THE REGULATION OF CILIARY NEUROTROPHIC FACTOR, LEUKEMIA INHIBITORY FACTOR AND MONOCYTE CHEMOATTRACTANT PROTEIN-1 IN INJURED PERIPHERAL NERVOUS TISSUE

by

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To God

and

to my parents,

ABSTRACT

Studies of Wallerian degeneration, the phenomenon wherein axons severed from the nerve cell body disintegrate, have revealed tightly regulated interactions between axons and Schwann cells. This present study focused on Schwann cell changes that occur following nerve injury. To tease out the different signals that influence the changes in Schwann cell phenotype, the regulation of three Schwann cell genes, namely ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF) and monocyte chemoattractant protein-1 (MCP-1), was investigated. Data showed that CNTF mRNA, regulated in a similar manner as myelin P_0, is induced by axonal contact even in the absence of transport from the nerve cell body. The mRNAs for LIF, which belongs to the same family as CNTF, and MCP-1 are induced in a biphasic manner in transected nerves – within 3-6 hours at the site of lesion and by 24 hours in the entire distal stump. Also, the induction of MCP-1 mRNA at the lesion site appears to be mediated by oxygen radicals. *In vitro*, Schwann cells produce MCP-1 mRNA in response to TNF-α stimulation through a pathway that can be inhibited by anti-oxidants and does not appear to require NF-κB activation. However, *in vivo* TNF-α is not crucial to the induction of MCP-1 mRNA in injured PNS. In contrast to the signal that decreases CNTF mRNA synthesis, the signals that induce MCP-1 and LIF mRNA are not strongly dependent on axonal degeneration.

RESUMÉ

La dégénérescence Wallérienne (dégénérescence antérograde) est une lésion de l'axone entraînant, en aval de celle-ci, une perte de la partie axonale distale qui n'est plus en continuité avec le corps cellulaire. Les études portant sur ce phénomène ont mis en évidence d'importantes interactions entre les axones et les cellules de Schwann (cellules gliales du système nerveux périphérique). Le travail présenté ici est centré sur les changements phénotypiques des cellules de Schwann qui se produisent après une lésion axonale. Afin d'isoler les signaux qui provoquent ces changements, nous avons choisi d'etudier la régulation de trois gènes qui induisent l'expression par les cellules de Schwann d'une part de deux cytokines de la même famille, la facteur neurotrophique ciliaire (ciliary neurotrophic factor; CNTF) et la facteur inhibant la leucémie (leukemia inhibitory factor; LIF) et d'autre part d'une chémokine, la protéine-1 chémoattracteur des monocytes (monocyte chemoattractant protein-1; MCP-1). Nous avons montré que le CNTF est induit par contact axonal même en l'absence de transport provenant du corps cellulaire. Par contre, le LIF et le MCP-1 apparaissent de manière biphasique dans le nerf sectionné, entre 3 et 6 heures au site lésionnel et au bout de 24 heures tout le moignon distale. Enfin, la présence d'ARNm du MCP-1 au site lésé semble être médiée par les radicaux libres. Le facteur de nécrose tumoral (tumor necrosis factor; TNF-α) provoque *in vitro* l'expression de l'ARNm du MCP-1 par les cellules de Schwann via une voie pouvant être inhibée par des anti-oxidants. Cependent, le TNF-α ne semble pas *in vivo* être crucial dans l'induction du MCP-1 lors d'une lésion dans le système nerveux périphérique. Enfin, dans le système nerveux périphérique, si la synthèse de CNTF est

régulée à la baisse après lésion, les signaux qui induisent l'expression des ARNm de MCP-1 et du LIF ne sont pas exclusivement dépendant de la dégénérescence axonale.

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CONTRIBUTIONS OF AUTHORS

As required when a manuscript-based thesis includes co-authored papers, the following section is included from the "Guidelines for Thesis Preparation" of McGill University.

"In general, when co-authored papers are included in a thesis the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the co-authored papers."

Dr. Peter M. Richardson is my supervisor and thus, is a co-author of all the manuscripts included in this thesis. He performed some of the sciatic nerve transections, helped in the collection of tissues, particularly the dorsal root ganglia, and edited all my written work. Dr. Mark A. Bisby, a co-author of the manuscript in chapter 4, performed the sciatic nerve ligations described in that said chapter and provided for all the C57BL/Wld mice used in this study. I performed the rest of the experiments.

PUBLICATIONS COMPLETED DURING

MY Ph.D. PROGRAM

1. **M.C. Subang**, M.A. Bisby and P.M. Richardson. (1997) "Delay of CNTF decrease following peripheral nerve injury in C57BL/Wld mice." Journal of Neuroscience Research 49: 563-568.

2. **M.C. Subang** and P.M. Richardson. (1999) "Tumor necrosis factor-alpha induces monocyte chemoattractant protein-1 mRNA in a Schwann cell line." Annals of the New York Academy of Sciences. 883: 523-525.

CLAIMS FOR ORIGINALITY

The loss of axons following nerve injury results in the loss of molecules normally secreted by neurons and the loss of contact between the Schwann cell membrane and the axolemma. The injury also results in the breakdown of the blood-nerve barrier and the subsequent exposure of the transected nerve to the various components of the blood and the activation of the resident immune cells. These events lead to the change in Schwann cell phenotype. The different signals have been teased out by studying three Schwann cell genes. This thesis project revealed the following:

1. CNTF, normally expressed by myelinating Schwann cells, appears to be regulated by components of the axolemma.
2. CNTF protein degradation in transected nerves of C57BL/Wld mice is delayed for many days.
3. LIF mRNA is expressed in the injured nerve in a biphasic manner – an early induction at the site of lesion within 6 hours after transection and a more sustained induction in the distal segments by 24 hours.
4. LIF mRNA is induced by IL-1β in cultured fibroblasts but not in cultured Schwann cells.
5. MCP-1 mRNA is expressed also in the injured nerve in a biphasic manner – an early induction at the site of lesion within 3 hours after transection and in the distal segments by 24 hours and persists for at least 16 days.
6. MCP-1 mRNA is induced in the ipsilateral DRG within 1 day after injury and persists for at least 16 days.

7. The early induction of MCP-1 mRNA in injured nerves is mediated by oxygen radicals.
8. TNF-α stimulates the synthesis of MCP-1 mRNA in cultured Schwann cells in a pathway that is mediated by oxygen radicals.
9. MCP-1 mRNA induction still occurs in the injured nerves of mice which lack types I and II TNF receptors.
10. MCP-1 mRNA expression is observed in the transected nerves of C57BL/Wld mice, which exhibit delayed Wallerian degeneration.

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ABBREVIATIONS

Ara-C	cytosine-β-D-arabinofuranoside
ARIA	acetylcholine receptor-inducing activity
BDNF	brain-derived neurotrophic factor
Ca^{++}	$calcium^{++}$
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CNTF	ciliary neurotrophic factor
CNTFR-α	ciliary neurotrophic factor receptor-alpha
CR3	complement receptor type 3
CT-1	cardiotrophin-1
Cyclo	cyclophilin
DMEM	Dulbecco's modified Eagle's medium
DRG	dorsal root ganglion
EGF	epidermal growth factor
FGF	fibroblast growth factor
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDNF	glial cell line-derived neurotrophic factor
GGF	glial growth factor
HRG	heregulin
IL-1β	interleukin-1β
IL-3	interleukin-3
IL-6	interleukin-6
IL-6R	interleukin-6 receptor
ICAM-1	intercellular ahdesion molecule-1
JNK	c-Jun N-terminal kinase
kDa	kilodalton
LIF	leukemia inhibitory factor
LIFR-β	leukemia inhibitory factor-beta
LNGFR	low affinity nerve growth factor receptor
LPS	lipopolysaccharide
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
MCP-1	monocyte chemoattractant protein-1
mM	millimolar
mRNA	messenger ribonucleic acid
NCAM	neural-cell adhesion molecule
NDF	neu differentiation factor
NF-κB	nuclear factor-κB
NGF	nerve growth factor
NRG	neuregulin
NT-3	neurotrophin-3

NT-4	neurotrophin-4
ORF	open reading frame
P_0	myelin protein P_0
PDGF	platelet-derived growth factor
PMP-22	peripheral myelin protein-22
PNS	peripheral nervous system
RLR	RNA localization region
RNase	ribonuclease
RT-PCR	reverse transcriptase-polymerase chain reaction
RTS	RNA transport signal
SCF	stem cell factor
SCG	superior cervical ganglion
SCIP	suppressed cAMP-inducible POU protein
TGF-β	transforming growth factor-β
TNFα	tumor necrosis factor-α
TNFR	tumor necrosis factor-α receptor
UTR	untranslated region
μM	micromolar
VCAM	vascular cell adhesion molecule

CHAPTER 1: INTRODUCTION

Traumatic nerve injuries, often resulting from vehicular accidents, and neurodegenerative diseases usually lead to permanent neurological dysfunctions (Hefti, 1994; Midha, 1997; Noble et al., 1998). To date, maintaining the survival of injured neurons, especially in the central nervous system (CNS), remains a big challenge.

I. WALLERIAN DEGENERATION

A. Morphological Changes in the Injured Axon

Nasse first observed the granular breakdown of the frog sciatic nerve below the injury site 5 months after transection (Nasse, 1839). Gunther and Schön reported that the structural changes occur as early as 1 week after injury (Günther, Schön, 1840). Waller later gave a detailed description of the physical changes that occur in the glossopharyngeal and hypoglossal nerves of the frog during the first 15 days after transection and noted that these changes occur also in other injured nerves (Waller, 1850). This breakdown of the axon after it has been severed from the nerve cell body is now called Wallerian degeneration. Electron microscopic examination revealed that the distal cut end of the nerve is characterized by swollen axon bulbs in the first 2 mm and a more normal looking fiber distally (Donat, Wisniewski, 1973). Twelve hours after transection, these swollen axons already contain subaxolemmal accumulation of mitochondria and dense bodies with the neurofilaments displaced to the center (Cravioto, 1969; Donat, Wisniewski, 1973). In young rodents, the highly organized neurofilaments and other components of the axoplasm become a mass of granular debris within 24-48

hours depending on the length of the severed nerve (Griffin, Hoffman, 1993b). This disintegration of axons occurs between 3-6 days in transected nerves of rabbits and cats (Donat, Wisniewski, 1973).

Peripheral nerve transection also results in the failure of motor neurons to transmit electrical stimuli (Miledi, Slater, 1970). Analysis of the motor end-plates showed that nerve terminals are degenerating at the time that electrical transmission is failing; and this nerve terminal breakdown occurs before the disintegration of the axons (Miledi, Slater, 1970).

