Schwann Cell Apoptosis and \(^{75}\text{NTR} \) siRNA

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ABSTRACT

The \(^{75}\text{NTR} \) pan-neurotrophin receptor (\(^{75}\text{NTR} \)) plays a pivotal role in linking the immune system with the nervous system. \(^{75}\text{NTR} \) is required for the development of several characteristic features of allergic asthma. Also \(^{75}\text{NTR} \) upregulated by reactive Schwann cells after peripheral nerve injury.

Moreover \(^{75}\text{NTR} \) and RhoA play a critical role in the regulation of apoptosis. To determine whether the designed siRNA for \(^{75}\text{NTR} \) can downregulates both \(^{75}\text{NTR} \) and Rho-A at RNA level in rats and, if so, at what magnitude, Schwann cell apoptosis occurs. Isolation and purification of neonate Schwann cells were prepared from rat sciatic nerve. Specific siRNA duplex was designed for \(^{75}\text{NTR} \).

To investigate the role of siRNA-mediated knockdown of \(^{75}\text{NTR} \), the gene expression in \(^{75}\text{NTR} \) was examined with reverse transcription–polymerase chain reaction (RT-PCR) and Real-Time RT-PCR. Schwann cell apoptosis was performed by Annexin and TUNEL assays after 24 hours. Following \(^{75}\text{NTR} \) Transfection siRNA, \(^{75}\text{NTR} \) gene, compared with control, was downregulated by 73%. Without using siRNA for Rho-A, Rho-A gene was downregulated by 89% at the same time. Based on Annexin assay, apoptosis of Schwann cells occurred in siRNA+NGF and control+NGF by 16.76±2.27 and 92.39±1.82, respectively.

TUNEL data showed that apoptosis of Schwann cells occurred in siRNA and control by 12.91±6.39 and 78.55±11.85, respectively. Thus, \(^{75}\text{-siRNA} \) downregulated both \(^{75}\text{NTR} \) and Rho-A at RNA level in rats and showed a role on decreased cell apoptosis compared to the controls.

Key words: Apoptosis; Rho-A; Schwann Cell Culture; siRNA; \(^{75}\text{NTR} \)
INTRODUCTION

The p75 pan-neurotrophin receptor (p75NTR) plays a pivotal role in linking the immune system with the nervous system. p75NTR is widely expressed by many kinds of hematopoietic and immune cells such as CD34+ cells and monocytes/macrophages and glial cells in nervous system. Many proinflammatory stimuli such as LPS, IL-1 and TNF-alfa can promote the expression of neurotrophic growth factor (NGF) and its receptors in immune cells.

Moreover, p75NTR is upregulated by reactive Schwann cells after peripheral nerve injury. Schwann cells have been transplanted to injured spinal cord in animal models and phase I spinal cord injury in humans. There is apoptosis in transplanted cells and one of molecules which has a role in cell apoptosis in nervous system and pathogenesis of allergic asthma is the pan-neurotrophin receptor (p75NTR) which is a member of the tumor necrosis factor (TNF) receptor family. Tumor necrosis factor receptor super family (TNFRSF) ligands are crucial to Natural Killer (NK) cell development, ability to kill and their cross talk to other immune cells. There is growing evidence that TNF family receptors play a role in death of NK cells. Rogers M.L showed that p75NTR mediated apoptosis can occur in immune-system cells.

The technique of small interfering RNAs (siRNAs) activate posttranscriptional gene silencing effectively and has become a great tool to knock down specific p75NTR gene expression. In addition, p75NTR can also activate the small GTPase RhoA. Rho family GTPases play a critical role in the regulation of apoptosis. The expression of p75NTR are dramatically up-regulated in activated Schwann cells after nerve injury. Sciatric nerve injury increases the amount of p75NTR which is an appropriate model to study. Nerve growth factor (NGF), the member of the neurotrophin family, stimulates neuronal death through the p75NTR-mediated signaling pathway.

It is possible that the interaction of neurotrophins with p75 NTR in some cells can lead to the activation of several signal pathways. NGF selectively binds with similar affinities to both the tyrosine kinase (Trk) receptors and to the p75NTR. However, few studies have focused on the various roles of p75NTR in Schwann cells which is involved in apoptosis. Following injury, axon growth regeneration is inhibited by binding of NOGO and other inhibitory molecules to the Nogo Receptor (NgR) and p75NTR, which activates intracellular pathways which induce neuronal apoptosis.

