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Damaged Neuronal Cells Induce 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 nerve injury, Schwann cells are activated and produce various proinflammatory mediators including IL-6, LIF and MCP-1, which result in the recruitment of macrophages and phagocytosis of myelin debris. However, it is unclear how the nerve injury induces Schwann cell activation. Recently, it was reported that necrotic cells induce immune cell activation via 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. To explore the possibility, we stimulated iSC, a rat Schwann cell line, with damaged neuronal cell extracts (DNCE). The stimulation of iSC with DNCE induced the expression of various inflammatory mediators including IL-6, LIF, MCP-1 and iNOS. Studies on the signaling pathway indicate that NF-κB, p38 and JNK activation are required for the DNCE-induced inflammatory gene expression. Furthermore, treatment of either anti-TLR3 neutralizing antibody or ribonuclease inhibited the DNCE-induced proinflammatory gene expression in iSC. In summary, these results suggest that damaged neuronal cells induce inflammatory Schwann cell activation via TLR3, which might be involved in the Wallerian degeneration after a peripheral nerve injury.

Keywords: Schwann cells, Toll-like receptor, IL-6, LIF, MCP-1, iSC

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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. Upon peripheral nerve injury, a specific and orchestrated sequence of histopathological events occurs, 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 and Gordon, 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 and Fournier, 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 and Gordon, 1997). In this cleaning process, the Schwann cells express various inflammatory cytokines and chemokines such as TNF-α, 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, it was reported that nerve damage induces iNOS gene expression in Schwann cells (Conti *et al.,* 2004). The production of NO in the PNS is implicated in the nerve injury-mediated neuropathic pain (Levy and Zochodne, 1998). However, it is unclear how the Schwann cells recognize the damage on the nerve and become activated.

Interestingly, it was recently reported that mRNA released from the necrotic cells activates the nearby innate immune cells by binding to the TLR3 (Kariko *et al.,* 2004). In addition, other members of the TLR family such as TLR2 and TLR4 were shown to recognize endogenous cytoplasmic molecules that are secreted upon tissue damage and elicit a danger signal in the cells of the innate immune system (Vabulas *et al.,* 2002). TLRs are type I transmembrane proteins with a TLR/IL-1R (TIR) homology domain in its cytoplasmic region and several leucine-rich repeats (LRR) in its extracellular region (Medzhitov *et al.,* 1997). 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). Thus far, more than 10 different TLRs with distinct ligand specificity have been identified (Takeuchi *et al.,* 2000; Hemmi *et al.,* 2000; Chuang *et al.,* 2000; Zhang *et al*., 2004). In the PNS, it was reported that TLR2 and TLR3 are expressed in the Schwann cells (Lee *et al.,* 2004; Oliveira *et al.,* 2003).

In this study, we postulated that damaged nerve-derived molecule induces inflammatory Schwann cell activation during Wallerian degeneration, and tested this hypothesis by using damaged neuronal cell extracts (DNCE). The results show that DNCE stimulation induces various proinflammatory gene expressions in Schwann cells via TLR3. This suggests that an endogenous TLR3 ligand, possibly RNA, released from the damaged nerve may bind to TLR3 on Schwann cells and induce proinflammatory gene expression, which is observed during Wallerian degeneration after peripheral nerve injury.

Methods

Reagents

The following reagents were purchased from the sources indicated: Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics and antimycotics were obtained from GIBCO BRL (Long Island, NY). TRI reagent and Griess reagent were acquired from Sigma (St. Louis, MO). Benzonase was obtained from Novagen (Madison, WI). Helenalin, SB202190, SP600125, and U0126 were obtained from Calbiochem (San Diego, CA). Neutralizing anti-TLR3 antibody (TLR3.7) was purchased from Apotech (Switzerland, Geneva). Moloney murine leukemia virus reverse transcriptase (M-MLV RTase) was purchased from Invitrogen (Carlsbad, CA), and the SYBR GREEN PCR Master mix was from Applied Biosystems (Foster City, CA).

Cell Cultures

The immortalized Schwann cells (Bolin *et al*., 1992) 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. Also, F11 cells (a hybrid cell line of mouse N18TG2 neuroblastoma and rat DRG sensory neuron) (Francel *et al.,* 1987) 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.