B. Axonal Degeneration Is Calcium Dependent

The degeneration of the axoplasm also occurs *in vitro* in organ culture of nerves maintained in a medium containing calcium (Schlaepfer, Bunge, 1973; Schlaepfer, 1974). Chelation of calcium in the bath medium, which results in the lowering of the intra-axonal Ca^{++} concentration below the physiological concentration of approximately 0.3 μM (Baker et al., 1971), preserves the axonal microtubules and neurofilament structures (Schlaepfer, Bunge, 1973; Schlaepfer, 1974; Schlaepfer, 1977). Conversely, increasing the calcium concentration or the addition of calcium ionophore or chemical detergent in the medium accelerates the granular disintegration of the axoplasm (Schlaepfer, Bunge, 1973; Schlaepfer, 1974; Schlaepfer, 1977). Also, the addition of calcium channel blockers retard the degeneration of axons after axotomy (George et al., 1995). These findings strongly suggest that axonal breakdown is triggered by an increase in intra-axonal concentrations of calcium.

Calpains, the calcium-activated proteases, have been shown to degrade neurofilaments, the major component of the axon cytoskeleton, in the presence of sufficient concentrations of calcium and have been found to be present in the peripheral nerve (Kamakura et al., 1983). Furthermore, the addition of calpain inhibitors to the medium inhibits neurite/axonal degeneration following axotomy (George et al., 1995). The action of calpains represents one of the nonlysosomal pathway of intracellular protein degradation (Croall, Demartino, 1991). There are two isoforms of calpain, μ and m, each is a dimer consisting of a 80 kDa and a 30 kDA subunits. In the brain, mcalpain is localized to a restricted subpopulation of neurons and glial cells (Hamakubo et al., 1986; Nixon, 1986). On the other hand, μcalpain is expressed primarily in the neurons (Hamakubo et al., 1986; Perlmutter et al., 1988).

II. SCHWANN CELL RESPONSES

A. Breakdown of Myelin Sheath

In a normal myelinating peripheral nerve, Schwann cells elaborate myelin sheath around their target axons. Within 24 hours after nerve transection, fragmentation of myelin lamellae occurs at the nodes of Ranvier and myelin ellipsoids start to form (Liu et al., 1995). By 48 hours, the distal nerve stump is characterized by the linear fragmentation of myelin (Schlaepfer, 1974). Two to three days after injury, Schwann cells have undergone nuclear and cytoplasmic hypertrophy and their processes start to phagocytose myelin ellipsoids and by 4-5 days, these myelin ellipsoids have been degraded (Liu et al., 1995).

The two isoforms (μ and m) of calpain are made by Schwann cells (Banik et al., 1991; Mata et al., 1991) and mcalpain colocalizes with myelin basic protein in the nerve (Li, Banik, 1995). Calpains can degrade myelin and has been suggested to take part in the normal turn-over of myelin proteins (Deshpande et al., 1995). The calpain activity in the myelin sheath is probably triggered by the increase in calcium concentration in the myelin compartment observed within 8 hours after transection (LoPachin et al., 1990).

B. Expression of Myelin Specific Genes is Reduced

The presence or absence of axonal contact has long been known to influence the expression of a number of Schwann cell genes. The myelin genes are some of these molecules regulated by axons. The changes in the expression of myelin-specific genes are summarized in Table 2.

1. The Proteins of Peripheral Myelin

The myelin sheath is an organelle that is made up of a large sheet of plasma membrane wrapped and tightly compacted around an axon. Schwann cells that elaborate this organelle express myelin-specific proteins (Jessen, Mirsky, 1992). These myelin proteins are divided into two. The first group, which includes P_0, myelin basic protein (MBP), proteolipid protein (PLP) and peripheral myelin protein 22 (PMP22), mediate myelin wrapping and compaction; and the second group, which includes myelin-associated glycoprotein (MAG) and other less abundantly expressed glial proteins, participate in the different stages of myelination (Lemke, 1988).

a. Myelin Protein P_0

Myelin protein P_0, a 28 kDa integral membrane glycoprotein, is the most abundant protein in PNS myelin, accounting for more than 50% of the proteins in the sheath (Greenfield et al., 1973). It is exclusively expressed in the PNS by myelinating Schwann cells (Brockes et al., 1980). The extracellular domain that is structurally similar to a prototypic immunoglobulin domain (Lemke et al., 1988b) can mediate both homotypic and heterotypic interaction with adjacent cell membrane surfaces (D'Urso et al., 1990; D'Urso et al., 1999). X-ray crystallography showed that P_0 molecules form tetramers on the cell membrane and interact with P_0 tetramers of the apposing membranes forming adhesive complexes and thus contribute to the compaction of myelin (Shapiro et al., 1996). Furthermore, direct membrane intercalation of the P_0 molecule via the tryptophan side chain has been proposed to determine the exact membrane spacing in the myelin (Shapiro et al., 1996). A recent study has shown that P_0 interact with myelin protein PMP-22 and form complexes at the cell membrane (D'Urso et al., 1999). The P_0 intracellular domain is rich in basic residues that can interact with negatively charged phospholipids of the adjacent cytoplasmic regions of the Schwann cell membrane and thus, contribute to the formation of the major dense line (Kirschner, Ganser, 1980; Lemke, Axel, 1988a; Lemke, 1988).

Mice lacking the P_0 gene have hypomyelinated peripheral nerves, display deficiencies in motor coordination and exhibit abnormal expression of p75 LNGFR, other myelin proteins and adhesion molecules (Giese et al., 1992). The myelin that is formed still has major dense lines but show poor compaction. Disruption of both P_0 and myelin basic protein (MBP) leads to severe hypomyelinated, loss major dense lines, mostly

uncompacted myelin and similar behavioral phenotype as shiverer mice (Martini et al., 1995).

b. Myelin Basic Proteins

Myelin basic proteins (MBPs) comprise a family of at least six soluble, highly cationic proteins, ranging in size from 14-22 kDa, that are transcribed from a single gene (Lemke, 1988). Products of the MBP gene are intracellular proteins that are generated by a combination of alternative exon splicing and alternative initiation start sites (Takahashi et al., 1985; Mathisen et al., 1993; Zelenika et al., 1993; Campagnoni et al., 1995). Together these MBP proteins account for 30-40 % of CNS myelin proteins and 5-15% of myelin proteins in the PNS (Lemke, 1988). Like the intracellular domain of P_0, MBP is rich in highly charged residues that can interact with the negatively charged phospholipid of the adjacent Schwann cell plasma membrane and thus contribute to the compaction of major dense line (Martini et al., 1995; Staugaitis et al., 1996).

The two mutant mouse strains, *shiverer* and *myelin deficient (mld)*, are deficient in MBP expression (Mikoshiba et al., 1991). The *shiverer* mice lack a large portion of the *MBP* gene, their CNS myelin are loosely wrapped and lack major dense lines but do not show major abnormalities in the PNS (Privat et al., 1979; Rosenbluth, 1980a; Rosenbluth, 1980b). The *mld* mutant mice have duplicated *MBP* gene. A large portion of the *MBP* gene is inverted upstream of the intact copy generating both sense and anti-sense mRNAs upon transcription (Popko et al., 1988). However, patches of MBP-containing myelin are still present in the CNS of these mice (Mikoshiba et al., 1991). In mice lacking both MBP

and P_0 genes, the PNS myelin lack the major dense line and most Schwann cells elaborate uncompacted myelin (Martini et al., 1995).

c. Peripheral Myelin Protein-22

Cloning of peripheral myelin protein-22 *(PMP-22)* or *SR13* revealed that it is identical to the previously cloned growth arrest specific gene 3 *(gas3)*, a protein which is induced in NIH3T3 cells following serum starvation or contact inhibition (Schneider et al., 1988; Welcher et al., 1991; Lemke, 1993). The open reading frame of *PMP-22* predicts a protein that is 160 amino acids long with a molecular weight of approximately 18 kDa (Welcher et al., 1991). It is a very hydrophobic molecule with four putative transmembrane domains and two extracellular domains (Manfioletti et al., 1990; D'Urso, Müller, 1997). PMP-22, a protein glycosylated at an asparagine residue located between the first and second membrane spanning regions (Manfioletti et al., 1990; Welcher et al., 1991; Spreyer et al., 1991), is localized to the compact regions of the peripheral myelin (Snipes et al., 1992). Recently, PMP-22 has been shown to associate with myelin P_0 forming complexes at the Schwann cell membranes (D'Urso et al., 1999).

Trembler and trembler^J mice, which have point mutations in the *PMP-22* gene, exhibit hypomyelination and neuronal deficits (Suter et al., 1992a; Suter et al., 1992b). Mice that carry extra copies of *PMP-22* gene also show deficiencies in PNS myelination and behavioral abnormalities (Magyar et al., 1996). *Tr/Tr* homozygotes can live for several months while Tr^J/Tr^J homozygotes die 2 weeks after birth (Suter et al., 1992a). In humans, the hereditary peripheral neuropathy Charcot-Marie Tooth disease type 1A (CMT1A) is associated with a duplication of the *PMP-22* gene while the autosomal

dominant hereditary neuropathy with liability to pressure palsies (HNPP) disorder is associated with a deleted *PMP-22* gene (Lemke, 1993; Snipes et al., 1993). Mice lacking the *PMP-22* gene exhibit delayed myelination, but at postnatal day 24 hypermyelination occurs, as determined by the presence of myelin tomacula, and 10 weeks after birth, myelin and axonal degeneration are observed (Adlkofer et al., 1995).

d. Myelin-Associated Glycoprotein

Myelin-associated glycoprotein (MAG), a heavily glycosylated 100 kDa integral membrane protein, is expressed at low levels both in the CNS and PNS (Lemke, 1988). Two MAG isoforms containing 607 and 563 amino acids are results of alternative splicing (reviewd by Quarles et al., 1992). The MAG gene has 13 exons and in exon 12 an in-frame termination codon has been identified (Mikoshiba et al., 1991; Quarles et al., 1992). Thus, the message containing exon 12 gives rise to the small isoform (S-MAG) while the mRNA that does not contain exon 12 gives rise to the large isoform (L-MAG) (Mikoshiba et al., 1991). In the CNS, the tyrosine kinase Fyn is activated during the early phase of myelination, around postnatal day 4 of the mouse, and is associated with L-MAG (Umemori et al., 1994). Moreover, in *fyn* deficient mice, the amount of MBP present in the brain is 50% of that of control mice and the CNS myelin is thinner and more irregular (Umemori et al., 1994; Umemori et al., 1999). Recently, Fyn has been shown to activate the transcription of MBP (Umemori et al., 1999). These observations strongly support the hypothesis that MAG is involved in the initiation of myelination (Trapp, 1990). However, L-MAG accounts for only 5% of the total MAG mRNA in the

PNS (Quarles et al., 1992). To date, it is not known whether the S-MAG associates with other signalling components and transactivate other myelin genes.