Thus, we performed Schwann cells culture following sciatic nerve injury in neonate rats and evaluated whether the designed siRNA for p75NTR can downregulate both p75NTR and Rho-A at RNA level in rat and assessed apoptosis of Schwann cells.

PATIENTS AND METHODS

Isolation and purification of neonate Schwann cells were achieved from rat sciatic nerve. Briefly, sciatic nerves of rat neonates were harvested and cut into 0.5–1 mm segments after being rinsed in Hank's buffered saline solution (HBSS), and stripped of epineurium and connective tissue.

Nerve segments were placed in DMEM (Sigma, USA) for 6 days during which half of their medium was replaced every 3 days (fasting period to eliminate fibroblasts). The culture medium was then replaced by DMEM plus 10% Fetal Calf Serum (FCS). The culture was maintained for 25 days. The whole process of cell culture was performed without providing enzyme and neurotrophic factors. The duration of cell culture was 25 days. Pure cell culture was prepared and confirmed by S100, p75NTR and Hoechst 33342 staining.

SiRNA Preparation

SiRNA was used to produce any change in p75NTR and Rho-A signaling pathway in Schwann cells. A siRNA and scramble (control siRNA) for p75NTR in rat were designed. Schwann cells transfected by siRNA and scramble.

To design specific siRNA duplex for p75NTR, we used target sequence 5'-AACCTCATCCCTGTCTATTGC-3'. The target sequence used for scramble was 5'-GTCCGTATTCTACCACTCTAT -3'. Transfection of siRNAs was performed using X-tremeGENE siRNA Transfection Reagent (Roche applied science cat no. o4476093001). For substance use, the protocol of Higuchi et al. was replicated.

Immunocytochemistry

Schwann cells grown on four-chamber tissue culture slides were washed with PBS three times, fixed in 4% paraformaldehyde for 10 min, permeabilized...
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with 0.2% Triton X-100 and again was washed three more times with PBS, and blocked for 1 hour with 5% goat serum and 2% bovine serum albumin (BSA). Cells were then incubated with primary antibodies overnight at 4C. Polyclonal anti-p75NTR antibody (SIGMA N3908) (diluted 1:1000), monoclonal anti RhoA antibody (diluted 1:1000) were used as the primary antibody. Cells were then washed three times with PBS, followed by incubation with fluorescein-conjugated secondary antibodies (invitrogen cat no.715-096-100) (diluted 1:1000) for 1 h at room temperature. After a second rinsing step with PBS, the cells were incubated with Hoechst 33342 (SIGMA), to counterstain cell nuclei for 5 min at room temperature. The coverslips were then washed three times in PBS for 5 min each. Labeled cells were examined with fluorescence microscopy.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR)
Total RNA was extracted from purified Schwann cells using the High Pure RNA Isolation Kit (Roche no. 11828665001) according to the manufacturer’s instructions. Total RNA from Schwann cells was extracted using Maxime RT-PCR Premix Kit (iNtRON) according to the manufacturer’s instructions. RT-PCR for RhoA evaluation was based on addition of RhoA primer to our previous solution of RNA extraction from Schwann cell transfected with siRNA on p75NTR but not siRNA for RhoA.

Real-Time Reverse Transcription–Polymerase Chain Reaction (Real-Time RT-PCR)
To investigate the role of siRNA-mediated knockdown of p75NTR, the gene expression in p75NTR was examined with Real-Time RT-PCR. Real-Time RT-PCR primers for p75, Rho-A and β-actin genes were designed and synthesized by MWG company. Schwann cell RNA was extracted with the High Pure RNA Isolation Kit).

cDNA was synthesized using PrimeScript™ RT reagent Kit (Perfect Real Time) Cat. #RR037T v0901Da Takara– SYBR Green Assay. Gene expression was measured by real-time PCR (Corbett, NSW, Australia) using 2 µl of cDNA with a three-step program: 95 °C for 20 s, 53 °C for 35 s and 72 °C for 45 s for 40 cycles. Duplicate samples without cDNA (no-template control) for each gene showed no contaminating DNA. β-actin was used as a normalizer.

