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Double-stranded RNA Induces Inflammatory Gene Expression in Schwann Cells: Implication in the Wallerian Degeneration

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Schwann cells play an important role in peripheral nerve regeneration. Upon neuronal injury, activated Schwann cells clean up the myelin debris by phagocytosis, and promote neuronal survival and axon outgrowth by secreting various neurotrophic factors. However, it is unclear how the nerve injury induces Schwann cell activation. Recently, it was reported that certain cytoplasmic molecules, which are secreted by cells undergoing necrotic cell death, induce immune cell activation via the toll-like receptors (TLRs). This suggests that the TLRs expressed on Schwann cells may recognize nerve damage by binding to the endogenous ligands secreted by the damaged nerve, thereby inducing Schwann cell activation. Accordingly, this study was undertaken to examine the expression and the function of the TLRs on primary Schwann cells and iSC, a rat Schwann cell line. The transcripts of TLR2, 3, 4, and 9 were detected on the primary Schwann cells as well as on iSC. The stimulation of iSC with poly (I : C), a synthetic ligand for the TLR3, induced the expression of TNF-α and RANTES. In addition, poly (I : C) stimulation induced the iNOS expression and nitric oxide secretion in iSC. These results suggest that the TLRs may be involved in the inflammatory activation of Schwann cells, which is observed during Wallerian degeneration after a peripheral nerve injury.

Key Words: Schwann cells, Toll-like receptor, iNOS, RANTES, TNF-α, iSC

INTRODUCTION

The incidence of facial nerve damage is often inevitable during surgery on the craniomaxillofacial area. Unlike in the central nervous system (CNS), neurons in the peripheral nervous system (PNS) have an intrinsic potential to regenerate upon axotomy. It is well known that a specific and orchestrated sequence of histopathological events is as a consequence of peripheral nerve injury, which eventually results in the full or partial regeneration of the injured nerve. In order to achieve a successful nerve repair, neuronal loss needs to be prevented, the axons have to re-grow and identify their correct target cells, and the myelin sheaths need to be re-synthesized (Fu et al, 1997). As a first step, the injured tissue needs to be cleaned up, and the axonal growth-inhibiting myelin debris must be removed (Grados-Munro et al, 2003). Schwann cells play an important role in this process, which is known as Wallerian degeneration. It is the Schwann cells that first respond to a nerve injury and become de-differentiated or activated (Stoll et al, 2002). The activated Schwann cells then retain their phagocytic capacity and begin cleaning the myelin and dead neuronal debris (Fu et al, 1997). In this cleaning process, the Schwann cells express various inflammatory cytokines and chemokines such as LIF, IL-6 and MCP-1, which induces the local inflammation and the recruitment of the macrophages from the blood vessel (Tofaris et al, 2002). In addition, the Schwann cells proliferate and align within the basal lamina tubes (bands of Bugner), providing a guidance substrate for the regrowth of axons. Those axons will eventually be remyelinated by the re-differentiated Schwann cells, which have aligned along them in a one to one ratio (Kury et al, 2001). However, it is unclear how the Schwann cells recognize the damage on the neurons and become activated. Similar to Schwann cells in a PNS injury, an injury to the CNS is accompanied by microglia activation. For example, activated microglia are easily detected in the brains of various neurodegenerative disease patients, including Alzheimer's disease and Parkinson's disease. Interestingly, it was recently reported that the heat shock protein released from the damaged neurons activates the nearby microglial cells by binding to the TLR4 expressed on the microglia (Kakimura et al, 2002). In addition, it was suggested that other members of the TLR family recognize

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ABBREVIATIONS: CNS, central nervous system; LPS, lipopolysaccharides; PAMP, pathogen-associated molecular pattern; PNS, peripheral nervous system; poly(I:C), polyinosinic acid-polycytidylic acid; TLR, toll-like receptor

the endogenous cytoplasmic molecules that are secreted upon tissue damage and elicit a danger signal in the cells of the innate immune system (Kariko et al, 2004).

