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Reduced inflammatory factor expression facilitates recovery after sciatic nerve injury in TLR4 mutant mice

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ABSTRACT

Toll-like receptors (TLRs) are extremely significant pattern recognition receptors. When nerve injury occurs, a variety of inflammatory factors are generated, leading to an exceedingly complex micro-environment. TLRs recognize damage-associated molecular patterns. To investigate the correlation between TLR4 and recovery after sciatic nerve injury, the model of sciatic nerve injury was conducted using TLR4-mutated mice (C3H/HeJ) and wild mice (C3H/HeN). Our goal was to identify short-stage and long-stage changes after sciatic nerve injury, mainly by checking the expression changes of inflammation factors in the short-stage and the differences in the recovery of the injured sciatic nerve in the long-stage. The results show that the increase of changes in the HeN group of IL-1β, IL-6, TNF-α and MCP-1 are more obvious than in the HeJ group, with caspase1 expression higher and Nlrp3 expression lower in the former group. Further results reveal intense inflammation occurred in the HeN group showing more neutrophils and macrophages. Nlrp3 and caspase1 showed little difference by Immunohistochemistry, with Nlrp6 expression differing between the HeJ group and the HeN group. The results led us to conclude that better recovery of the injured sciatic nerve occurred in the HeJ group because the expression of GAP-43 and p75NTR was higher and had a better SFI figure. TLR4 mutation can decrease the expression of inflammatory factors and enhance the speed of recovery after sciatic nerve injury. The changes in the expression of Nlrp6, which are related to the TLR4 mutation, may influence recovery of the injured sciatic nerve. Further studies will be conducted to confirm these results.

1. Introduction

Sciatic nerve injury caused by trauma to a sciatic nerve stem or branch is an ordinary traumatic complication. This injury is a dysfunctional disorder of the somatic sensory, motor and autonomic nerves. Nerve damage can lead to nerve-dominated feelings and muscle dysfunction, pain, and even lifelong disability [1,2]. Diagnosis and treatment can benefit from elucidation of the precise mechanisms of sciatic nerve injury.

Toll-like receptors (TLRs), membrane-spanning receptors, and NOD-like receptors (NLRs), cytoplasmic receptors are extremely important pattern recognition receptors in the innate immune response. TLRs identify not only pathogen-associated molecular patterns (PAMP) but also damage-associated molecular patterns (DAMP) [3,4]. TLRs were discovered in the brain and are expressed by microglia, astrocytes, oligodendrocytes, and neurons [5]. TLRs are pattern recognition receptors activated by diverse ligands, which are secreted by microorganisms or lesioned organisms. It is conceivable that such ligands are also present in a damaged sciatic nerve, thereby recruiting macrophages and Schwann cells into the microenvironment, contributing to the clearance of axon and myelin debris [6–8].

Existing studies suggest that injured nerve repair is interconnected with the inflammatory reaction, although the specific molecular mechanisms are ambiguous. TLR-mediated NF-κB activation is required for the production of pro-IL-1β, while cleavage of pro-IL-1β to its mature form is dependent on NLR-mediated caspase-1 activation. TLRs induce transcriptional activation of pro-IL-1β, which is processed via inflammasome activation. IL-1β expression is a well-characterized outcome of TLR and inflammation reactions.

TLR4, the first discovered TLR related protein, is recognized as playing an essential role in the recognition of microbial components, such as lipopolysaccharide (LPS), which are generally seen in gram-negative bacteria [9]. Transformation in the internal microenvironment can activate TLR4. Activation of TLR4 will further trigger factors in the

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Table 1
Primer sequences for RT-qPCR.

Genes	Forward-primers	Reverse-primers
IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
IL-1β	GCAACTGTTCCTGAACTCAACT	ATCTTTTGGGGTCCGTCAACT
TNF-α	CCCTCACACTCAGATCATCTTCT	GCTACGACGTGGGCTACAG
MCP-1	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
Caspase-1	ACAAGGCACGGGACCTATG	TCCCAGTCAGTCCTGGAAATG
Nlrp3	ATTACCCGCCCGAGAAAGG	TCGCAGCAAAGATCCACACAG
Nlrp6	CTCGCTTGCTAGTGACTACAC	AGTGCAAACAGCGTCTCGTT

signal pathway, causing the release of inflammatory factors [10]. Tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-1β are the three major inflammatory factors in the early inflammatory stage. Changes in their expression can signal the degree of damage to the sciatic nerve [11,12].

Currently, the precise function and interactions, and complex molecular mechanisms between TLRs and inflammation in the development of sciatic nerve injury remain elusive. For validating the role of TLR4 in inflammation and nerve repair mechanism, this experiment was conducted on a TLR4 mutated C3H/HeJ mice to develop a sciatic nerve injury model and used a wild type of C3H/HeN mice as the control group. The intention of the experimental basis for the nerve damage is to explore the relationship between changes in inflammatory factor expression and recovery after sciatic nerve injury, and to further reveal whether TLR4 mediates the relationship between inflammation and recovery after sciatic nerve injury.