Like neural-cell adhesion molecule (NCAM), MAG belongs to the immunoglobulin superfamily having five Ig-like domains in the extracellular region (Lemke, 1988). In addition, the first Ig-like domain contains the Arg-Gly-Asp (RGD) sequence, which may be involved in binding to integrin-type receptors (Rouslahti, Piersbacher, 1987). MAG is localized in periaxonal membranes of central and peripheral myelin sheath and absent from compact myelin (Sternberger et al., 1979; Trapp, Quarles, 1982). It is expressed also in Schmidt-Lanterman incisures, paranodal loops and mesaxons (Martini, Schachner, 1988b; Trapp et al., 1989) where they exhibit homophilic interactions (Trapp et al., 1999).

The generation of mice with null mutations in myelin specific genes has confirmed the roles of these myelin proteins in the formation and maintenance of the myelin sheath. In summary, myelin proteins in the PNS have overlapping functions. P_0, the most abundant protein in PNS myelin, appears to be involved at all stages of myelination (Martini, Schachner, 1997). In addition, PMP22 and to lesser extent MAG, appear to be important for initial ensheathment of axons (Magyar et al., 1996; Martini, Schachner, 1997), MBP is also important for compaction of major dense line, MBP and PMP-22 regulate myelin thickness (Martini et al., 1995), and except for MBP, most are involved in the maintenance of myelin (Martini, Schachner, 1997).

e. Changes in Myelin Protein Concentrations

The expression of a number of Schwann cell genes depends on the presence or absence of axons (Table 2). The loss of axonal influence during Wallerian degeneration results in the decreased synthesis of myelin proteins P_0, MBP, MAG and PMP-22 and the increased synthesis of the p75NGFR (Trapp et al., 1988; Gupta et al., 1988; Kuhn et al., 1993; Gupta et al., 1993). Following axonal regeneration, the re-expression of myelin proteins occurs concomitant with the decrease in the expression of low affinity receptor for nerve growth factor (p75 LNGFR), neural-cell adhesion molecule (NCAM) and L1 (Martini, Schachner, 1988a; Gupta et al., 1988; Kuhn et al., 1993; Gupta et al., 1993).

To determine the putative neuron-derived factors regulating the expression of myelin specific genes, Schwann cells have been cultured in the absence of serum and treated with agents that elevate the intracellular concentrations of cAMP. Schwann cells that are grown in the defined medium (serum-free) and treated with forskolin synthesize the P_0 mRNA and reduce the concentrations of the mRNAs for GFAP, NCAM and p75NGFR (Morgan et al., 1991). Thus, the elevation of intracellular cAMP may mediate the axon to Schwann cell signaling that triggers myelination. The effects of forskolin on the expression of myelin protein mRNAs is inhibited by FGF-2, TGFβ, GGF or serum factors (Morgan et al., 1991; Stewart et al., 1995). However, in Schwann cell cultures that have been established without prior exposure to serum during the culture process, P_0 and MBP are constitutively expressed and addition of GGF/neuregulin inhibits the expression (Cheng, Mudge, 1996).

2. Synthesis Myelin-Specific Transcription Factors is Decreased

To date, there are at least two transcription factors that might be involved in the regulation of myelination: suppressed cAMP-inducible POU protein (SCIP) and krox-20.

a. Suppressed cAMP-Inducible POU Protein (SCIP)

Tst-1 or Oct-6 or suppressed c-AMP-inducible POU protein (SCIP) belongs to the Class III POU domain family of transcription factors, which are expressed primarily in subsets of neurons and myelin-forming glia of the brain and the PNS (Monuki et al., 1989; He et al., 1991). SCIP mRNA concentrations in Schwann cells are normally low and increase markedly 1-3 hours after treatment with forskolin, an agent that increases intracellular cAMP concentrations (Monuki et al., 1989). The concentrations of SCIP mRNA remain elevated for as long as the intracellular cAMP concentrations in Schwann cells are high. In comparison, the mRNAs for the myelin genes are induced 18-24 hours after forskolin treatment (Monuki et al., 1989). Furthermore, in co-transfection experiments, SCIP binds to the P_0 promoter through its POU domain and its amino terminal domain and represses the transcription of P_0 (He et al., 1991; Monuki et al., 1993a; Monuki et al., 1993b).

During rat embryogenesis, SCIP is first observed at E17 in the nuclei of Schwann cells expressing LNGFR and S100 in the dorsal root proximal to the spinal cord and by E19 expression spreads distally within the dorsal root (Zorick et al., 1996). Furthermore, SCIP-iimunoreactivity is detected in Schwann cell nuclei of neonatal rats (Scherer et al., 1994). The expression of SCIP mRNA in the sciatic nerve is most robust at P1 to P4 then declines thereafter and is no longer detectable in the adult (Scherer et al., 1994; Zorick et

al., 1996). Furthermore, in nonmyelinating peripheral glia like the satellite cells in the DRG and the nonmyelinating Schwann cells in the superior cervical ganglion postganglionic nerve, SCIP mRNA is expressed transiently from the end of the first postnatal week to P20 (Zorick et al., 1996). In adult rats, sciatic nerve transection results in the slight transient rise, from 1 to 12 days post-lesion, of SCIP mRNA while nerve crush leads to a rise in SCIP mRNA concentrations in the distal stump from 8 days post-injury and persists for at least 58 days (Scherer et al., 1994). However, in neonatal rats transection of the sciatic nerve results in the reduction of SCIP, P_0, and histone H3 mRNA by 2 days. These patterns of SCIP mRNA expression in degenerating and regenerating nerves strongly support the hypothesis that SCIP is involved in the regulation of myelin genes.

b. Krox-20

The gene *krox-20* or *EGR-2* was first described as a serum-responsive immediate early gene that encodes a protein with Cys_2/His_2 zinc-finger motif and is closely related to the transcription factor Sp1 (Chavrier et al., 1988; Chavrier et al., 1989; Cortner, Farnham, 1990; Nardelli et al., 1991; Nardelli et al., 1992). The gene product of *krox-20* is a transcription factor that modulates *hox* genes that are involved in hindbrain development (Chavrier et al., 1990; Schneider-Maunoury et al., 1993; Sham et al., 1993). Disruption of the *krox-20* gene leads to the reduction or loss of rhombomeres 3 and 5 of the hindbrain and die during the first two weeks after birth (Schneider-Maunoury et al., 1993). In addition, the mice display lack of myelinated peripheral nerves revealing that krox-20 is involved in myelination (Topilko et al., 1994).

In the PNS, krox-20-immunoreactivity is detected in the cytoplasm of dorsal root ganglia neurons and in the nucleus of satellite cells of the DRG and Schwann cells (Herdegen et al., 1993). Immunohistochemical studies showed that krox-20 expression in nerves first appears at P1 in the dorsal roots and follows a proximal to distal distribution similar to that of SCIP but with a delay of 1-2 days (Zorick et al., 1996). However, the use of transgenic mice carrying a *lacZ* insertion in the *krox-20* gene, which do not present phenotypes in the heterozygous state (*krox-20* $^{\pm}$), showed that krox-20 is first expressed at mouse embryonic day 10.5 (E 10.5) only in the nerves adjacent to the neural tube (Topilko et al., 1997). At E15.5, krox-20 is expressed in the entire peripheral nerves. In contrast to SCIP expression, krox-20 expression is maintained in adulthood, coincident with that of myelin protein P0, and no transient expression is observed in nonmyelinating Schwann cells and satellite cells in the DRG (Zorick et al., 1996; Topilko et al., 1997). A sciatic nerve transection or crush in rodents leads to a fall in krox-20 expression and a similar decrease is observed in the nerves of patients with peripheral neuropathies (Zorick et al., 1996; Topilko et al., 1997). Moreover, in a patient recovering from axonal neuropathy krox-20-immunoreactivity is detected in Schwann cells undergoing the remyelination process (Topilko et al., 1997).

In vitro studies showed that cultured Schwann cells do not synthesize krox-20 and that a direct axonal contact is necessary for the krox-20 expression in explants of DRG taken from E15.5 mouse embryos (Murphy et al., 1996). Neurons at various stages of development have the capacity to induce krox-20 expression in Schwann cells from neonatal animals (Murphy et al., 1996). Thus, the signal for krox-20 expression is always present in neurons and that the krox-20 expression is regulated by the developmental

stage of the Schwann cells. However, explants of DRG from E12.5 embryos, which do not normally synthesize krox-20, are able to express krox-20 in the presence of diffusable factors from the neural tube; this action can be mimicked by treatment with NRG-β2 or the combination of FGF2 and either CNTF or LIF (Murphy et al., 1996).

C. Changes in Expression of Integrins

Axons control myelination by regulating the expression of the different Schwann cell genes including those of the receptors of the components of the basal lamina. Integrins are cell surface receptors that mediate cell-cell and cell-extracellular matrix interactions (Hynes, 1992). All integrins are composed of heterodimers of α and β subunits (Hynes, 1992). To date, Schwann cells have been shown to express $\alpha_1\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$ integrins, the receptors for laminin (Fernandez-Valle et al., 1994; Niessen et al., 1994; Feltri et al., 1994). $\alpha_6\beta_1$ is widely expressed while $\alpha_6\beta_4$ expression is restricted to epithelial cells, some types of endothelial cells, perineural fibroblasts and Schwann cells (Niessen et al., 1994). Schwann cells in culture synthesize $\alpha_1\beta_1$ and $\alpha_6\beta_1$ integrins; however, when cultured in the presence of DRG neurons, myelinating Schwann cells reduce the synthesis of $\alpha_1\beta_1$ and $\alpha_6\beta_1$ and increase the synthesis of $\alpha_6\beta_4$ integrin (Fernandez-Valle et al., 1994). In addition, antibodies against β_1 inhibit myelination by blocking the differentiation of Schwann cells into a myelinating phenotype (Fernandez-Valle et al., 1994).

$\alpha_6\beta_4$ integrins are commonly found in hemidesmosomes, which are specialized structures of keratinocytes and other epithilial cells that mediate adhesion to underlying basement membrane (Stepp et al., 1990; Sonnenberg et al., 1991). In the peripheral

nerve, $\alpha_6\beta_4$ immunoreactivity is described as a thin ring around the myelin sheath and developmentally regulated (Niessen et al., 1994; Feltri et al., 1994; Quattrini et al., 1996). In nonmyelinating Schwann cells, it is localized at contact regions between axons and the Schwann cells (Niessen et al., 1994). Following sciatic nerve crush, α_6 and β_1 mRNA expression are not changed while that of β_4 changes as does P_0 mRNA (Feltri et al., 1994). In contrast, in transected sciatic nerves, β_4 mRNA expression is markedly reduced by 4 days after injury and then increases progressively from 8 to 58 days following transection (Feltri et al., 1994). Although β_4 mRNA is re-expressed in chronically denervated Schwann cells, it is no longer restricted to the abaxonal region rather but is distributed in a diffuse, non-polarized pattern (Feltri et al., 1994; Quattrini et al., 1996). To date, this re-expression of β_4 mRNA in transected nerves remains unexplained.