Preparation of Damaged Neuronal Cell Extract

To induce necrotic neuronal cell damage, F11 were washed with PBS and resuspended in a glial cell culture media at 10^6 cells/ml. Neuronal cell death was induced by four cycles of freezing on liquid nitrogen followed by thawing in water bath at 37°C. Such treatment generally resulted in 95% lysis of the cells as judged by trypan blue inclusion. The cells were spun down and the supernatant was used as DNCE for the following studies. The protein concentration of the DNCE prepared as above ranged 4-5 µg/ml, which was measured by Bradford method. The DNCE as well as other putative glial cell activators were tested for endotoxin contamination before use.

Real-Time Reverse Transcription Polymerase Chain reaction (RT-PCR)

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 3 µg of the total RNA by incubating for 1 h at 37°C in a reaction mixture containing 0.5 µg of oligo $(dT)_{15}$, 0.5 mM dNTP mix, 1X first-strand buffer, RNase inhibitor (5 units), 5mM DTT, and M-MLV reverse transcriptase (5 units). The transcripts of the IL-6, iNOS, LIF, MCP-1, RANTES 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 20 µl 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. The mRNA levels of each target gene were normalized to the levels of GAPDH and are represented as fold induction. The PCR primer sequences used are shown in Table 1.

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 540 nm after 15 min. The nitrite concentration in each sample was calculated by using a standard curve.

Statistical Analysis

The statistical significance of differences in different groups was tested using the unpaired Student's t-test with a threshold of $p<0.001$, *.

Table 1. The sequence of the primers used for real-time PCR.

Primer	Forward	Reverse
IL-6	GCC CTT CAG GAA CAG CTA TG	CAG AAT TGC CAT TGC ACA AC
iNOS	TCT GTG CCT TTG CTC ATG ACA	TGC TTC GAA CAT CGA ACG TC
LIF	TAA ATG CCA CCT GCG CCA TA	TCA TGT TTG GCG CAC ATA GC
MCP-1	GGC CTG TTG TTC ACA GTT GCT	ACA CCT GCT GCT GGT GAT TCT
RANTES	ATC CCT CAC CGT CAT CCT C	CTT CTT CTC TGG GTT GGC AC
GAPDH	AGG TCA TCC CAG AGC TGA ACG	CAC CCT GTT GCT GTA GCC GTA T

Results

To investigate the mechanisms of Schwann cell activation upon nerve injury, we stimulated iSC, an immortalized rat Schwann cells, with DNCE derived from DRG neuronal cell line F11, and tested if the damaged nerve-derived molecules induce proinflammatory gene expression in Schwann cells. To begin with, DNCE stimulation induced IL-6, LIF, MCP-1, and RANTES gene expression by 8, 4, 9, and 45 fold, respectively (Fig. 1). In addition, DNCE induced iNOS gene expression by 860 fold in iSC (Fig. 2A). To test the induction of iNOS transcripts are coupled to the secretion of NO, we measured the level of nitrite in the media upon DNCE stimulation by Griess reaction. We found 23 μM of nitrite was secreted from the iSC upon DNCE stimulation for 24 h (Fig. 2B). These data demonstrate that damaged neuronal cell-derived molecules induce Schwann cell expression of proinflammatory cytokine, chemokines, and iNOS.

To characterize the intracellular signaling pathways induced by DNCE stimulation in iSC, we utilized pharmacological inhibitors for NF-κB and MAP kinases. Pretreatment of iSC with 5 μM helenaline, an inhibitor of NF-κB, completely inhibited the DNCE-induced LIF and MCP-1 gene expression (Fig. 3A and B). Similarly, the addition of MAP kinase inhibitors (SB202190, p38 inhibitor; SP600125, JNK inhibitor; U0126, ERK inhibitor) abrogated the DNCE-induced LIF gene expression (Fig. 3A). In the meanwhile, the addition of these MAP kinase inhibitors inhibited the induction of MCP-1 transcripts by 30-45% (Fig. 3B). However, the treatment of above inhibitors alone did not affect the basal level LIF or MCP-1 gene expression. These data indicate