TLRs are type I transmembrane proteins that are evolutionarily conserved between insects and humans (Medzhitov et al, 1997). Each TLR has a TLR/IL-1R (TIR) homology domain in its cytoplasmic region and several leucine-rich repeats (LRR) in its extracellular region. Through their extracellular domains, the TLRs recognize the relatively conserved motifs of various molecules from microorganisms, which are known as pathogen-associated molecular patterns (PAMPs). Several PAMPs have been identified, including lipopolysaccharides (LPS) from Gram-negative bacteria, peptidoglycan (PGN) and lipoteichoic acid (LTA) from Gram-positive bacteria, microbial lipoproteins and lipopeptides, bacterial flagellin, double-stranded viral RNA (dsRNA), as well as bacterial DNA. Thus far, more than 10 different TLRs with a distinct ligand specificity have been identified (Takeuchi et al, 2000; Hemmi et al, 2000; Chuang et al, 2000; Zhang et al, 2004).

In the CNS, various TLRs are expressed on the microglia and astrocytes, and induce inflammatory responses as a result of bacterial/viral infection or tissue damage. However, the expression and the function of the TLR in the immune cells in the PNS have not been determined. In this study, it was postulated that the TLRs expressed on Schwann cells initiate the inflammatory responses in the PNS, which are observed upon peripheral nerve damage. Therefore, the expression and the function of TLRs on the Schwann cells were examined to test this hypothesis. The results showed that various TLRs, including TLR2, 3, 4, and 9, are expressed on Schwann cells, and that the TLR3-mediated signal induces a strong inflammatory signal in the iSC. This suggests that the TLRs might be involved in the inflammatory activation of Schwann cells, which is observed during Wallerian degeneration after peripheral nerve damage.

METHODS

Reagents

The following reagents were purchased from the sources indicated: Dulbecco's modified Eagle's medium (DMEM), Modified Eagle's medium (MEM), fetal bovine serum (FBS), antibiotics and antimycotics were obtained from GIBCO BRL, USA. Lipopolysaccharide (LPS), poly (I : C), TRI reagent and Griess reagent were acquired from Sigma, USA. Moloney murine leukemia virus reverse transcriptase (M-MLV RTase) was purchased from Life Technologies, USA, the Taq polymerase was from Stratagene, USA, and the SYBR GREEN PCR Master mix was from Applied Biosystems, USA.

Cell cultures

Primary Schwann cells were prepared from the dorsal root ganglions (DRGs) of 1 day-old Sprague-Dawley (SD) rat pups. A pellet of DRGs was incubated in 1 ml of 0.25% collagenase A and 0.1 ml of 0.2% DNase I at 37°C for 1 hour, and then treated again with 1 ml of 0.25% trypsin-EDTA for 10 min. The DRGs were then dissociated by repeated pipetting with a Pasteur pipette in the culture medium containing MEM, 5% heat-inactivated FBS, 2 mM L-glutamine, 0.5% glucose, and antibiotics. After filtering in a 70μl-cell strainer, the cells were pelleted by spinning at 800 rpm for 10 min. The cell pellet was resuspended in the culture medium, seeded on poly-D-lysine (20μg/ml)-coated dishes and maintained in a 5% CO_2 incubator at 37°C. After 1 day, 10μM cytosine arabinoside (Ara-C) was added to the medium to suppress the fibroblast proliferation, and the glial cell line-derived neurotrophic factor (GDNF) (10 ng/ml) was added to the medium after 2 days. The primary Schwann cells were used for the experiments after passage 2 to 3. The immortalized Schwann cells were cultured in DMEM containing 10% heat-inactivated FBS, 2 mM L-glutamine, and antibiotics. The iSC cells were maintained in a 5% CO_2 incubator at 37°C.