Relative mRNA levels were quantified in three samples per group using the comparative Ct method.22

TUNEL Assay
For detection of DNA fragmentation in Schwann cells in vitro, Schwann cells grown on four well chamber slides. In situ detection of DNA fragments by terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) was performed using in situ DNA fragmentation assay Kit and manufacturer’s instructions was followed (In Situ Cell Detection kit; Roche cat no. 11684795910). Briefly, Schwann cells treated with siRNA or scramble as control for 24h, then were incubated with NGF for 18h, were fixed with 4% (wt/vol) paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated with equilibration buffer. Slides were layered with TUNEL reaction mixture containing terminal deoxynucleotidyltransferase-mediated dUTP fluorescein nick end labeling (TUNEL) reaction mixture supplied in the in situ Cell Death Detection Kit, incubated the cells for 1 hour at 37°C in a humid chamber and visualized by fluorescence microscopy.

Dual Staining by Apoptotic kit Annexin V and Propidium Iodide
For additional estimate of cell death, the translocation of phosphatidylserine was assessed from the inner side to the outer layer of the plasma membrane and also the loss of membrane integrity using Annexin-V-Flous Staining kit (Rochecat no. 11988549001) and by fluorescence microscopy. Schwann cells were transfected with p75-siRNA or scramble as control after 24h, and then cells were incubated with 2.5S NGF (Invitrogen cat no.13257-019) for 18 h. At last, cells were fixed, analyzed for Annexin and stained with propidium iodide (PI) to label whole cells. Reagents were added in 1 ml incubation buffer HEPES, washed slides with PBS, incubated with reagent mixture for 10-15 min at 15-25°C and these were PI images of the same field of view. The Schwann cells apoptosis were compared with p75-siRNA and Control using fluorescence microscopy. TUNEL positive cells were counted and represented as the percentage for PI stain.

Statistical Analysis
Schwann cell apoptosis is expressed as mean ± standard error of mean (SEM) from eleven fields in 24
hours. Statistical significances of differences were tested using the independent Student’s t test.

RESULTS

Pure Schwann cell culture was prepared. The purity of Schwann cells were 100%.

RT-PCR and Real-Time RT-PCR

We showed that, siRNA could suppress both p75\textsuperscript{NTR} (Figure 1) and Rho-A gene by RT-PCR. Real-Time RT-PCR data demonstrated that following p75\textsuperscript{NTR} siRNA, p75\textsuperscript{NTR} gene compared with control down regulated by 73% after 48 hours. On the other hand, without using siRNA for RhoA, Rho gene was down regulated by 89% at the same time.

Figure 1. Reverse transcription-polymerase chain reaction (RT-PCR) of p75NTR and RhoA 48 hour after transfection with p75NTR siRNA and scramble ; 1: scramble (RhoA); 2: siRNA (RhoA); 3: scramble (p75NTR); 4: siRNA (p75NTR)

Figure 2. The inhibitory effect of p75-siRNA on p75 expression in primary Schwann cell culture. Schwann cells have been stained with antibody against p75. (A) p75 expression in Schwann cells transfected with p75-siRNA. (B) p75 expression in Schwann cells transfected with scramble. Nuclei have been stained with Hoechst 33342.

Figure 3. The inhibitory effect of p75-siRNA on Rho-A expression in primary Schwann cell culture. Schwann cells have been stained with antibody against Rho-A. (A) Rho-A expression in Schwann cells transfected with p75-siRNA. (B) Rho-A expression in Schwann cells transfected with scramble. Nuclei have been stained with Hoechst 33342.
Schwann Cell Apoptosis Assays by Annexin V and Tunnel

Schwann cell apoptosis was performed by Annexin and TUNEL assays. Quantitative assays were performed by Annexin V and Tunnel assay kits for 24 hours. Annexin data showed that apoptosis of Schwann cells occurred in siRNA and control by 4.42% ± 2.38 and 32.39% ± 1.82, respectively. TUNEL data showed that apoptosis of Schwann cells occurred in siRNA and control in 7.06% ± 1.31 and 23.33% ± 3.00, respectively.

Induction of apoptosis by NGF in siRNA and control were performed. Annexin data showed that apoptosis of Schwann cells occurred in siRNA+NGF and control+NGF by 16.76% ± 2.27 and by 92.39% ± 1.82, respectively. TUNEL data showed that apoptosis of Schwann cells occurred in siRNA and control by 12.91% ± 6.39 and 78.55% ± 11.85, respectively.

