectively. Likewise, there was a reduction in the area of activated glial cells. In addition, the glial cells had a less activation in the TLR2 knock-out mice. The authors propose that TLR2 contributed to the glial cell activation and HO-1 gene expression associated with TBI. However, in
their study, the secondary brain injury was not evaluated, nor was the cerebral inflammation noted. In the current research, the data proved that TLR2 plays an important role in mediating the inflammatory signals in the brain after TBI, and knocking out TLR2 could prevent secondary brain injury following TBI. In this study, we found that the levels of TLR2 protein in the contused brain were increased following TBI; however, the mechanism underlying the initial effect on the TLR2 signaling pathway following TBI remains unclear. Otherwise, as mentioned in the previous study [4], some endogenous stimuli of TLR2, such as heat shock proteins 60 and 70, fibrinogens, and fibronectin, were reported to have been found or elevated in the CSF and brain after TBI. The main TLR2 ligands in the brain after TBI and the whole mechanism related to simvastatin call for further research.

A number of previous studies provide evidence that apoptosis is a prominent form of cell death associated with secondary brain injury after TBI [19]. Brain edema often leads to a rise in intracranial pressure and is an important contributor to the morbidity and mortality associated with TBI. Indeed, increased apoptotic cell death and brain edema that correlated with neurological deficits as indices of brain injury were found 24 hours after TBI in this study. However, these pathohological changes were more severe in TLR2(+/-) mice than in TLR2(-/-) mice. These findings illustrate the pro-inflammatory role of TLR2 in TBI-induced secondary brain injury. While the precise mechanisms underlying secondary brain injury are complex, it is clear that the inflammatory response contributes to disease progression. Although the CNS differs from other organ systems because of its near-complete isolation from the systemic bloodstream by the blood–brain barrier, the primary steps of immune activation within the brain follow a scenario similar to that seen in other organs [20]. TBI initiates the inflammatory response via disruption of the blood–brain barrier, triggering edema and infiltration of inflammatory cells [21]. Numerous inflammatory cytokines such as TNF-α, IL-1β, and IL-6, released within minutes of the primary injury, can initiate the infiltration of inflammatory cells into the brain [22]. The elevation of levels of inflammatory cytokines is also a major cause of increased cerebral edema and blood–brain barrier dysfunction following TBI [23]. In the present study, the absence of TLR2 function significantly inhibited TBI-induced TNF-α, IL-1β, and IL-6 production in the brain, whereas the presence of TLR2 function resulted in greatly increased cerebral inflammatory cytokine production after TBI. Therefore, it appears that TLR2 plays an important role in the regulation of cerebral inflammation after TBI, and may explain the potential effect of TLR2 in inducing inflammation as described previously.

In conclusion, to the best of our knowledge, this study is the first to demonstrate the possible role of TLR2 in the secondary brain damage induced by TBI and the effect of TLR2 on modulating the expression of cerebral pro-inflammatory cytokines. We found that knocking out TLR2 could play a role in protecting against TBI-induced secondary brain injury, possibly by regulating inflammatory cytokines. These findings raise the possibility that TLR2 may become a new therapeutic target for the treatment of TBI.

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References