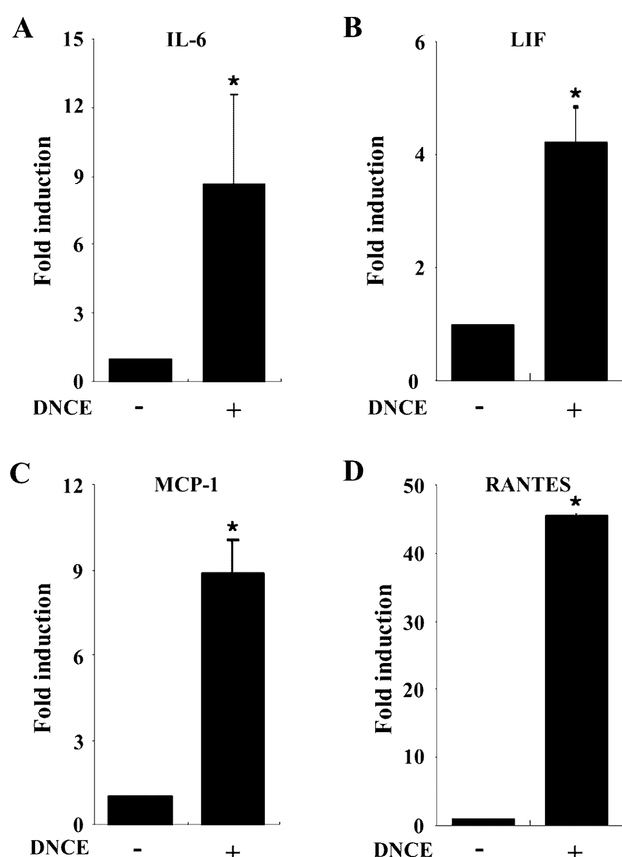

Fig. 1. DNCE stimulation induces proinflammatory gene expression in Schwann cells. iSC cells (4×10^5 cells/ml) were treated with DNCE (8×10^5 cells/ml) for 3 h. Total RNA was isolated from each sample and used to determine IL-6 (A) , LIF (B), MCP-1(C) and RANTES (D) gene expression by real-time RT-PCR. The mRNA levels of each sample were normalized to the levels of GAPDH and are represented as fold induction. The means±S.E.M of three independent experiments are shown. (*, $p< 0.001$)

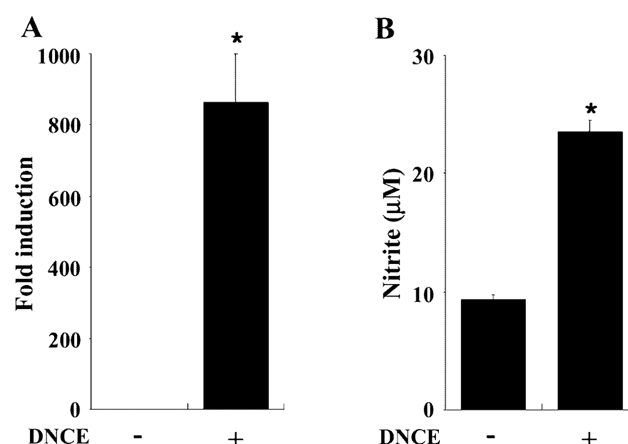

Fig. 2. DNCE stimulation induces iNOS gene expression and NO production in iSC cells. (A) The iSC cells were treated with DNCE (1:2) for 3 h. Total RNA was isolated and used to determine the levels of iNOS transcript by real-time PCR. (B) iSC cells were cultured in the presence or absence of DNCE for 24 h. NO production was determined by measuring the nitrite concentration of the culture supernatant of each sample by the Griess assay. The means ± S.E.M of three independent experiments are shown. (*, $p< 0.001$)

