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TNFα mediates Schwann cell death by upregulating p75^{NTR} expression without sustained activation of NFκB

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Administration of tumour necrosis factor α (TNFα) to axotomised mouse neonatal sciatic nerves increased Schwann cell apoptosis in the distal nerve segments, 5-fold greater than axotomy alone. TNFα upregulated the low affinity neurotrophin receptor, p75^{NTR}, indicative of phenotype reversion in Schwann cells. Furthermore, re-expression of p75^{NTR} and downregulation of the pro-myelinating transcription factor, Oct 6, in Schwann cells occurred by treatment with TNFα, even after the maturation of these cells with brain derived neurotrophic factor (BDNF). TNFα treatment of Schwann cells produced only a transient activation of NFκB. More importantly, in NFκB (p65) mutant mice, axotomy increased Schwann cell apoptosis further than that seen in mice expressing NFκB (p65), implicating a survival role for NFκB. Collectively, these data suggest that TNFα can potentiate Schwann cell death through the modulation of their phenotype. Immature Schwann cells express a high level of p75^{NTR} and as a consequence are susceptible to extracellular death stimuli because of the lack of sustained NFκB translocation.
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Introduction

During development of the peripheral nervous system (PNS), Schwann cells are produced in excess number to ensure the establishment of a one-to-one association between the Schwann cell and the nerve axon. Those Schwann cells that do not establish an axonal connection are referred to as 'supernumerary' Schwann cells, which are eliminated by apoptosis (Grinspan et al., 1996; Nakao et al., 1997; Syroid et al., 2000). Axonal contact is one of the most important regulatory events not only in Schwann cell viability but also in maturity (Dong et al., 1995). For instance, the apoptotic death of these cells can be further potentiated by axotomy (Syroid et al., 2000), and susceptibility to apoptosis is heavily reliant on axonally derived trophic support (Winseck et al., 2002) as well as Schwann cell maturity (Dong et al., 1995). Interestingly, an induced peripheral nerve injury such as axotomy will potentiate the pro-inflammatory cytokine response (Shamash et al., 2002), initiate alterations in the Schwann cell cycle, alter Schwann cell phenotype, and possibly initiate Schwann cell death (Bonetti et al., 2000; Chandross et al., 1996; Lisak et al., 2001; Myers et al., 2003).

Two signalling mechanisms known to result in Schwann cell death include the TNFα/TNFR1 (Hall et al., 2000) and NGF/p75^{NTR} pathways (Syroid et al., 2000). Schwann cells have been shown to express both p75^{NTR} and TNFR1 (Skoff et al., 1998; Syroid et al., 2000), and we have shown upregulated Schwann cell apoptosis in vivo upon administration of exogenous NGFβ to axotomised sciatic nerves in wild-type neonatal mice but not in p75^{NTR} -mutant mice, suggesting that p75^{NTR} may potentiate Schwann cell apoptosis (Petratos et al., 2003). Furthermore, in vitro evidence has defined p75^{NTR} -mediated Schwann cell death as independent of Bcl-2 (Soilu-Hanninen et al., 1999). However, the role of p75^{NTR} in initiating Schwann cell death is dependent on many extracellular and intracellular factors which govern the development and survival of these cells (Bhakar et al., 1999; Khursigara et al., 2001).

The molecular mechanisms which determine Schwann cell maturation during development also determine their survival and relate primarily to axon–Schwann cell and Schwann cell–Schwann cell interactions. These unique associations drive myelination through various transcription factors which are activated as a consequence of growth factor/cytokine signalling mechanisms, and modulation of these mechanisms has profound

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effects on Schwann cell development, survival, and death (for review, see Wegner, 2000). For instance, when the transcription factor nuclear factor (NF) κB, an upstream regulator of Oct 6 (SCIP or tst1, a POU domain containing protein which has been associated with the progression of the immature Schwann cell toward a mature myelinating phenotype), is inhibited in myelinating co-cultures, myelination is prevented (Nickols et al., 2003).

In this study, we have focused on one of the prime candidates of peripheral nerve disease and injury, the pro-inflammatory cytokine, TNFα, and its ability to regulate molecular and cellular events in Schwann cells in the context of axonal injury. We have found that TNFα can upregulate the expression of $p75^{NTR}$ and render Schwann cells susceptible to apoptosis since the activation of NFκB is transient and not sustained. These data have direct implications in inflammatory demyelinating conditions where pro-inflammatory cytokines such as TNFα may in fact drive demyelination through phenotype reversion and subsequently render Schwann cells susceptible to death due to the lack of sustained endogenous activation of specific survival factors such as NFκB, integral for Schwann cell maturation and survival.

Materials and methods

Antibodies

The following primary antibodies were used to examine receptor and transcription factor levels in Schwann cells by Western immunoblotting, immunocytochemistry, and immunohistochemistry: mouse anti-rat/mouse Thy1.1 IgG, mouse anti-S-100α and β IgG (Chemicon International; CA); goat anti-soluble TNFR1 (sTNFR1) IgG, mouse anti-β-actin IgG (Sigma-Aldrich Co.; MI); rabbit anti-human $p75^{NTR}$ IgG (Promega Corporation; WI); rabbit anti-TNFR1 Ig, mouse anti-human NFκB (p65 subunit) (Santa Cruz Biotechnology; CA), mouse anti-activated caspase-3 IgG (Cell Signalling Technology Inc.; MA), rabbit anti-SCIP (Oct 6) [gift from Dr. Todd Zorick of the Salk Institute, CA; Zorick et al., 1996].

