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# Fas ligand acts as a counter-receptor in Schwann cells and induces the secretion of bioactive nerve growth factor

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ABSTRACT

Fas ligand (FasL) is best known for its role in apoptosis. Membrane-bound FasL can signal in FasL-bearing cells, a process known as reverse signalling. The biological and functional consequences of FasL reverse signalling in Schwann cells were studied. FasL engagement induced the secretion of soluble mediator(s) that stimulated neurite growth in PC12 cells, NGF secretion, and NGF mRNA levels. ERK1/2 and Src phosphorylation was rapidly increased and inhibition of their activation affected NGF synthesis and release. FasL can therefore act as a signal-transducing molecule in Schwann cells, leading to the secretion of NGF, and may contribute to peripheral nerve regeneration.



## 1. Introduction

Fas ligand (FasL/CD95L/TNFSF6) is a member of the tumor necrosis factor superfamily (TNF) (Suda et al., 1993). FasL is a type II homotrimeric transmembrane protein with both cell surface and secreted isoforms (Suda et al., 1993; Tanaka et al., 1995). FasL is mostly studied as a ligand for Fas receptor, and was first described as a trigger for apoptotic cell death in Fas-expressing cells in the immune system (Trauth et al., 1989; Yonehara et al., 1989). The Fas/FasL pair has been extensively studied in the immune system, and is involved in clonal deletion of thymocytes and downregulation of the immune response (Aggarwal, 2003). More recently, Fas has been implicated in non-apoptotic functions in the immune system, including stimulation of T-cell responses and enhancement of proinflammatory cytokine secretion (Peter et al., 2007).

Although FasL is primarily studied as a ligand for the Fas receptor, FasL itself is able to transduce intracellular signals, thus acting as a counter-receptor. This feature is common to many members of the TNF superfamily, which have been considered "bi-directional" receptors (reviewed in (Eissner et al., 2004). The intracellular domain of FasL is highly conserved across species and presents several features of a signalling molecule (Ghadimi et al., 2002; Linkermann et al., 2003; Sun and Fink, 2007; Wenzel et al., 2001). It contains a proline rich region that interacts with proteins containing Src homology 3 (SH3) and WW domains (Ghadimi et al., 2002; Hane et al., 1995; Voss et al., 2009; Wenzel et al., 2001). In T-cells, FasL can transduce "reverse" or "counter" signals that regulate cell cycle progression and cytokine secretion (Desbarats et al., 1998; Suzuki and Fink, 1998), (reviewed in (Newell and Desbarats, 1999)) as well it can also regulate its own expression and trafficking (Sun and Fink, 2007).

Fas and FasL are not restricted to the immune system, but are also co-expressed in the reproductive and nervous systems (Bonetti et al., 2003; French et al., 1996; Moalem et al., 1999; Suda et al., 1993). In the nervous system, Fas and FasL are constitutively expressed by neurons and glial cells *in vivo* and *in vitro*, and both molecules are upregulated after nervous system injury (Choi and Benveniste, 2004). We have recently shown that FasL engagement on Schwann cells leads to mobilization of a number of intracellular signalling pathways (Thornhill et al., 2007), suggesting that FasL reverse signalling may occur in the nervous system, as well as in the immune system. Schwann cells are the main glial cells of the peripheral nervous system (Jessen and Mirsky, 1998). They originate from the neural crest during embryogenesis and differentiate into myelinating and non-myelinating mature Schwann cells (Jessen and Mirsky, 2005). The transition from immature Schwann cells to mature Schwann cells (myelinating or non-myelinating) is marked by a stop in proliferation and an exit