D. Schwann cell proliferation

Autoradiographic studies showed that the labelling index of mouse Schwann cells in the degenerating nerve peaks on the third day after transection and declines thereafter (Bradley, Asbury, 1970). The peak of mitosis in rats and cats occurs 3-4 days after injury (Pellegrino et al., 1986; Oaklander et al., 1987; Griffin, Hoffman, 1993b; Liu et al., 1995). The signal for Schwann cell proliferation during Wallerian degeneration is not clearly understood.

Schwann cell proliferation has been studied extensively *in vitro*. The first soluble and potent Schwann cell mitogen identified is glial growth factor (GGF) (Raff et al., 1978). GGF treatment does not result in the elevation of intracellular concentrations of cAMP but stimulates Schwann cell proliferation through the activation of protein kinase

C (Yoshimura et al., 1993). Fibroblast growth factor-1 (FGF-1), FGF-2 and platelet-derived growth factor (PDGF) have weak mitogenic activities which can be potentiated with the addition of forskolin, an agent that elevates intracellular cAMP, (Davis, Stroobant, 1990; Chen et al., 1991). Increasing the concentration of intracellular cAMP up-regulates PDGF receptor and may possibly modulate receptors of other growth factor in a similar manner (Weinmaster, Lemke, 1990). TGF-β can also synergize with FGF2 or PDGF to stimulate Schwann cell proliferation but the combination of TGF-β and cAMP elevation is not mitogenic (Schubert, 1992).

E. Changes in the Expression of Trophic Factors

The molecules made by target tissue in limited quantities that promote survival of neurons are called neurotrophic factors (Barde, 1988). However, recent findings showed that the "classical" neurotrophic factors might have actions outside of the nervous system (Horigome et al., 1993; Kawamoto et al., 1995) and cytokines that primarily have immune function act also on neurons (Yamamori et al., 1989; Kushima et al., 1992; Cheema et al., 1994a; Hirota et al., 1996).

There are several families of neurotrophic factors known to act on neurons and glial cells in the PNS. These trophic factors are the neurotrophin family (reviewed by Ebendal, 1992), the IL-6 family of cytokines or the neuropoietic cytokines (Ernsberger et al., 1989; Yamamori et al., 1989; Ikeda et al., 1996; Pennica et al., 1996) and the transforming growth factor (TGF) superfamily (Stewart et al., 1995; Guénard et al., 1995; Rosenthal, 1999). Perhaps several combinations of trophic factors might act in concert to

promote survival and maintenance of the neurons. The changes in trophic factor expression following axonal degeneration are summarized in Table 3.

1. The neurotrophin family

The prototype for the neurotrophic factors is the nerve growth factor (NGF), the first of this class of molecules to be discovered and characterized (Levi-Montalcini, 1987). NGF and the other members of the neutrophin family have common structural features – each comprising of seven β-strands which form three anti-parallel pairs (Ebendal, 1992). The neurotrophins primarily act on the cells in the nervous system (Mendell, 1995). They are synthesized as precursors and proteolytically cleaved by pro-protein convertases (Bresnahan et al., 1990; Steiner et al., 1992).

a. Nerve Growth Factor

NGF was initially isolated and characterized as the molecule secreted by mouse sarcoma 180 that supports the survival of neurons in the neural crest-derived sensory and sympathetic ganglia (Levi-Montalcini, 1987). Production of NGF in the adult nerve is normally very low but following nerve transection, NGF mRNA concentrations increase in a biphasic manner (Heumann et al., 1987a; Heumann et al., 1987b). The initial rapid rise in NGF mRNA concentration is transient and peaks within 6 hours and is followed, 2 days later, by a sustained rise (Heumann et al., 1987b). This initial rapid rise in NGF mRNA can be reproduced in organ cultures but the second phase of increase is only seen when activated macrophages are added (Heumann et al., 1987b). In Wld mice which exhibit subnormal recruitment of macrophages, sciatic nerve transection does not lead to

a marked increase in NGF synthesis in the distal stump (Brown et al., 1991). Macrophage-derived molecules like IL-1β (Lindholm et al., 1987) or TNF-α (Hattori et al., 1993) stimulate the expression of NGF mRNA in fibroblasts *in vitro*. Cultured Schwann cells synthesize NGF mRNA only in response to stimulation be agents that elevate intracellular cAMP (Matsuoka et al., 1991).

In adult rat lumbar DRG high affinity binding to NGF is restricted to neurons that are immunopositive for calcitonin gene-related peptide (CGRP) and for substance P (Verge et al., 1989). The high affinity binding and signal transduction of NGF involve two molecules – trk and p75 receptors. The first receptor to be described was the p75 low-affinity NGF receptor (LNGFR) (Johnson et al., 1986), which is capable of binding to all the neurotrophins at a low affinity (reviewed by Ebendal, 1992). Although p75 is not necessary for neurotrophin signaling (Ibañez et al., 1992), it increases ligand binding affinity and modulates the signal transduced by trks (Hempstead et al., 1991). In the PNS, p75 LNGFR expression is not limited only to neurons. The p75 LNGFR is expressed early during development in emigrated neural crest cells (Shah et al., 1994) and expression is maintained in the Schwann cell lineage until the cells have differentiated and started to express myelin proteins (Jessen, Mirsky, 1991; Jessen et al., 1994). Sciatic nerve injury results in the decrease in P75 LNGFR expression in DRG neurons (Zhou et al., 1996) and an increase in expression in Schwann cells (Taniuchi et al., 1988; Zhou et al., 1996). Disruption of *p75* gene results in the loss of heat sensivity and associated with ulcers in distal extremities (Lee et al., 1992). Also, loss of innervation to the pineal gland and reduced or absence of sweat gland innervation have been observed in mice lacking in p75 NGF receptor (Lee et al., 1994).

The high-affinity receptor of NGF is a 140 kDa tyrosine kinase receptor that is a product of the protooncogene *trk* (Martin-Zanca et al., 1989). Trk mRNA has been localized to neural crest-derived trigeminal sensory, dorsal root, and paravertebral sympathetic ganglia (Martin-Zanca et al., 1990). The binding of NGF to Trk activates the tyrosine kinase resulting in the tyrosine phosphorylation of Trk (Kaplan et al., 1991a; Kaplan et al., 1991b). In rat pheochromocytoma PC12 cells, formation of activated Trk homodimers mediates neuronal survival and differentiation (Rovelli et al., 1993). In addition, injection of antibody against NGF *in utero* results in the loss of Trk expressing neurons in the DRG (Carroll et al., 1992). Trk mRNA concentrations in lumbar DRG neurons decrease following sciatic nerve transection (Verge et al., 1992). Mice deficient in the gene for *NGF* or *trk* are insensitive to pain and temperature and severe reduction in sympathetic and sensory neurons, particularly the small-sized neurons in the spinal and trigeminal ganglia (Smeyne et al., 1994; Crowley et al., 1994).

In humans, mutations in *trkA* are implicated in congenital insensitivity to pain with anhidrosis (CIPA), an autosomal-recessive disorder characterized by recurrent fever due to anhidrosis (absence of sweating), lack of reaction to noxious stimuli, self-mutilating behavior, and mental retardation (Indo et al., 1996; Yotsumoto et al., 1999). Patients with CIPA exhibit defects in *trkA* ranging from point mutation at nucleotide 1825 (A→G transtition), single base C deletion at nucleotide 1726 (Yotsumoto et al., 1999), and deletion-, splice- and missense mutation in the tyrosine kinase domain (Indo et al., 1996).

b. Brain-derived Growth Factor

The second member of the neurotrophin family was isolated from pig brain and is called brain-derived neurotrophic factor (BDNF). It is expressed mainly in the nervous system and in the periphery it is made only in heart, lung and skeletal muscle (Ebendal, 1992). The mature BDNF protein has approximately 50% amino acid sequence similarity with NGF (Barde et al., 1982; Leibrock et al., 1989). Included in the conserved regions are 6 cysteine resides that form the disulfide bonds important for the stabilization of the 3-D structure (Thoenen, 1991).

BDNF supports the survival of trigeminal sensory neurons at the time that they innervate their targets; these neurons later lose their BDNF-responsiveness and become dependent on NGF (Buchmann, Davies, 1993). During mouse embryogenesis, BDNF mRNA is expressed in the dorsal root, trigeminal, and sympathetic ganglia as well as in the vestibular ganglia and cochlea (Schecterson, Bothwell, 1992). BDNF supports also the survival of motor neurons (Koliatsos et al., 1993).

The amount of BDNF made in adult rat nerve is normally very low and sciatic nerve transection leads to a slow increase of BDNF mRNA concentrations in the distal stump that starts at 3 days post-injury and reaches the maximum expression after 3-4 weeks (Meyer et al., 1992; Funakoshi et al., 1993). In addition, cultured Schwann cells constitutively express BDNF mRNA and addition of ionomycin or a combination of ionomycin and TPA increases the expression (Meyer et al., 1992).

The discovery of TrkA, a product of the *trk* protooncogene, as the high affinity receptor for NGF led to the identification of the other members of the Trk family of receptor tyrosine kinases as the high affinity receptors for the other neurotrophins

(reviewed by Segal, Greenberg, 1996). In situ hybrization studies showed that while *trkA* mRNA is localized in majority of the neurons in the DRG, *trkB* mRNA is present in some DRG neurons and also in Schwann cells (Carroll et al., 1992). The mRNA for full length *trkB* is expressed in the neurons while the mRNA for the truncated *trkB* is expressed in satellite cells of the DRG (Wetmore, Olson, 1995). Following sciatic nerve or dorsal root lesion, *trkB* mRNA increases in the DRG (Ernfors et al., 1993). Moreover, the mRNA for truncated *trkB* , which is localized in Schwann cells, increases in the proximal nerve segment but decreases in the distal nerve stump (Funakoshi et al., 1993).

Loss of *BDNF* gene results in severe problems in coordination and balance associated with loss of neurons in sensory ganglia, particularly most neurons in the vestibular ganglion (Enfors et al., 1994a). Examination of the *trkB* deficient mice showed that trigeminal sensory neurons depend on TrkB signaling in the early stages of development until most neurons have extended their axons to their fields and could compete for the target-derived NGF (Piñón et al., 1996).

c. Neurotrophin-3

The third member of the family, neurotrophin-3 (NT-3) was cloned using the partial sequence similarity between NGF and BDNF (Thoenen, 1991). NT-3 supports the survival of sympathetic neuroblasts before they become NGF-responsive (Verdi, Anderson, 1994). The neurons in the developing DRG are supported also by NT-3 before they are dependent on NGF (Wright et al., 1992; Hory-Lee et al., 1993). In addition, NT-3 drives the differentiation of cultured neural crest-derived cells from the gut into neurons and glia (Chalazonitis et al., 1994). In mouse embryos, NT-3 mRNA concentrations are

high in motor neurons, moderate in sympathetic ganglia, and almost undetectable in sensory dorsal root and trigeminal neurons (Schecterson, Bothwell, 1992; Wetmore, Olson, 1995). NT-3 mRNA is present in sciatic nerves of adult rats and following axotomy, this expression decreases in the distal stump 6-12 hours post-injury and is gradually restored to normal levels 2 weeks after injury (Funakoshi et al., 1993).