The following secondary antibodies were used: sheep anti-mouse horseradish peroxidase [HRP]-conjugated affinity purified IgG, goat anti-rabbit HRP-conjugated affinity purified IgG, donkey anti-sheep/goat HRP-conjugated affinity purified IgG, goat anti-mouse rhodamine isothiocyanate (RITC)-conjugated affinity purified IgG, Cy3-conjugated anti-mouse IgG (Chemicon); sheep anti-mouse biotin-conjugated affinity purified IgG, sheep anti-rabbit biotin-conjugated affinity purified IgG (Vector Laboratories; CA); donkey anti-goat Alexa Fluor 546-conjugated IgG, fluorescein isothiocyanate (FITC)-conjugated streptavadin (Molecular Probes Inc.; OR).

Animals, genotyping, and surgery

Sciatic nerve Schwann cell isolation from post-natal days 2–3 wild-type C57Bl/6 mouse and Wistar rat pups was approved by the Monash University Departments of Biochemistry, Anatomy and Microbiology Animal Ethics Committee. Mice lacking p65 (RelA) die at mid-gestation due to TNFα-mediated hepatocyte apoptosis (Beg et al., 1995; Doi et al., 1999). To examine the role of p65 in Schwann cell survival, viable $p65^{-/-}$ mice were generated on a *TNFα*-deficient background as described previously (Gugasyan et al., 2004). Post-natal day 0–1 wild type, $p65^{-/-}/TNF\alpha^{-/-}$ mice, $p65^{+/-}/TNF\alpha^{-/-}$ mice, and $p65^{+/+}/TNF\alpha^{-/-}$ mice were generated on a 129/B6 background and genotyped for authenticity. DNA was isolated from tails of post-natal day 0–1 mice from $p65^{+/-}/TNF\alpha^{-/-}$ heterozygous matings, and the p65 genotype was determined by PCR. PCR primers for p65 were (p65 5′) CCTATAGAGGAGCAGCGCGGG; (p65 3′) AATCGGATGTGAGAGGACAGG; and (HH-Neo) AAATGTGTCAGTTTCATAGCCTGAAGAACG. The left sciatic nerve of these mice was axotomised as previously described (Petratos et al., 2003) in accordance with the Royal Melbourne Hospital Animal Ethics Committee. Gelfoam (Upjohn companies; MI) soaked in either 1 μg tumour necrosis factor-α [TNFα] (Peprotech; Rocky Hill, NJ) or 1 μg nerve growth factor-β [NGFβ] (Peprotech) was applied to the transected nerve segments of wild-type 129/Sv × BalbC mice. All animals were sacrificed 24 h post-axotomy under ice anaesthesia followed by transcardial perfusion with PBS and 4% paraformaldehyde (pH 7.4). Sciatic nerve segments (proximal, distal, and contralateral) were excised and processed for immunohistochemistry or alternately dissected out, and the segments frozen in liquid nitrogen for Western immunoblotting.

Schwann cell culture

Sciatic nerves from a cohort of mouse and rat pups (n = 8 for rats and n = 11 for mice) were pooled into 2 ml basal Schwann cell medium [Dulbecco's Modified Eagles Medium [DMEM] (Gibco, Invitrogen Corporation; CA) supplemented with 10% foetal calf serum [FCS] v/v, 1% v/v penicillin/streptomycin (Commonwealth Serum Laboratories; Victoria, Australia), 2 μM Forskolin, and 10 μg/ml bovine pituitary extract [BPE] (Sigma-Aldrich) then digested with 2 mg of collagenase-D (Roche Applied Science; Mannheim, Germany) and 10% v/v of trypsin (Commonwealth Serum Laboratories) until the tissue was degraded. The Schwann cell suspension was then applied to an anti-rat/mouse Thy 1.1 antibody-coated 10 cm plate for immunopanning at 37°C with 5% CO_2 for 30 min in order to remove contaminating fibroblasts. Following immunopanning, the cell suspension was collected and centrifuged at 180 × g for 5 min. Schwann cells were plated into poly-L-lysine (25 μg/ml in sterile PBS; Sigma-Aldrich)-coated T75 flasks (CellStar; CA) and incubated at 37°C with 5% CO_2 for 24 h before changing the medium. The medium was replenished every 48 h to ensure growth to confluence before passaging. The fibroblast cell line 3T3 was utilised in all experiments as a control for any fibroblast contamination remaining in the culture following immunopanning and to determine whether the cytokines and growth factors administered exhibited any molecular effects on fibroblasts.

Immunofluorescence

(i) Anti-S-100 α + β, TNFR1, $p75^{NTR}$ in vitro

Schwann cells were seeded at a density of 100,000 cells/well into 8-well chamber slides (LabTech; Uckfield, U.K) and allowed to grow for 3 days, changing the medium every 24 h. The cells were fixed with 4% paraformaldehyde at RT for 10 min. Cells were washed (3 × 10 min with PBS, pH 7.4) and blocked with blocking buffer (10%FCS, 0.3%Triton X-100 in PBS, pH 7.4) for 30 min at RT. Schwann cells were probed with either rabbit anti-human $p75^{NTR}$ IgG (1:200 in blocking buffer; Promega), goat anti-mouse-TNFR1 antibody (1:200 in blocking buffer; Sigma-Aldrich), and mouse anti-S-100α and β antibody (1:200 in blocking buffer;

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