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from the cell cycle. This cell cycle arrest is reversible and following injury, the Schwann cells are able to de-differentiate and re-enter the cell cycle (Jessen and Mirsky, 2005). While Schwann cell precursors need to be in close proximity with axons for survival, immature and mature Schwann cells can survive independently from axons thanks to the establishment of an autocrine survival loop (Jessen and Mirsky, 2005; Meier et al., 1999). Schwann cell development is controlled by extracellular signals including neuregulin-1, endothelin, and Notch. The extracellular matrix also plays a role in Schwann cell proliferation and myelination. In addition, the myelination program is controlled by the transcription factors Sox-10, Krox-20 (Egr-2), and Oct-6 (Mirsky et al., 2008). Schwann cells are the neurotrophin-producing cells of the PNS (Bunge, 1994; Reynolds and Woolf, 1993) and they secrete the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), ciliary neurotrophic factor (CNTF), and glial cell-derived neurotrophic factor (GDNF) (Bunge, 1994; Frostick et al., 1998; Reynolds and Woolf, 1993; Watabe et al., 1995), as well as components of the extracellular matrix and cell adhesion molecules (Mirsky et al., 2008; Reynolds and Woolf, 1993). Following PNS injury, Schwann cells de-differentiate and adopt a proliferating phenotype. During Wallerian degeneration they also upregulate the synthesis of growth factors, extracellular matrix proteins, and cell adhesion molecules (Mirsky et al., 2008; Reynolds and Woolf, 1993). PNS regeneration following injury cannot occur without Schwann cells (Bunge, 1994) but even in the presence of Schwann cells, recovery is never complete (Frostick et al., 1998). Therefore, uncovering new signals that may enhance regeneration is of significant importance.

Here we explore the biological and functional consequences of FasL reverse signalling on Schwann cells. It is shown that FasL engagement in Schwann cells leads to the release of bioactive NGF, and the increased production of NGF mRNA in murine cells. Furthermore, these results are extended to primary human Schwann cells, suggesting that the Fas/FasL system modulates growth factor synthesis and release in human peripheral nervous system.

## 2. Materials and methods

### 2.1. Mice

C3H and C57BL/6 (*wt*) mice were purchased from Charles River Canada and B6.MRL-Fas$^{lpr}$ (*lpr*) mice were bred in our animal facility. All experiments followed Canadian Council on Animal Care ethical guidelines and were approved by the McGill University Animal Care Committee.

### 2.2. Schwann cell preparation and culture

Schwann cells from different origins were used. Primary Schwann cells were isolated from newborn mice (in developmental stage) or from the sciatic nerve of adult mice 7 days post a crush injury at a stage when they de-differentiated and switched to a proliferative phenotype (following Wallerian degeneration). Schwann cells from human origin were also used to assess whether FasL reverse signaling is of physiological relevance to humans. A transformed mouse Schwann cell line, MSC80, was finally used for the availability of large number of cells. Expression of FasL was confirmed in primary Schwann cells and MSC80 cells by RT-PCR (data not shown).

Primary mouse Schwann cells were prepared from dorsal root ganglia of C3H newborn mice (post-natal day 0 to 4) or sciatic nerves of adult C57BL/6 (*wt*) and B6.MRL-Fas$^{lpr}$ (*lpr*) mice. Schwann cells from newborn mice were isolated following a method modified from Weinstein and Wu (Weinstein and Wu, 2001). In brief, dorsal root ganglia were digested in 0.25% collagenase (EC 3.4.24.34; Sigma), followed by 0.125% trypsin (EC 3.4.21.4; Worthington Biochemicals) and 4 U/mL DNase I (EC 3.1.21.1; Worthington Biochemicals). Cell suspensions were plated on 60-mm BD Primaria™ cell culture dishes (BD Biosciences) in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO® Invitrogen) plus 10% fetal calf serum (FCS; HyClone), 100 U/mL penicillin/100 μg/mL streptomycin (GIBCO® Invitrogen), and 0.1 mM non-essential amino acids (GIBCO® Invitrogen). Cells were treated for 2 days with cytosine arabinosine (AraC; Sigma; 10 μM) and then grown in DMEM supplemented with 10% FCS, penicillin/streptomycin, non-essential amino acids, bovine pituitary extract (Sigma; 10 μg/mL) and forskolin (EMD Chemicals; 2 μM). At confluency, Schwann cells were purified by antibody-mediated cytolysis with anti-Thy 1.2 (AbD Serotec) and Low-Tox®-M rabbit complement (Cedarlane) to eliminate contaminating fibroblasts (Weinstein and Wu, 2001). For preparation of conditioned medium (CM), cells were plated in DMEM/2% FCS and treated for 48 h with 5 μg/mL anti-FasL antibodies (clone MFL3), or isotype-matched control antibodies (BD Biosciences).