Reverse transcription polymerase chain reaction (RT-PCR)

The total RNA was isolated by using a TRI reagent according to the manufacturer's instruction, treated with RNase-free DNase I for 30 min, and then heat-inactivated for 30 min at 65°C. The cDNA was synthesized from 3μg of the total RNA by incubating for 1hr at 37°C in a reaction mixture containing 0.5μg of oligo $(dT)_{15}$, 0.5 mM dNTP mix, 1X first-strand buffer, RNase inhibitor (5 units), 5 mM DTT, and M-MLV reverse transcriptase (5 units). The mRNA expression of the TLRs, TNF-α RANTES, iNOS, and GAPDH were measured using the following PCR reaction; initial denaturation at 94°C for 3 min, a 30 sec denaturing step at 94°C, a 30 sec annealing step at 60°C (for TLR3, 4, TNF-α, and GAPDH) or at 63°C (for TLR2, 9 and RANTES), and then a 30 sec extension at 72°C. A total of 35 reaction cycles were performed. The PCR primer sequences used are shown in Table 1. The amplified DNA products were resolved in a 2% agarose gel and the images

Table 1. The sequence of the primers for the rat TLRs, TNF-α RANTES, iNOS, and GAPDH

Primer	Forward	Reverse
TLR2	GGA GAC TCT GGA AGC AGG GA	CGC CTA AGA GCA GGA TCA AC
TLR3	AAT CCC GGT CAA GGT GTT CAA	GGC CCG AAA ACA CTC TTC TCA
TLR4	TGC TCA GAC ATG GCA GTT TC	TCA AGG CTT TTC CAT CCA AC
TLR9	TAA CTG GAG CAG TCT GGC CT	CGT CAG GTT CAT CAC AAT GG
RANTES	ATC CCT CAC CGT CAT CCT C	CTT CTT CTC TGG GTT GGC AC
TNF-α	TGT CCG CAT GTT GAG ATC AT	GGG TAA AGG GAG GTG GAG
iNOS	TCT GTG CCT TTG CTC ATG ACA	TGC TTC GAA CAT CGA ACG TC
GAPDH	CAC CCT GTT GCT GTA GCC GTA T	AGG TCA TCC CAG AGC TGA ACG

were analyzed by using LAS-1000 CH (FUJIFILM).

Real-time PCR

The transcripts of the iNOS and GAPDH genes were quantified by real-time PCR analysis, using ABI PRISM 7900 Sequence Detection System Thermal Cycler (PE Applied Biosystems, USA). Using a 20μl reaction mixture containing first strand cDNA, primers, and the SYBR GREEN Master mix, real-time PCR was performed by incubating each sample at 50°C for 2 min, at 95°C for 10 min, which was followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C.

Nitrite assay

Nitric oxide (NO) production was measured, using a Griess assay kit according to the manufacturer's instruction. Briefly, equal volumes of the culture supernatants and the Griess reagent were mixed, and absorbance was measured at 570 nm after 15 min. The nitrite concentration in each sample was calculated by using a standard curve.

RESULTS

The mRNA expression levels of various TLRs, including TLR2, 3, 4, and 9, in the primary Schwann cells and the iSC, a Schwann cell line derived from rat sciatic nerve, were first examined. As shown in Fig. 1, the transcripts of the above TLRs were expressed in the primary Schwann cells as well as in the iSC. In order to examine the role of the TLRs expressed on the Schwann cells, the iSCs were stimulated with either LPS or poly (I : C), a ligand for TLR4 and TLR3, respectively. As shown in Fig. 2A, stimulation of the iSC with LPS or poly (I : C) induced the TNF-α and RANTES mRNA expression. The induction levels of the proinflammatory cytokine and chemokine gene after poly (I : C) stimulation were quantified by real-time PCR analysis (Fig. 2B). Stimulation of the iSC with poly (I : C) increased the RANTES and TNF-α mRNA levels by 230-, and 360-fold, respectively. Similarly, stimulation with LPS, a TLR4 ligand, increased the mRNA levels of the above cytokine and chemokine genes by more than 600-fold. These results indicate that the TLR3 and TLR4 are expressed in the Schwann cells and transduce the inflammatory signal upon binding to its ligand.