The actions of NT-3 are mediated by its high affinity binding to TrkC tyrosine kinase receptor (Lamballe et al., 1991) and lower affinity binding to TrkB receptor (Windisch et al., 1995). Like *trkB*, *trkC* gene also encodes an isoform that lacks the tyrosine kinase domain, the truncated TrkC (Palko et al., 1999). Mice that overexpress this truncated form suffer from severe developmental defects in the heart and the PNS and thus, die early (Palko et al., 1999). Mice lacking in *trkC* exhibit abnormalities in movement and postures and lack Ia muscle afferent projections to spinal motor neurons and have fewer large myelinated axons in the dorsal root and posterior column of the spinal cord (Klein et al., 1994). On the other hand, mice deficient in *NT-3* gene exhibit abnormal limb movements (Ernfors et al., 1994b), have severe loss of sensory and sympathetic neurons (>70%) (Farinas et al., 1994; Liebl et al., 1997), group Ia proprioceptive afferents and muscle spindles are particularly absent (Kucera et al., 1995) and most die after birth (Ernfors et al., 1994b).

d. Neurotrophin-4

Additional members of the neurotrophin family were cloned by PCR with the use of the conserved regions of NGF, BDNF and NT-3 as primers (Thoenen, 1991). Neurotrophin-4 (NT-4), which is which is identical to neurotrophin-5 (Berkemeier et al.,

1991; Ip et al., 1992a), exerts its actions by binding to TrkB and p75 LNGFR (Klein et al., 1992; Windisch et al., 1995; Ryden et al., 1995). In *in vitro* studies, NT-4 supports the survival of vestibular neurons protects them against ototoxic agents (Zheng et al., 1995). Administration of NT-4 rescues axotomized facial motor neurons of neonatal rats (Koliatsos et al., 1994). NT-4 is widely expressed in the nervous system as well as in nonneural tissues (Timmusk et al., 1993). In the peripheral nerves, NT-4 mRNA concentrations decrease in the distal stump 6-12 hours after transection and then progressively increase to concentrations eight fold of normal by 2 weeks (Funakoshi et al., 1993).

Loss of *NT-4* gene in the mouse leads to deficits in sensory neurons in the nodose-petrosal and geniculate ganglia but facial motor neurons and sympathetic neurons in the SCG do not appear to be affected (Liu et al., 1995; Conover et al., 1995). Disruption of both *BDNF* and *NT-4* genes increases the severity of the sensory deficits while facial motor neurons remain unaffected (Liu et al., 1995; Conover et al., 1995).

2. The neuropoietic cytokines

The second family of molecules that can support the survival of neurons is a group of distantly related cytokines that have predicted structural similarities (Bazan, 1991; Yamamori, Sarai, 1992) and utilize multi-component receptors with gp130 as the signal transducing subunit (Taga, 1996). This family of cytokines includes ciliary neurotrophic factor (CNTF), interleukin-6 (IL-6), leukemia inhibitory factor (LIF), oncostatin-M (OSM), interleukin-11 (IL-11), and cardiotrophin-1 (CT-1) (Zhang et al., 1994; Taga, 1996).

a. Interleukin-6

IL-6 is a pleiotropic cytokine that is made by a number of cell types including B-cells (Hirano et al., 1986), monocytes (Aarden et al., 1987), fibroblasts (Guba et al., 1992), and endothelial cells (Fabry et al., 1993). In the central nervous system (CNS), IL-6 mRNA is detected in granule cells of the cerebellum, pyramidal neurons of the hippocampus, and selected neuronal populations in the cortex and hypothalamus (Schobitz et al., 1993). IL-6 mRNA is not detectable in the normal peripheral nerve but following sciatic nerve transection, IL-6 mRNA is expressed in Schwann cells of the distal nerve stump within 6 hours after transection but is already at low concentrations by 24 hours (Bolin et al., 1995; Zhong, Heumann, 1995; Bourde et al., 1996). Furthermore, IL-6 mRNA is made in medium to large neurons of the DRG after sciatic nerve transection (Murphy et al., 1995).

The actions of IL-6 in the nervous system include a survival-promoting activity for cultured catecholaminergic neurons from fetal and neonatal rat midbrain (Kushima et al., 1992) and cultured embryonic DRG neurons (Hirota et al., 1996). Co-administration of IL-6 and soluble IL-6 receptor (sIL-6R) results in the delay of progression of motor deficits in wobbler mutant mice (Ikeda et al., 1996). Transgenic mice carrying both the human *IL-6* and *IL-6R* genes exhibit accelerated axonal regeneration following injury to the hypoglossal nerve (Hirota et al., 1996). The mice deficient in *IL-6* gene appear healthy but are not able to mount a normal immune response following a bacterial challenge (Dalrymple et al., 1995). In these mice, sciatic nerve transection leads to a higher number of DRG neurons undergoing cell death (Murphy et al., 1999).

The effects of IL-6 are mediated by signaling through its receptor complex (reviewed by Taga, 1996). IL-6 binds to the extracellular domain of the IL-6 receptor α (IL-6Rα) subunit, either the soluble or the membrane bound form (Taga et al., 1989; Taga, Kishimoto, 1992; Rose-John, Heinrich, 1994). The IL-6-IL-6Rα complex then associates with gp130, enabling it to form a homodimer (Taga et al., 1989; Murakami et al., 1993). The homodimerization of gp130 leads to the activation of the members of the JAK family of kinases (Narazaki et al., 1994), which are attached to the gp130 subunits, and starts the signaling cascade (Lütticken et al., 1994; Stahl et al., 1994a).

b. Ciliary Neurotrophic Factor

Ciliary neurotrophic factor (CNTF) was first described and isolated based on its ability to support the survival of chick ciliary neurons (Lin et al., 1989; Lin et al., 1990). Purification and cloning revealed that CNTF is a 200-amino acid protein with a molecular weight of 20-24 kDa (Stöckli et al., 1989; Lin et al., 1990). Furthermore, CNTF lacks the consensus sequence for signal peptide and the mode of release of CNTF from its cell of synthesis is presently not known (Stöckli et al., 1989).

To date, CNTF is known to have other potent effects on several populations of neurons and glia. CNTF drives the differentiation of neurotransmitter phenotype of sympathetic neurons (Ernsberger et al., 1989; Saadat et al., 1989), supports the survival of motor (Sendtner et al., 1992a; Mitsumoto et al., 1994), hippocampal (Ip et al., 1991), sensory and preganglionic sympathetic neurons (Sendtner et al., 1994). Also, CNTF rescues degenerating substantia nigra dopaminergic neurons (Hagg, Varon, 1993). In addition, CNTF stimulates the differentiation of type 2 astrocytes from O-2A progenitor

cells (Hughes et al., 1988) and supports the survival of oligodendrocytes (Louis et al., 1993; Mayer et al., 1994). Like IL-6 and LIF, CNTF also induces the expression of acute phase proteins in hepatocytes (Dittrich et al., 1994).

In the PNS, CNTF is expressed postnatally and is localized to a subset of Schwann cells (Rende et al., 1992; Dobrea et al., 1992). Although CNTF is expressed abundantly in adult myelinated peripheral nerves, it decreases markedly following nerve injury (Sendtner et al., 1992b; Dobrea et al., 1992; Rabinovsky et al., 1992; Seniuk et al., 1992). Re-expression of CNTF in the nerve occurs after axonal regeneration once remyelination has commenced (Sendtner et al., 1992b). To date the physiological function of CNTF in the PNS remains unclear.

Cloning of the CNTF binding protein, CNTFRα, revealed that it is a glycosyl-phosphatidylinositol (GPI) linked molecule and has similarities with IL-6 receptor α (Davis et al., 1991; Davis, Yancopoulos, 1993). Further studies showed that CNTF responsiveness is mediated by the binding of CNTF to CNTFRα, which in turn associates with the functional LIF receptor (the LIF receptor β and gp130 dimer) (Baumann et al., 1993; Davis et al., 1993; Stahl, Yancopoulos, 1994b). Gp130, the signal transducing subunit of the IL-6 receptor complex, is the common signal transducer for the members of this cytokine family (Nishimoto et al., 1994; Ip, Yancopoulos, 1996). CNTFRα immunoreactivity is present in adult neurons in hippocampal formation, piriform cortex, olfactory bulb, cerebellar Purkinje cells, pons, red nucleus and other nuclei associated with motor control including ventral horn neurons of the spinal cord (MacLennan et al., 1996). Expression is also observed in skeletal muscle and adult DRG neurons, both in the perikarya and in the axons (MacLennan et al., 1996). During development, CNTFRα

immunoreactivity is localized in differentiating neurons, including those in DRG, sympathetic ganglia, ventral horn, intermediolateral column of the spinal cord and developing brainstem nuclei (MacLennan et al., 1996).

c. Leukemia Inhibitory Factor

Leukemia inhibitory factor (LIF) was initially described in the nervous system as the molecule secreted by cultured heart cells which promoted the cholinergic differentiation of rat sympathetic neurons (Yamamori et al., 1989). LIF is now known to be important in blastocyst implantation, bone resorption, fat metabolism, maintenance of hematopoietic stem cells and in the induction of hepatic acute phase response (Gearing, 1991). A recent investigation showed that LIF also stimulates survival of Schwann cells in culture (Dowsing et al., 1999). LIF and CNTF have overlapping actions on sympathetic, sensory and motor neurons (Cheema et al., 1994a; Cheema et al., 1994b) probably because they share receptor components (Ip et al., 1992b; Gearing, 1993; Baumann et al., 1993; Taga, 1996). The functional LIF signaling receptor complex consists of gp130 and LIF receptor β (LIFRβ) (Taga, 1996).

LIF is normally present in peripheral nerves at very low concentrations and induced rapidly following nerve injury in the ipsilateral DRG, and in segments distal and immediately proximal to the lesion (Curtis et al., 1994; Banner, Patterson, 1994). The message for LIFRβ rapidly decreases in the distal stump and persists for at least 2 weeks (Banner, Patterson, 1994). Also, LIFRβ mRNA concentration decreases in organ cultures of SCG and DRG (Banner, Patterson, 1994). However, studies investigating axonal

transport showed that LIF is retrogradely transported to the cell bodies of sensory and motor neurons and this transport is increased after nerve crush (Curtis et al., 1994).