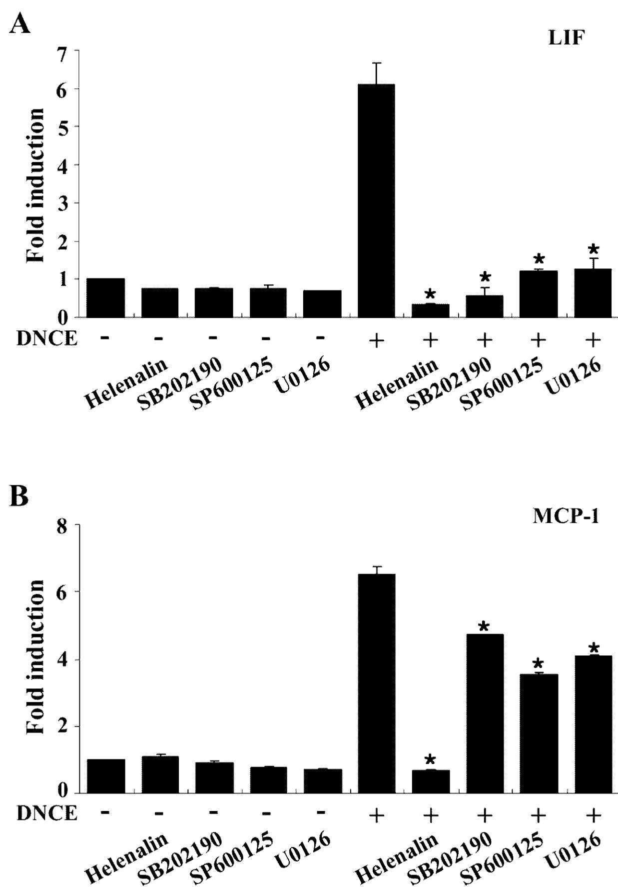

Fig. 3. The activation of NF-κB, p38, JNK and EKR is required for the DNCE-induced LIF and MCP-1 gene expression in iSC. iSC cells were pretreated with NF-κB (5 μM), p38 (10 μM), JNK (10 μM), and EKR (10 μM) inhibitors for 1 h and then stimulated with DNCE (1:2) for 3 h. Total RNA was isolated from each sample and used to determine the level of LIF (A) and MCP-1 (B) transcripts by real-time-PCR. (*, $p < 0.001$; compared to the DNCE-stimulated sample)

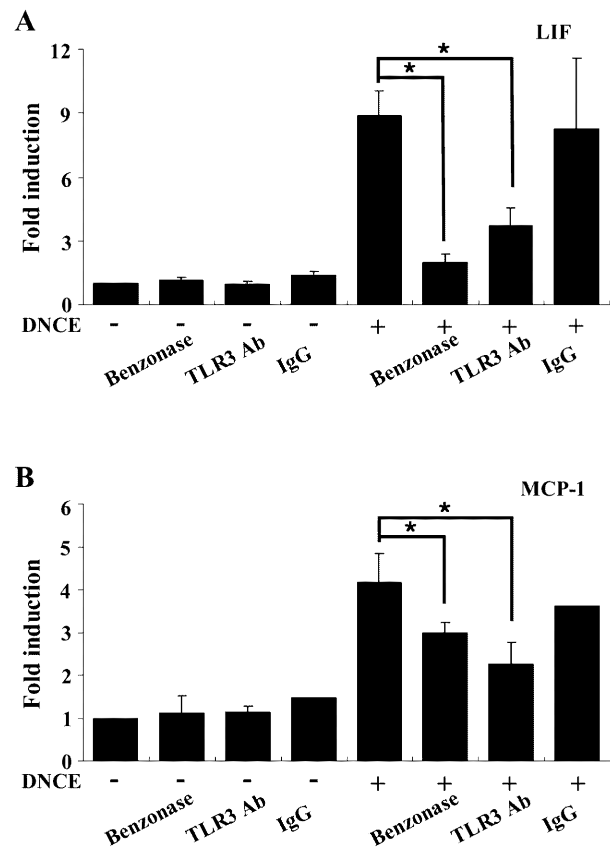

Fig. 4. DNCE stimulate Schwann cells via TLR3. iSC cells were pretreated with benzonase (10 U), IgG (20 μg/ml), or neutralizing TLR3 antibody (20 μg/ml) for 1 h, and then stimulated with DNCE (1:2) for 3 h. Total RNA was isolated and used to determine the level of LIF (A) and MCP-1 (B) transcripts by real-time-PCR. (*, $p < 0.001$)

that the activation of NF-κB, p38, JNK and ERK is required for the DNCE-induced LIF and MCP-1 gene expression in iSC.