It has previously been reported that, following a chronic constriction peripheral nerve injury, iNOS is expressed within and distal to the injury site (Levy et al, 1999). In this study, iNOS was expressed in the activated Schwann cells as well as in the nerve-infiltrating macrophages. The aberrant expression of iNOS in the Schwann cells has been implicated in the development of experimental allergic neuritis (EAN) and the neuropathic pain (Conti et al, 2004; Levy et al, 1999). Thus far, the stimulating signals that induce iNOS expression in the Schwann cells upon nerve damage have not been determined. Therefore, the TLR3-

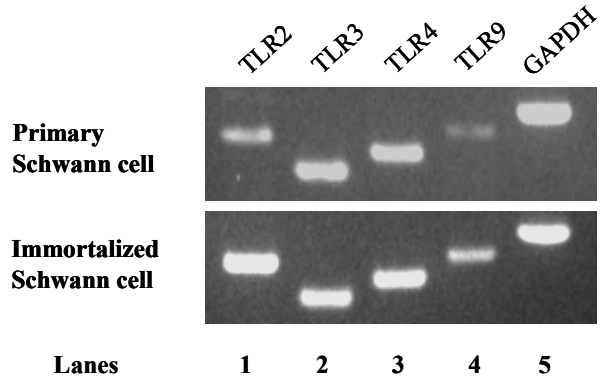

Fig. 1. The expression of the TLRs on rat schwann cells. The total RNA was prepared from primary Schwann cells and iSC. The cDNA of each sample was generated by using M-MLV Reverse Transcriptase and used to amplify TLR genes (lanes 1 to 4) and GAPDH gene (lane 5). The samples were resolved in a 2% agarose gel.

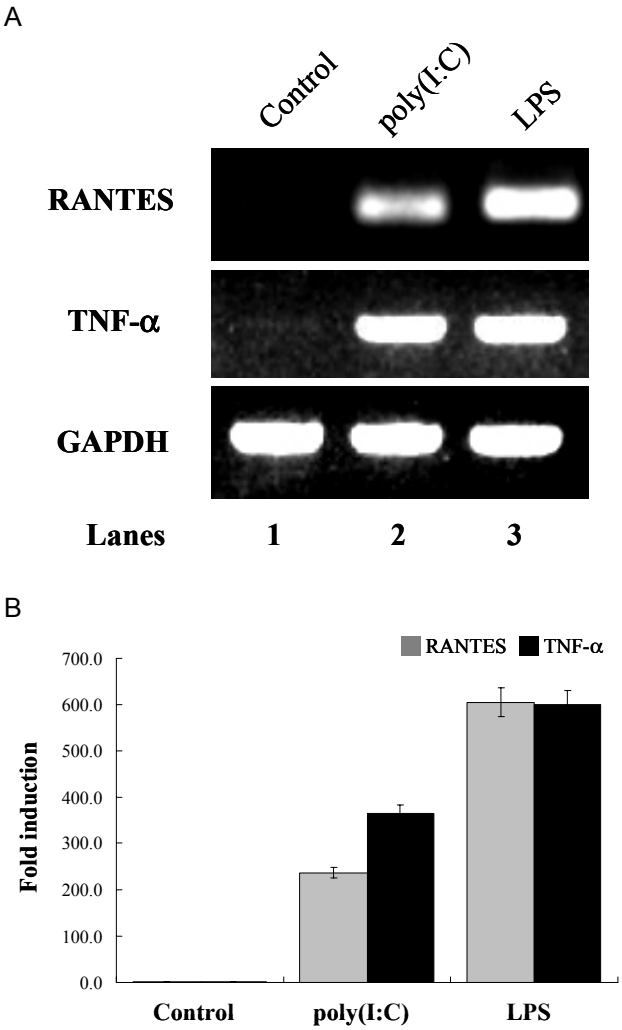

Fig. 2. The TNF-α and RANTES gene expression induced by the TLR ligands in iSC cells. The iSC cells were treated for 3 h with or without the poly (I : C) (20μg/ml) and LPS (100 ng/ml). The total RNA was isolated and used to determine the levels of TNF-α and RNATES mRNA expression by either RT-PCR (A) or real-time RT-PCR (B). Means ± S.E.M of duplicate samples from a representative of three independent experiments are shown.