Mice deficient in either *LIF* or *CNTF* gene are viable and do not show apparent neurological abnormalities but *CNTF* null mice display mild motor dysfunction associated with a slight reduction in motor neurons later in adulthood (Masu et al., 1993; Rao et al., 1993). In addition, 2% of the Japanese population lack the *CNTF* gene but have no overt neurological abnormalities (Takahasi et al., 1994). However, disruption of both *LIF* and *CNTF* genes results in a dramatic loss of motor neurons and an earlier onset of motor dysfunction (Sendtner et al., 1996). Mice lacking either *CNTFRα* or *LIFRβ* gene exhibit dramatic loss of motor neurons and die soon after birth (DeChiara et al., 1995; Li et al., 1995) suggesting that CNTFRα has other ligand(s) besides CNTF that is (are) important for normal development of motoneurons. Disruption of *gp130* gene resulted in mice with severe hematological and myocardial disorders that died before birth (Yoshida et al., 1996).

d. Cardiotrophin-1

Cardiotrophin-1 (CT-1) was discovered by expression cloning and found to induce cardiac myocyte hypertrophy (Pennica et al., 1995a). Like other members of this family, CT-1 also stimulates hepatocyte production of acute phase proteins (Peters et al., 1995). The trophic action of CT-1 on rat DRG neurons *in vitro* is similar to that of CNTF and LIF (Thier et al., 1999). CT-1 mRNA is expressed in the embryonic limb bud and is secreted by differentiated myotubes (Pennica et al., 1996). Treatment with CT-1 rescues neonatal rat motor neurons after axotomy (Pennica et al., 1996). Binding studies showed

that CT-1 utilizes the functional LIF receptor complex for signal transduction (Pennica et al., 1995b). In addition, CT-1 has been shown to bind to a GPI-linked membrane protein that is distinct from CNTFR∀ (Pennica et al., 1996). It is possible that CT-1 is one of the molecular signals from the target that promotes the survival of motor neurons during embryogenesis.

3. The Transforming Growth Factor Superfamily

Transforming growth factor (TGF) and related proteins belong to a superfamily of related molecules that modulate cell growth, differentiation, migration and death (Wahl, 1992; Massagué et al., 1994). These molecules are grouped into different clusters: TGF-β, activins/inhibins, M✚llerian inhibitory substance (MIS), bone morphogenic proteins (BMPs), glial-derived neurotrophic factor (GDNF) and other related proteins (Wahl, 1992; Massagué et al., 1994). Synthesized as large precursor proteins, these molecules dimerize and are proteolytically cleaved by furin peptidase resulting in dimers linked by disulfide bonds with the monomers oriented anti-parallel to each other (Massagué et al., 1994; Dubois et al., 1995). While TGF-βs are homodimers (Wahl, 1992; McCartney-Francis, Wahl, 1994) and activins are dimers of related β_A and β_B chains, inhibins are composed of a β chain and a distantly related α chain (Massagué et al., 1994). Among the members of this superfamily, TGF-β and GDNF have potent actions in the nervous system.

a. Transforming Growth Factor-β

The three mammalian TGF-β isoforms are encoded by different genes but the mature proteins share 70-80% homology in their amino acid sequence, bind to the same receptors and have similar actions (Wahl, 1992; McCartney-Francis, Wahl, 1994). The mature TGF-β remains inactive as it is associated, through a noncovalent interaction, with the 75-kDa latency-associated protein (LAP), which is covalently bound to the 135-kDa modulator/binding protein (Wahl, 1992; McCartney-Francis, Wahl, 1994). *In vitro*, TGF-β activated by denaturing conditions (e.g., extremes of pH and heat), ionizing radiation, plasmin, thrombospondin, and calpain (Schultz-Cherry, Murphy-Ullrich, 1993; Munger et al., 1997; Abe et al., 1998).

TGF-βs act on Schwann cells by promoting the nonmyelinating phenotype – inhibits P_0 and galactocerebroside synthesis and stimulates the synthesis of p75 LNGFR, NCAM, L1, and glial fibrillary acidic protein (GFAP) (Mews, Meyer, 1993; Stewart et al., 1995). In addition, in cocultures of rat embryonic DRG neurons and Schwann cells from neonatal rat sciatic nerve maintained under myelinationg conditions, addition of TGF-β blocks myelin formation (Guénard et al., 1995). In the TGF-β-treated cocultures many of the Schwann cells ensheath multiple axons, an ensheathment pattern that is characteristic of the nonmyelinating phenotype (Guénard et al., 1995).

TGF-βs exert their actions by binding to three cell surface proteins. The type I (53 kDa) and type II (70-83 kDa) TGF receptors are serine-threonine kinases and both participate in signal transduction while the type III (250-350 kDa) receptor, also called betaglycan, binds and presents TGF-β to the functional signaling complex (McCartney-Francis, Wahl, 1994).

In the PNS, TGF-β1, -β2, and -β3 have been detected by immunohistochemistry in Schwann cell cytoplasm as well as in the perineurium and the endoneurial blood vessels (Scherer et al., 1993; Rufer et al., 1994). In addition, TGF-β2 and -β3 are also detected in the axoplasm of most axons (Unsicker et al., 1991). Northern blot analysis showed that TGF-β1 and-β3 mRNA concentrations in rat sciatic nerve remain constant from P1 to P90 but TGF-β2 mRNA concentration is below the detection limit (Scherer et al., 1993). After sciatic nerve crush and transection, TGF-β-immunoreactivity increased within 12 hours after lesion in the proximal stump and by 48 hours in the distal stump (Rufer et al., 1994). The increased TGF-β staining in the lesioned sciatic nerves persisted for at least 14 days (Rufer et al., 1994). The concentrations of TGF-β1 mRNA starts to increase 1 day post-axotomy and remains elevated in transected nerves for at least 24 days. Following nerve crush, TGF-β1 mRNA concentrations increase in the distal stump 1-8 days post-lesion and gradually return to baseline concentrations between 8-24 days (Scherer et al., 1993). In contrast, TGF-β3 mRNA concentrations decrease following axotomy and in crushed nerves, start to rise between 8-24 days. The patterns of distribution of TGF-β1 and TGF-β3 mRNA are comparable to that of p75 LNGFR and P_0 mRNA, respectively (Scherer et al., 1993). Cultured Schwann cells produce TGF-β1 mRNA, which is reduced by forskolin treatment (Scherer et al., 1993).

b. Glial-Derived Neurotrophic Factor

Glial-derived growth factor (GDNF), a distant member of TGF-β family purified from the supernantant of the glial cell line B49, was initially identified for its ability to support survival and maturation of cultured rat embryonic dopaminergic neurons (Lin et

al., 1993). It has been shown to promote survival of embryonic rat motor neurons *in vitro* and *in vivo*, and it prevents degeneration of motor neurons of neonatal rats following facial nerve transection (Henderson et al., 1994). In addition, GDNF treatment of chick embryos *in ovo* rescues motor and sympathetic ganglia neurons but not DRG, nodose, or ciliary ganglia neurons from programmed cell death (Oppenheim et al., 1995). GDNF mRNA is expressed in the developing skeletal muscle, peripheral nerve, DRG, ventral roots and spinal cord (Henderson et al., 1994; Trupp et al., 1995). Its expression in peripheral nerves persists into adulthood (Naveilhan et al., 1997). Transection of peripheral nerves results in an increase of GDNF mRNA in the distal nerve stump and the muscle but not in the proximal nerve stump (Naveilhan et al., 1997).

Many of the actions of GDNF appear to overlap with those of neurotrophins and neuropoietic cytokines, especially, its actions on motor neurons (Chavrier et al., 1989; Koliatsos et al., 1993; Koliatsos et al., 1994; Henderson et al., 1994; Schmalbruch, Rosenthal, 1995). These effects are mediated by the GDNF receptor complex - the ligand binding receptor subunit GFRα1, a glycosyl-phosphotidylinositol- (GPI-) linked protein (Jing et al., 1996) and the transmembrane tyrosine kinase Ret, the signal transducer (Trupp et al., 1996).

In adult mice, GFRα1 mRNA is expressed in the peripheral nerve and the spinal cord while Ret is expressed in the spinal cord motor neurons (Naveilhan et al., 1997). Following sciatic nerve crush, GFRα1 mRNA concentrations are increased in the distal stump but not in the proximal stump and in the spinal cord (Naveilhan et al., 1997). Ret mRNA, on the other hand, increases rapidly in the spinal motor neurons and the DRG neurons (Naveilhan et al., 1997).

The other members of the GDNF family thus far are neurturin, which binds to GFRα2 (Widenfalk et al., 1997), artemin, which binds to GFRα3 (Baloh et al., 1998), and persephin, which binds to GFRα4 (Milbrandt et al., 1998; Enokido et al., 1998). These molecules also signal through the tyrosine kinase Ret (Trupp et al., 1998). Disruption of the neurturin and GFRα2 genes revealed that these molecules are essential for the development of the enteric nervous system and for the parasympathetic cholinergic innervation of the lacrimal and the submandibular salivary glands (Rossi et al., 1999; Heuckeroth et al., 1999). It is still not known how these GDNF related molecules are distributed in injured peripheral nerve.

F. Neuregulins: the putative neuronal signals that regulate Schwann cell phenotype?

Neuregulins constitute a family of molecules encoded by a single gene and are classified according to their distinct amino termini (Marchionni et al., 1993; Fishbach, Rosen, 1997). Glial growth factor (GGF) isoforms, the potent mitogens for Schwann cells, were initially purified from bovine pituitary extracts (Raff et al., 1978). Cloning of GGFs revealed that a single gene encodes these Schwann cell mitogens as well as acetylcholine receptor-inducing activity (ARIA), neu differentiation factor (NDF) and heregulins (HRG) (Marchionni et al., 1993). Alternative splicing gives rise to the different α and β isoforms of neuregulin (Marchionni et al., 1993). All these molecules signal through receptors belonging to the EGF receptor family. Neu/HER2/erbB2 is the signal transducing subunit (Holmes et al., 1992) while HER3/erbB3 and HER4/erbB4 are the ligand binding subunit (Vartanian et al., 1997). Heterodimerization of either erbB3 or

erbB4 with erbB2 is necessary for signal transduction (Chen et al., 1996; Vartanian et al., 1997).

In situ hybridization studies showed that during mouse embryogenesis, the mRNAs for neuregulins are localized in the sensory, motor and sympathetic ganglion neurons starting at midgestation (Meyer, Birchmeier, 1994). On the other hand, erbB2 receptor has been localized in migrating neural crest cells (Shah et al., 1994) while erbB3 receptor is produced by developing Schwann cells and in a subset of cells in the DRG but not in motor neurons (Vartanian et al., 1997). These observations strongly indicate that neuregulins are involved in the development of peripheral nerves by signalling through the erbB2 and erbB3 receptor complex.