Recently, it was reported that mRNA released from necrotic cells activate innate immune cells via TLR3 (Kariko *et al.,* 2004). In an attempt to test the involvement of RNA in the DNCE-induced iSC activation, we degraded RNA in the DNCE by incubated the DNCE with benzonase, a kind of ribonuclease. Upon treatment with benzonase, the DNCE-induced LIF and MCP-1 gene expression was inhibited by 75 and 30%, respectively (Fig. 4A and B). This suggests that RNA released from the necrotic neuronal cells may contribute to the DNCE-mediated LIF and MCP-1 gene expression. In addition, the addition of anti-TLR3 neutralizing antibody inhibited the DNCE-mediated LIF and MCP-1 gene expression by 60 and 45 %, respectively (Fig. 4A and B), indicating that TLR3 on iSC is responsible for the DNCE-induced Schwann cell activation. Taken together, the data suggest that ribonuclease-sensitive damaged neuron-derived molecule activate Schwann cells via TLR3.

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 that inhibits nerve growth. The activated Schwann cells then secrete inflammatory chemokines such as IL-6, LIF, 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, 1999). These neurotrophic factors protect damaged neurons from apoptosis and induce neuronal outgrowth. Therefore, nerve damage-induced Schwann cell activation is a critical step for the successful nerve regeneration. However, it is unclear how the Schwann cells recognize nerve injury and become activated.

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 showed that necrotic cell-derived cytoplasmic molecule such as mRNA and heat shock protein can also bind to TLRs (Kariko *et al.,* 2004; Kakimura *et al.,* 2002). Considering that peripheral nerve injury will also release the axoplasmic molecules, it was reasoned that the TLRs expressed on the Schwann cells might function as receptors that recognize nerve injury. In order to examine this hypothesis, we first tested if Schwann cells are activated by damaged neurons. In our study, the stimulation of iSC induced the mRNA expression of IL-6, LIF, MCP-1, and RANTES. The expression of these proinflammatory genes upon nerve injury in the PNS has been well documented. After nerve injury, IL-6 is immediately upregulated by activated Schwann cells (Bolin *et al*, 1995). Schwann cell production of IL-6 induces the secretion of LIF and MCP-1 from the activated Schwann cells and recruits macrophages from the blood vessel, which is a key feature of Wallerian degeneration (Tofaris et al., 2002). We also found that DNCE induced iNOS gene expression and NO production in iSC. It is well known that NO contributes to inflammation and neuronal cell death (Akaike *et al.,* 1996; Dawson and Dawson, 1996). Additionally, NO production during peripheral nerve injury is implicated in the development of neuropathic pain (Zochodne *et al.,* 1999; Levy *et al.,* 1999). These reports suggest that DNCE-induced NO produced by Schwann cells in the PNS may contribute to the development of neuropathic pain that is often observed in peripheral nerve damage.

Studies on the DNCE-mediated intracellular signaling pathways revealed that the activation of NF-κB, p38, JNK and ERK are required for the DNCE-induced LIF and MCP-1 gene expression. The activation of above signaling molecules in DRG Schwann cells upon peripheral nerve injury has been previously reported (Ma and Bisby, 1998; Myers *et al.*, 2003). Although it is speculative, it is possible that damaged nerve-derived molecules stimulate nearby Schwann cells to activate NF-κB, and MAP kinases. The activation of these signaling molecules, then, induces the production of proinflammatory chemokines and iNOS that are involved in Wallerian degeneration.

It was previously reported that mRNA released from the necrotic cells can activate innate immune cells (Kariko et al., 2004). To investigate the putative molecules in the DNCE that activates Schwann cells, we treated the DNCE with benzonase before use. The data imply that RNA in the DNCE contributes to the activation of iSC. These results suggested that TLR3 on the Schwann cells may function as receptor for the putative Schwann cell activator in the DNCE. Previous study in our lab has shown that TLR3 are expressed on the cytoplasmic and intracellular membrane of primary Schwann cells as well as iSC (not shown data). Indeed, blocking the TLR3 on iSC using neutralizing antibody significantly inhibited the DNCE-induced LIF and MCP-1 gene expression. Taken together, these data argue that RNA released from the necrotic neuronal cells may induce iSC activation via TLR3.

In this study, we discovered that Schwann cells are activated by necrotic neuronal cells. In addition, we found that the DNCE-induced Schwann cell activation is mediated by TLR3. Based on these data, 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 RNA. 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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