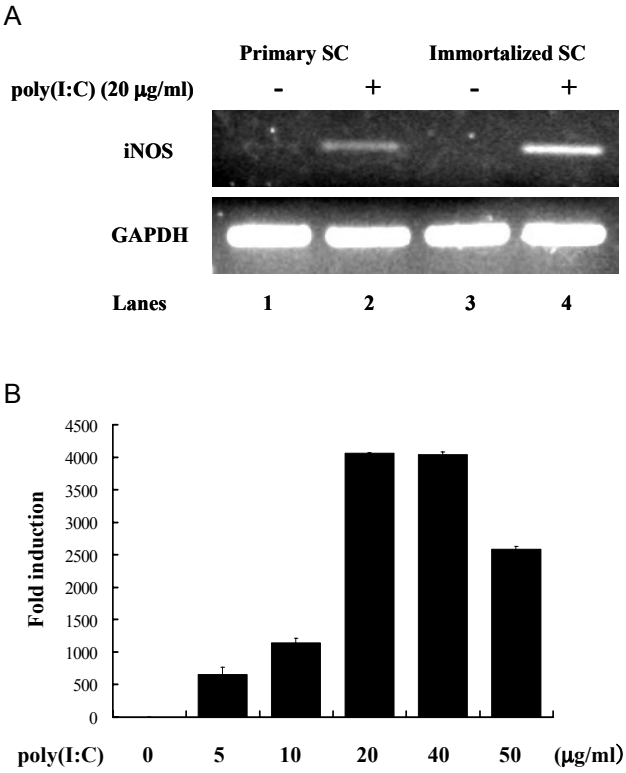

Fig. 3. Poly (I : C) stimulation induces the expression of the iNOS gene in rat schwann cells. A. The primary Schwann cells (lanes 1 and 2) and iSC (lanes 3 and 4) were treated for 3 h with (lanes 2 and 4) or without (lanes 1 and 3) poly (I : C) (20μg/ml). The total RNA was isolated and used to determine the level of iNOS mRNA accumulation by RT-PCR. B. The iSC cells were stimulated with the indicated concentrations of poly (I : C). The total RNA was prepared from each sample and used for real-time PCR to determine the iNOS mRNA levels. Means±S.E.M of duplicate samples from a representative of three independent experiments are shown.

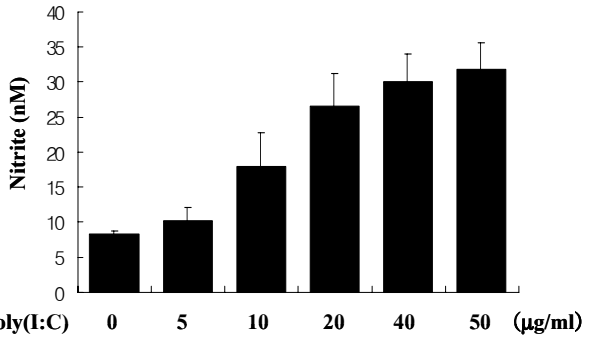

Fig. 4. Poly (I:C) stimulation induces NO production in iSC cells. The iSC cells were treated with indicated concentrations of poly (I:C) for 24 h. NO production was determined by measuring the nitrite concentration of the culture supernatant of each sample by the Griess assay. Means±S.E.M of duplicate samples from a representative of three independent experiments are shown.

mediated inflammatory signal was examined to determine if it induced iNOS expression in the Schwann cells (Fig. 3A). Stimulation of the rat primary Schwann cells with 20 μg/ml poly (I : C) induced the iNOS gene transcript, demonstrated by RT-PCR (Fig. 3A, lanes 1 and 2). Similarly, poly (I : C) stimulation increased the iNOS transcription in the iSC (Fig. 3A, lanes 3 and 4), whereas it had no effects on the expression of rat GAPDH, which is a house keeping gene (Fig. 3A. lower panel). The induction level of the iNOS transcript upon poly (I : C) stimulation in the iSC was measured by real-time PCR analysis (Fig. 3B). Stimulation of the iSC with poly (I : C) dose-dependently increased the iNOS mRNA level: The level of induction was increased as the concentration of poly (I : C) was increased up to 20μg/ml, and the level then declined at 50μg/ml (Fig. 3B). The 20μg/ml poly (I : C) stimulation induced the iNOS transcript by up to 4000 fold. The induction of the iNOS gene is usually coupled to NO production, which can be assayed by measuring the nitrite levels in the culture medium. As expected, the production of NO was also increased upon poly (I : C) stimulation in the iSC (Fig. 4). The basal nitrite concentration in the culture supernatant was below 10 nM, which increased up to 30 nM upon poly (I : C) (20μg/ml) stimulation for 24 h. These results suggest that TLR3 stimulation induces NO production in rat Schwann cells.