Neural crest cells arise from the dorsal aspect of the neural tube and migrate to different regions of the embryo giving rise to the various cells of the peripheral nervous system (PNS), bone, smooth muscle, pigment and endocrine cells (Bronner-Fraser, 1994). Clonal analysis of neural crest cultures revealed that neuregulins promote glial differentiation and inhibit the generation of neurons (Shah et al., 1994). Survival and further development of Schwann cell precursors into immature Schwann cells ($LNGFR^-$, $S100^-$) require the presence of neurons (Jessen et al., 1994). In the absence of neurons, fibroblast growth factor-2 (FGF2) and neuregulin-β1 (NRG-β1) can mediate the survival and maturation of the precursors into $S100^+$ Schwann cells (Dong et al., 1995; Jessen et al., 1994).

The importance of neuregulins as the neuronal signals that mediate Schwann cell development and function *in vivo* has been investigated with the generation of mice with null mutations in the neuregulin gene as well as in the genes encoding the functional

neuregulin receptors (Meyer, Birchmeier, 1995; Gassman et al., 1995; Riethmacher et al., 1997). Mice with targeted mutations in the *neuregulin* gene die during embryogenesis and Schwann cell precursors and cranial ganglia do not develop normally (Meyer, Birchmeier, 1995). Most mice with targeted mutations in *erbB3* gene die during embryogenesis and the embryos that survive develop to term (Riethmacher et al., 1997). More detailed study of the embryonic development of *erbB3* mutant mice showed that Schwann cell precursors and Schwann cells that associate with axons of the sensory and motor neurons are lacking (Riethmacher et al., 1997). Sensory neurons in the DRG as well as motor neurons appear normal at E12.5 but by E18.5 significant number of cells have undergone cell death (Riethmacher et al., 1997). In contrast, mice deficient in *erbB2*, the signal transducer, die at midgestation (E11) due to failure of the heart to develop normally and display defects in cranial neural crest-derived sensory ganglia while trunk neural crest-derived sensory neurons in the DRG appear normal at E10.5 (Lee et al., 1995). Recently, the cardiac defect of these erbB2 null mice has been genetically rescued and the mouse embryos lacking the *erbB2* gene are able to develop to term but die at birth (Morris et al., 1999). In these mice Schwann cells lacking, the motor and sensory neurons are dramatically reduced in numbers, and the axons of surviving motor and sensory neurons are severely defasciculated and have aberrant projections (Morris et al., 1999). These observations suggest that neuregulins made by neurons are important signals for normal Schwann cell development.

Schwann cells are not only the target cells of neuregulins but are also the source of synthesis of these molecules in the nerve during Wallerian degeneration (Carroll et al., 1997). Neuregulins are not detected normally in uninjured nerves but following nerve

lesion, the mRNA and protein concentrations of the GGF subfamily of neuregulins, erbB2, and erbB3 increase beginning at 3 days post-axotomy (Carroll et al., 1997). Tyrosine phosphorylation of erbB2, which marks the activation of the receptor, increases in the sciatic nerve undergoing Wallerian degeneration (Kwon et al., 1997). The time course of the increase in concentration of GGF and that of the increase in the activation of erbB2 correlate with the time course of Schwann cell proliferation in the distal nerve stump (Carroll et al., 1997; Kwon et al., 1997).

III. FIBROBLAST RESPONSE

In the normal nerve fibroblasts are found in the epineurium, perineurium and endoneurium, the diffusion barrier that serves to regulate the microenvironment of the axons (Thomas, 1966; Ohara et al., 1986). Very little is known about the responses of fibroblasts after axotomy. The nerve epineurium, perinuerium, and endoneurium undergo restructuring after injury, especially in cases where axonal regeneration has been prevented (Thomas, 1966; Latker et al., 1991; Popovic et al., 1994; Bradley et al., 1998). At the lesion site, Schwann cells and endoneurial and perineurial fibroblasts grow out of the transected end and become embedded in the network of collagen fibrils (Thomas, 1966). In the distal nerve segments, endoneurial fibroblast-like cells proliferate at 2 weeks post-axotomy (Salonen et al., 1988; Popovic et al., 1994) and their thin processes encircle the persisting basal lamina (Thomas, 1964; Popovic et al., 1994). A month after nerve lesion the endoneurial cells completely surround and separate the basal lamina tubes from each other (Popovic et al., 1994; Bradley et al., 1998). The message of

fibroblast growth factor (FGF)-1 and -2 increases in the proximal and distal stump after nerve injury (Meisinger, Grothe, 1997) and may stimulate the proliferation of fibroblasts.

Another important response of fibroblasts to nerve injury is the production of molecules that could help in the regeneration of axons (Lindholm et al., 1987) or influence other non-neuronal cells (Horigome et al., 1993; Kawamoto et al., 1995). Fibroblasts stimulated by interleukin-1, which may be derived from macrophages, and by tumor necrosis factor (TNF)-α produce NGF (Lindholm et al., 1987; Gordon, Galli, 1990). This fibroblast-derived NGF promotes survival of neurons (Goedert et al., 1984; Levi-Montalcini, 1987) and stimulates the mast cells to synthesize cytokines and to degranulate (Bullock, Johnson, Jr., 1996).

IV. MACROPHAGE INFILTRATION

Monocytes/macrophages are considered to be important players in the repair of damaged nervous tissue. In the normal peripheral nervous system (PNS), they constitute 2-9% of all endoneurial cells (Oldfors, 1980; Griffin et al., 1993a) and are found around blood vessels, among nerve fibers in the endoneurium and around sensory neuronal cell bodies (Lu, Richardson, 1993; Vass et al., 1993). The normal turn-over of these mononuclear cells up to 80% in the DRG and 60% in the peripheral nerve within 3 months (Vass et al., 1993). Physical insult to a peripheral nerve results in Wallerian degeneration, which elicits an inflammatory response that is restricted to mononuclear phagocytes (reviewed by Brück, 1997). In rats, infiltrating mononuclear cells are first detected by immunohistochemistry three days following injury and their numbers increase thereafter (Stoll et al., 1989; Chumasov, Svetikova, 1991; Taskinen, Röyttä, 1997). They form perivascular infiltrates in the epineurium and are found around the vasa nervosa and between myelinated and unmyelinated fibers (Chumasov, Svetikova, 1991).

These macrophages remove axonal and myelin debris (Müller, Minwegen, 1987; Perry et al., 1995; Brück et al., 1996). In the late stages of Wallerian degeneration, when the mass of myelin have been phagocytosed by the macrophages and regenerating nerve fibers appear, the number of mononuclear cells declines sharply and only clusters and chains of "foam" cells remain subsequently between the fibers (Chumasov, Svetikova, 1991). These chains of foam cells are observed even 1.5 to 2 months following injury (Chumasov, Svetikova, 1991; Lu, Richardson, 1993).

A. The molecular signals for monocyte homing

The mechanism of macrophage recruitment following nerve lesion is still not well understood. However, the initial "signal" appears to depend on the degeneration of the axons (reviewed by Griffin et al., 1993a). In general, the extravasation of leukocytes both soluble and membrane-bound molecules have been implicated including adhesion ligands on the leukocytes and their counter-receptors on the endothelial cells (reviewed by Springer, 1994; Springer, 1995). Migration has been proposed to occur in at least three steps involving the interactions of selectin-mucin-like molecule, chemoattacrant- receptor and integrin-immunoglobulin family (Springer, 1994). These interactions act in sequence, not in parallel.

1. Selectins

The word selectin is derived from lectin and select from the same Latin root which means to select by picking out (Springer, 1995). Selectins mediate the initial tethering of leukocytes to the vessel wall and subsequently the formation of labile adhesions that enable leukocytes to roll in the direction of flow (Springer, 1994). The three members of the selectin family all contain a N-terminal region similar to mammalian C-type lectin followed by an epidermal growth factor (EGF)-like domain, a variable number consensus repeats of complement regulatory proteins, a transmembrane

domain and a short cytoplasmic tail (Pober, Cotran, 1991). The expression of selectins is limited to the cells of the vasculature (Springer, 1995). Thus, the role of selectins in the migration of monocytic cells into the injured PNS has not been studied.

2. Chemoattractants

Chemoattractants mediate the activation of integrin adhesiveness and in directing the migration of leukocytes (Springer, 1994; Springer, 1995). The are two ways by which cells can migrate. In the process of chemotaxis, leukocytes move in the direction of increasing concentration of the chemoattractant. Alternatively, cells can move towards increasing adhesiveness. The classical chemoatttractants, like formylated peptides, complement fragments and arachidonic acid metabolites act broadly on the leukocytes (Springer, 1994). However, almost a decade ago, a family of chemoattractive cytokines has been shown to specifically act on subsets of leukocytes (Leonard, Yoshimura, 1990). This family of chemokines is made up of small, basic and heparin-binding molecules, with molecular weights ranging from 8 to 12 kDa, and is further classified based on their sequence homology around two cysteine residues (Rollins, 1997). The α chemokines have the CXC motif and mostly act on neutrophils and nonhemotopoietic cells involved in wound healing while the β chemokines have the CC motif and mostly act on monocytes, lymphocytes and eosinophils (Springer, 1995). The limited cellular distribution of chemokine receptors contributes to their specificity (Rollins, 1997). The chemokine receptors are 7 transmembrane spanning, G-protein – coupled receptors (Baggiolini et al., 1997). Although these receptors have overlapping specificities, their ligand specificity does not cross the CC versus CXC boundaries (Springer, 1994).

The monocyte chemoattractant protein-1 (MCP-1) is a member of the β-chemokine family. Human MCP-1, which was originally cloned from myelomonocytic and glioma cell lines, is synthesized by several immune and non-immune cells (Leonard, Yoshimura, 1990). In the PNS, MCP-1 mRNA expression has been observed in the nerve

following tellurium-induced primary demyelination, nerve crush and nerve transection (Toews et al., 1998). Recently, MCP-1 mRNA was shown to be induced at the site of trauma within 1.5 hours and in the distal stump within 16 hours (Carroll, Frohnert, 1998). In addition, the peak of expression occurred 1day after nerve crush while in transected nerve it was observed 2 days after injury (Toews et al., 1998). MCP-1 mRNA expression has been localized to Schwann cells (Ransohoff, 1997). Thus, it is likely that Schwann cells contribute to the homing of monocytic cells by synthesizing chemokines.