Schwann cells from adult C57BL/6 (*wt*) and B6.MRL-Fas$^{lpr}$ (*lpr*) mice were isolated from sciatic nerves 7 days post crush injury following a method modified from Pannunzio et al. (Pannunzio et al., 2005). In short, sciatic nerves were dissected, epineurium was removed, and the nerves were digested in 0.05% collagenase (Sigma)/0.1% hyaluronidase (EC 3.2.1.35; Sigma). The resulting cell suspension was plated on laminin-coated plates in DMEM supplemented with 10% FCS, 100 U/mL penicillin/100 μg/mL streptomycin, and non-essential amino acids. Cells were kept in culture for 5 days after isolation, and medium was changed every 2 days. Before treating the cells for FasL engagement, the cells were serum-starved for 18 h in DMEM supplemented with F12 nutrient mixture (Sigma) and N2 supplement (GIBCO® Invitrogen). Cells were then treated with 5 μg/mL murine FasFc protein chimera (mFasFc; R&D Systems) or 5 μg/mL control antibody (BD Biosciences or Jackson Immunoresearch Laboratories, Inc).

The transformed Schwann cell line, MSC80, was provided by Dr. Anne Baron-Van Evercooren (INSERM, Paris, France) (Boutry et al., 1992). MSC80 cells were serum-starved for 18 h and then treated for the indicated times with 5 μg/mL murine FasFc protein chimera (mFasFc; R&D Systems) or 5 μg/mL control antibody (BD Biosciences or Jackson Immunoresearch Laboratories, Inc). When indicated, the cells were pretreated for 30 min with the following inhibitors: PD98059 (30 μM, EMD Chemicals), PP2 (10 μM; EMD Chemicals), actinomycin D (ActD; 5 μg/mL; Sigma), or cycloheximide (CHX; 10 μg/mL; Sigma).

Human Schwann cells were obtained from embryonic sciatic nerves from late term abortuses as previously described (Weinstein and Wu, 2001; Wu et al., 2001). All procedures were reviewed and approved by the Institutional Review Boards at the Albert Einstein College of Medicine and the Bronx Municipal Medical Center. Human Schwann cells were grown in DMEM supplemented with 10% FCS, penicillin/streptomycin, non-essential amino acids, bovine pituitary extract (Sigma; 10 μg/mL) and forskolin (EMD Chemicals; 2 μM). For experiments, cells were plated for 18 h in DMEM supplemented with 5% FCS and then treated with anti-FasL antibody clone NOK1 or clone MFL3 (5 μg/mL; BD Biosciences).

Rat Schwann cells were provided by Shireen Hossain (McGill University, Montreal, Canada). They were obtained from dorsal root ganglia of embryonic (E15) Sprague–Dawley rats as previously described (Fragoso et al., 2003). Rat Schwann cells were serum-starved for 18 h before treatment with anti-FasL antibody clone MFL4 (BD Biosciences) or mFasFc at 5 μg/mL.

### 2.3. PC12 neurite outgrowth assay

PC12 cells were obtained from the American Type Culture Collection (ATCC) and grown in DMEM supplemented with 10% FCS and 5% horse serum (HyClone). For assays, PC12 cells were seeded in 96-well plates in CM from Schwann cells stimulated with anti-FasL antibody clone MFL3 (BD Biosciences), isotype-matched control