DISCUSSION

Schwann cell activation is one of the hallmarks of nerve degeneration after a peripheral nerve injury. Upon activation, Schwann cells become de-differentiated and clean up the myelin debris, which are known to inhibit nerve growth. The activated Schwann cells then secrete inflammatory cytokine and chemokine such as TNF-α and MCP-1, which recruits the peripheral macrophages at the site of nerve injury and facilitates the cleaning process. In addition, the activated Schwann cells express various neurotrophic factors, including NGF and CNTF (Terenghi et al, 1999). These neurotrophic factors protect damaged neurons from apoptosis and induce neuronal outgrowth. However, it is unclear how the Schwann cells recognize nerve damage and become activated.

Recent studies on the TLR have shed some new light on this issue. The TLRs were originally regarded as receptors for the pathogen-derived molecules. However, recent reports suggest that the TLRs are used as receptors for endogenous molecules as well such as mRNA or heat shock protein (Kakimura et al, 2002; Kariko et al, 2004). Considering that peripheral nerve damage will also induce the exposure of the axoplasmic molecules, it was reasoned that the TLRs expressed on the Schwann cells might function as receptors, which recognize nerve damage.

In order to examine this hypothesis, the expression of various TLRs on primary Schwann cells was examined. It is known that the TLR2 are expressed on the Schwann cells and mediate *M. leprae*-mediated Schwann cell death (Oliveira et al, 2003). In addition, the expression of the functional TLR4 was also indicated, because stimulation with LPS, a TLR4 ligand, induced strong inflammatory cytokine expression in the Schwann cells (Skundric et al, 1997). In this study, in addition to TLR2 and 4, the TLR3 and 9 transcripts were detected in the rat Schwann cells, suggesting that the Schwann cells have the intrinsic potential to recognize various infectious agents and endo-

genous molecules, and then initiate the inflammatory responses in the PNS.

In order to determine the role of the TLRs expressed on the Schwann cells, the iSCs were stimulated with the ligand for each TLR, and the mRNA expression level of the various pro-inflammatory cytokines and chemokine was measured. In this study, stimulation with poly (I:C) or LPS, a TLR3 agonist and a TLR4 agonist, respectively, upregulated the mRNA expression level of TNF-α and RANTES. This suggests that TLR3 expressed on the Schwann cells might induce the expression of the above cytokines and chemokine upon binding to the mRNAs escaping from the injured nerve. TNF-α is rapidly upregulated after a facial nerve injury and plays important roles in nerve regeneration (Streit et al, 1998). For example, TNF-α inhibits the cyclic AMP-induced Schwann cell differentiation (Lisak et al, 2001). In addition, studies using TNF-α-deficient mice have shown that TNF-α is required for macrophage recruitment following a nerve injury and the removal of myelin debris (Liefner et al, 2000). The proliferation of human Schwann cells upon TNF-α stimulation have also been reported (Scarpini et al, 1999). Overall, these reports suggest that, after a peripheral nerve injury, the TLR3-mediated signal can induce Schwann cell de- differentiation and the recruitment of macrophages.

The study on the iNOS expression indicates that the TLR3-mediated signal induces iNOS expression and subsequent NO production in Schwann cells. The expression of iNOS has been detected in Schwann cell after a nerve injury, and implicated in the development of neuropathic pain and EAN (Levy et al, 1999; Conti et al, 2004). NO production by Schwann cells is also implicated in HIV-associated sensory neuropathy (Keswani et al, 2003). Considering these earlier reports, it is possible that the mRNAs released from the damaged nerve tissue induces NO production from Schwann cells via TLR3, which is involved in the development of neuropathic pain. In this study, we propose a novel hypothesis to explain the molecular mechanisms underlying the activation of Schwann cells after peripheral nerve injury; Schwann cells become activated through TLR3 binding to endogenous mRNA. Thus far, there is no *in vivo* data to support this hypothesis. Therefore, future studies, using TLR3-deficient mice, will be needed to validate this hypothesis.

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