2. Materials and methods

2.1. Mice work

All animal procedures were approved by the Institutional Ethics Committee of Qingdao University Medical College, under a permit of animal use (SCXK40090007) in the Center of Experimental Animal at Qingdao. The permit is in compliance with the Experimental Animal Regulations set by the National Science and Technology Commission, China. The all SPF (specific-pathogen-free) male mice of C3H/HeN and C3H/HeJ were aged 6–8 weeks (23–26 g). The HeN group was purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). The HeJ group was procured from Model Animal Research Center of Nanjing University (Nanjing, China). The HeJ group is a single amino acid mutation of TLR4, the 712th amino acid from proline to histidine, which makes the function of TLR4 receptor completely different and unable to produce inflammatory cytokines [13,14]. The mice were housed in the animal facility with a 12 h light/dark cycle, the temperature at 22–23 °C and the relative humidity at 60%, with food and water. The mice adapted to the environment within one week. The experiment proceeded subsequently.

2.2. Surgical procedures and tissue preparation

The experiment contained three groups. The HeN group was randomly divided into a control group ($n = 16$), and a normal injury group (HeN group, $n = 32$). The HeJ group was a mutation injury group (HeJ group, n = 32). A solution of 5% chloral hydrate (350 mg/kg) was used to anesthetize the mice through an intraperitoneal injection. A 75% alcohol disinfectant was then employed to sterilize the cleanly shaven furl of the thigh and its surrounding area.

Skin layers and muscle were separated through surgery using sterile operational conditions. The incision of the skin was approximately 1.5 cm. The skin and muscle connections were then bluntly separated, using hemostatic forceps to expose the sciatic nerve. The length of separated muscle was approximately 0.8 cm. The HeN group and the HeJ group mice were also operated in the following manner. The sciatic nerve was exposed at mid-thigh location and was crushed at full pressure for 60s with a pair of jeweler's forceps (no. 4), thereby allowing comprehensive transection of neural fibers without breaking the epineurium [15,16]. The control group mice had the sciatic nerve freed but not crushed.

After repositioning the sciatic nerve and muscle, the skin and muscle incision were sutured with 6–0 no damage thread. These procedures were performed by a single person to ensure experimental consistency. The animals were kept in the animal housing facility for the appropriate survival times (6, 12, 24 and 48 h) for the duration of the test.

2.3. RNA isolation and RT-qPCR

The mice were euthanized at 6, 12, 24 or 48 h after sciatic nerve injury. The ischiadicus were extracted cautiously, snap frozen, and stored at − 80 °C until use. The nerves were homogenized in RNAiso Plus (TaKaRa, Dalian, China), and the tissue was ground until no fragments remained, using disposable tissue grinding pestles. Total RNA was extracted using RNAiso Plus according to the manufacturer's protocol.

RNA integrity was assessed using a BioPhotometer (Eppendorf, Hamburg, GER). DNAase treatment and cDNA synthesis were done with Super Mix for qPCR + gDNA wiper (Vazyme, Nanjing, China). RT-qPCRs were performed with 100 ng cDNA in SYBR Green qPCR reaction PCR Kit (Qiagen, GER) and run on a CFX96 Real-Time PCR Detection System (Bio-Rad, USA). All PCR reactions were done three times. Primers were designed by Primer Bank. Primer sequences are listed in Table 1. The RT-qPCR data were normalized according to geometric averaging of multiple internal control genes. Processing of raw data and calculation of normalized relative quantities was done by the $2^{-\Delta\Delta Ct}$ method.

2.4. Western blot analysis

For Western blot analysis, 10-mm distal segments of the injured sciatic nerve were removed, snap frozen, and stored at − 80 °C until use. Protein lysates were digested by RIPA buffer (50 mmol/L Tris-HCl; 150 mmol/L NaCl; 1% Nonidet; 0.5% deoxycholate; 1 mmol/L EDTA; and 1 mmol/L PMSF) with protease inhibitors (1 g/mL each of pepstatin, aprotinin and leupeptin) on ice for 30 min. The total protein concentration was established using a BCA Protein Assay Kit (Beyotime, China). Equal amounts of protein lysates (30 to 40 μg) were separated using 8% SDS-PAGE, then transferred to PVDF membranes with a diameter of 0.45 μm. Membranes were blocked using a blocking buffer (5% milk in PBS containing 0.1% Tween-20 or 5% BSA in TBS containing 0.1% Tween-20) and incubated overnight at 4 °C with a primary antibody. Secondary HRP conjugated antibodies (1:5000, CST) were used to interact with the primary antibody at room temperature for 1 h. The antibodies used were anti-Nlrp3 (1:400, CST), anti-Nlrp6 (1:500, Abcam), anti-caspase-1 (1: 400, Abcam), anti-p75NTR (1:200, Bioss), anti-NF-κB (1:200, Bioss) and anti-β-actin (1: 10,000, Abcam). The proteins were detected visually using the ECL reagent and showed by an imager (UVP Biospectrum 810, USA).