Histogram of percent of apoptotic Schwann cells is seen in figure 4 which demonstrated that NGF induced Schwann cell apoptosis, and siRNA can decrease apoptosis compared with control group. Meanwhile, siRNA alone can decrease apoptosis compared with control group. Immunocytochemistry for P75 NTR (Figure 2) and Rho-A were also demonstrated (Figure 3).

DISCUSSION

We showed that downregulation of p75 using siRNA protected Schwann cells against NGF-induced cell death. Downregulation of p75 mRNA also caused an indirect downregulation of RhoA mRNA. These results demonstrated the feasibility of reducing the vulnerability of Schwann cell death to neurotrophin-induced apoptosis and suggest that this may act via the RhoA signaling pathway in these cells.

Higuchi et al. also had demonstrated that apoptosis was induced by NGF through p75NTR in mice. They had showed that NGF-induced cell death in Schwann cells which is induced by myelin associated glycoprotein are attenuated by the siRNA.9

Induction of cell death through p75NTR has been described in Schwann cells also in other papers.23-25

Khursigara et al. described an interaction between p75NTR and receptor-interacting protein 2 (RIP2). RIP2, an adaptor protein with a serine threonine kinase and a caspase recruitment domain (CARD), is highly expressed in dissociated Schwann cells. RIP2 binds to the death domain of p75 via its CARD domain in an NGF-dependent manner.23

Hirata et al. showed that p75NTR signals differentiation and apoptosis through intracellular...
ceramide elevation. The final response is dependent on the intracellular ceramide level and Schwann cells adjust their response by changing expression level of p75NTR which is selective for NGF.24

The study by Soilu-Hanninen et al. demonstrated the existence of two separate pathways that accelerate apoptosis in Schwann cells: a Bcl-2-blockable pathway initiated on loss of NGF support, and a Bcl-2-independent, the cytokine response modifier A (CrmA)-blockable pathway mediated via the p75NTR.25

In addition, induction of apoptosis by NGF through p75NTR has been described in oligodendrocytes26 and retinal neurons.27

Apoptosis is directly adjusted by different members of the Ras and Rho GTPases. Under serum-starved conditions Rac1 stimulates signals in fibroblast cell line that result in an increase of ceramide levels and Fas ligand gene expression, which lead to apoptosis. Both JNK and nuclear factor kappa B (NF-κB) are concerned in transformation downstream of Rho GTPases, suggesting that both these signals might tilt the balance towards apoptosis or transformation depending on the extracellular signals. It means that it depends on serum starvation versus presence of growth factors.12

On the other hand, in the absence of TrkA, NGF binding to p75NTR induces the apoptotic cell death via activation of NF-kB in Schwann cells and oligodendrocytes.25,26

In our study, RT-PCR showed the downregulation of p75NTR and Rho-A expression after 24 hours. Ahmed et al. showed siRNA-mediated silencing of components of the inhibitory signalling cascade, including p75NTR, NgR and Rho-A mRNA, of 70%, 100% and 100% of the related protein, respectively. They concluded that Rho-A knockdown might be the most successful object for a disinhibition strategy to encourage CNS axon regeneration in vivo.28

There were several differences between our and Ahmed’s study. First, our target cell was Schwann cell instead of dorsal root ganglion cell. Second difference was in siRNA, which our designed specific siRNA for p75NTR downregulated RhoA without using siRNA for RhoA. Third, Ahmed et al., used CNS myelin extract in their experiment as inhibitory molecules, therefore they evaluated NgR. The last difference was the time which we performed our experiments one day and compared expression of p75NTR and Rho-A in this time period rather than 3 days in Ahmed’s study.

In conclusion, these results demonstrated that p75 using siRNA protected Schwann cells against NGF-induced cell death. Decreased Schwann cell apoptosis could be explained by downregulated p75NTR and Rho-A following using p75NTR-siRNA. Thus, for future studies, it is recommended to transfet cells such as Schwann or Olfactory Ensheathing Cells with p75NTR-siRNA before transplantation to the injured spinal cord in a rodent and see whether apoptosis decreases in vivo.

REFERENCES

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