3. Leukointegrins

Integrins are cell surface receptors that mediate cell-cell and cell-extracellular matrix adhesion (Hynes, 1992). In co-cultures of nerve segments and macrophages, antibodies to complement receptor type 3 (CR3) which is also known as Mac-1 or $\alpha_M\beta_2$ integrin has been reported to inhibit the recruitment of phagocytes and the removal of myelin debris (Lunn et al., 1989; Brück, Friede, 1990a). In addition, depletion of serum complement in rats reduced recruitment of macrophages into the degenerating nerve and macrophage activation (Levi-Montalcini, 1987). CR3 binds to intracellular adhesion molecule-1 (ICAM-1) (Hynes, 1992). ICAM-1, a 90 kDa glycoprotein that belongs to the immunoglobulin superfamily, is primarily expressed in endothelial cells and is up-regulated after cytokine stimulation (Springer, 1995). In the PNS, in spite of the colocalization of ICAM-1 with Ia antigen on macrophages in the nerve during the early stages of experimental autoimmune neuritis, it was not initially detected on endothelial cells and macrophages in transected nerves (Stoll et al., 1993b). However, another group has observed that ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) are expressed on endothelial cells at the lesion site and in the distal stump of injured nerves (Castano et al., 1996). Furthermore, ICAM-1 deficient mice sciatic exhibit a reduction in recruitment of macrophages in the distal stump and in the removal of myelin after sciatic nerve transection (Vougioukas et al., 1998).

All these observations suggest that MCP-1 as well as the interaction of ICAM-1 and CR3 contribute to the homing of monocytes into the injured peripheral nervous tissue.

B. Macrophages remove myelin and axonal debris

The studies of Ramon y Cajal (Ramon y Cajal, 1928) and Nageotte (as cited by (Ramon y Cajal, 1928)) suggested the involvement of hematogenous cells in the phagocytosis of degenerating myelin. This concept was later supported by the experiments using the Millicell diffusion chambers which revealed that non-resident phagocytic cells are responsible for the removal of myelin (Beuche, Friede, 1984). Selective inhibition of macrophages by the intraperitoneal injection of silica quartz dust results in delayed myelin degradation (Müller, Minwegen, 1987). Inhibition or limiting the recruitment of marcophages into the degenerating nerve by whole body irradiation or intravenous injection of dichloromethylene (Cl_2MDP) also leads to the reduction of myelin removal (Perry et al., 1995; Brück et al., 1996). As some myelin degradation still occurs even in the absence of infiltrating macrophages, the initial removal of myelin could be attributed to Schwann cells and resident macrophages and that recruited macrophages are required for the complete myelin degradation (Perry et al., 1995).

Immunohistochemical studies revealed that these infiltrating cells are positive for Mac-1 (complement receptor type 3 or CR3) and Fc-receptors (Scheidt, Friede, 1987). However, the macrophage Fc-receptor does not appear to participate in myelin degradation as myelin removal still occurs in the absence of immunoglobulins (Hann et al., 1988). In co-cultures of non-resident macrophages and nerve segments exposed to β-mannosidase the infiltration of macrophages is inhibited while treatment with L-fucosidase blocks myelin removal (Brück, Friede, 1990b). A binding site for polysaccharides is also present on CR3 (Lambris, 1988) and a fucose-bearing surface molecule enhances the activation of macrophage C3 receptor (Griffin, Mullinax, 1984).

When the co-cultures of macrophages and nerve segments are maintained in a medium supplented with C3-deficient serum, myelin phagocytosis is inhibited (Brück, Friede, 1991). Furthermore, TNF-α treatment down-regulates CR3 expression and dramatically reduces myelin uptake by phagocytic cells (Brück et al., 1992). *In vivo*, depletion of serum complement a day before sciatic nerve crush reduces the recruitment of macrophages and their subsequent activation (Dailey et al., 1998b). Thus, CR3 appears to be important for normal migration, activation, and phagocytic function of macrophages in the degenerating nerves.

V. MAST CELL RESPONSE

Mast cells, which are often associated with hypersensitivity, release a number of bioactive compounds, including histamine, serotonin and TNF, upon stimulation (reviewed by Purcell, Atterwill, 1995; Dines, Pwell, 1997). These cells are found in connective tissues and could be identified histologically by the presence of metachromatic cytoplasmic granules (Enerbäck et al., 1965). Mast cells in the normal nerve are numerous in the epineurium and perineurium where they are often aggregated into small groups near blood vessels; and in the endoneurium, these cells are few and are found between nerve fibers and are not associated with blood vessels (Enerbäck et al., 1965; Olsson, 1967).

A. Degranulation of Mast Cells

Mechanical injury to the nerve, from a slight compression without microscopic damage to the nerve, triggers the rapid degranulation of mast cells at the site of the lesion (Enerbäck et al., 1965; Olsson, 1967). Metachromatic cytoplasmic granules have been found around mast cells near blood vessels in the epineurium and perineurium and among nerve fibers in the endoneurium (Olsson, 1965; Olsson, 1967). In the distal nerve stump,

no sign of degranulation has been observed for up to one week after injury (Olsson, 1965; Olsson, 1967).

1. Mast cell degranulation affects the blood-nerve barrier

The blood-nerve barrier (BNB), which is comprised of the tight junctions between the endothelial cells of the blood vessels in the endoneurium and the perinuerial cells in the perinuerium, protect the axons by regulating the endoneurial microenvironment (Latker et al., 1991). One week after nerve injury, some serum proteins cross the BNB and by 2 weeks, essentially all macromolecules cross the BNB and enter the endoneurium (Seitz et al., 1989; Latker et al., 1991). In crush lesions the integrity of the BNB gradually recovers while transection of the nerve renders the barrier permanently leaky (Seitz et al., 1989).

Mast cell cytoplasmic granules contain proteases and biogenic amines, including histamine and 5-hydroxytryptamine or serotonin (reviewed by Dines, Pwell, 1997). These cells degranulate following injury thereby releasing these molecules (Olsson, 1965; Enerbäck et al., 1965). Histamine concentrations in the nerve sheath, which contains the mast cells, decrease within 2 hours after injury and then increases by 7 days and remains elevated for at least 2 weeks in the distal nerve stumps (MacDonald et al., 1981). This distribution correlates with the increase in mast cell numbers in the distal stump (Enerbäck et al., 1965; Windebank et al., 1985). In addition, administration of histamine and compound 48/80, which promotes mast cell degranulation, into rat peripheral nerves alters the vascular permeability and promotes edema formation (Powell et al., 1980). Thus, molecules released by mast cells contribute to the changes in vascular permeability in the degenerating nerve.

Biogenic amines may also affect Schwann cells as these cells appear to express the 5-HT_{2A} serotonin receptor (Yoder et al., 1996). Concentrations of intracellular Ca^{++} in Schwann cells increase in response to serotonin application (Yoder et al., 1996).

However, it is not known how activation of this receptor in Schwann cells contribute to the changes during Wallerian degeneration.

2. Mast cell proteases contribute to myelin degradation

Proteases are some of the enzymes present in the cytoplasmic granules of mast cells (Johnson et al., 1988; Dietsch, Hinrichs, 1991; Compton et al., 1998). Incubation of CNS myelin with mast cell degranulation supernatant results in the degradation of MBP (Johnson et al., 1988; Dietsch, Hinrichs, 1991). In addition, MBP itself can stimulate the degranulation of mast cells (Johnson et al., 1988). It is possible that proteases released from mast cells contribute to the degradation of myelin after nerve injury.

B. Mast Cell Proliferation

After the initial degranulation following nerve injury, very few mast cells are found around the site of lesion, and their number increases by 4 to 6 weeks (Olsson, 1965; Enerbäck et al., 1965). In the distal nerve segment on the other hand, mast cells number in the endoneurium increases starting at 2 days post-transection and remains elevated for at least 40 weeks (Olsson, 1965; Enerbäck et al., 1965; Latker et al., 1991). No significant changes in epineurial mast cell number have been observed (Enerbäck et al., 1965).

Interleukin-3 (IL-3) and stem cell factor (SCF) are the classical regulators of mast cell survival and proliferation (reviewed by Galli et al., 1993). However, neonatal rats treated daily with NGF have increased mast cell numbers in a number of different tissues (Aloe, Levi-Montalcini, 1977). Conversely, administration of antibodies against NGF results in the reduction of mast cell numbers *in vivo* (Aloe, 1988). Rat peritoneal mast cells express the high affinity receptor for NGF, trkA, but not p75 LNGFR (Horigome et

al., 1993). Hence, NGF, which increases in the distal nerve stump after injury (Heumann et al., 1987a; Heumann et al., 1987b) may contribute to the increase in endoneurial mast cell numbers in the distal segments (Olsson, 1965; Enerbäck et al., 1965). However, in rat peritoneal mast cells, NGF does not stimulate proliferation but supports survival by preventing apoptosis of IL-3 deprived mast cells (Kawamoto et al., 1995). In addition, NGF treatment of mast cells induces the mRNA expression of IL-3, IL-10, TNF-α and GM-CSF (Bullock, Johnson, Jr., 1996). Thus, in the injured nerves, macrophage-derived IL-1 (Lindholm et al., 1987) and mast cell-derived TNF-α (Gordon, Galli, 1990) may stimulate fibroblasts to produce NGF, which in turn stimulates the production of IL-3 by mast cells. IL-3 is a potent growth factor for mast cells (Lantz, Huff, 1995).

VI. C57CL/Wld MICE AS A TOOL TO STUDY WALLERIAN DEGENERATION

The mutant mouse strain C57BL/Wld, formerly called C57BL/Ola or Ola, is characterized to have delayed Wallerian degeneration, a property that is intrinsic to the axon (Glass et al., 1993). Associated with the delayed Wallerian degeneration is the retarded myelin breakdown and subnormal recruitment of myelomonocytic cells (Lunn et al., 1989). Concomitant with axonal breakdown, cell numbers in the distal stump of control outbred ULP mice increase within three days following axotomy while in the distal stump of Wld mice no change in cell numbers is observed even 10 days after lesion (Lunn et al., 1989). However, Wld nerve explants exhibit disintegration of axonal cytoskeleton and when co-cultured with non-resident macrophages, non-resident macrophages migrate into the nerve (Brück et al., 1995). *In vitro*, a higher concentration of calcium is necessary for calpain to degrade neurofilaments (Glass et al., 1994). However, cloning of the 80 kD ubunit of m-calpain showed that m-calpain in Wld mice is not different from those of control mice (Glass et al., 1998). The mutation has been

mapped to mouse chromosome 4 (Lyon et al., 1993) and within the candidate region an 85-kb tandem triplication has been identified (Coleman et al., 1998).

Studies on axonal regeneration in Wld mice showed that the delayed degeneration of axons in these mice is associated with delayed regeneration of sensory neurons and to a lesser extent, the motor neurons (Bisby, Chen, 1990; Brown et al., 1992). The "cell body response" to injury as determined by an increase in GAP-43 mRNA concentration and Jun-immunoreactivity in facial and sensory neurons is similar to that of control mice (Bisby et al., 1995). Hence, the delayed regeneration of sensory axons in Wld mice is most likely due to the retarded degeneration of axons and not to the lack of neuronal cell body response to injury.

Table 1. Some of the cellular events in the nerve during Wallerian degeneration.

Time	Event	Reference
0-12 h	▪ degranulation of mast cells at the lesion site ▪ ↑ in Na, K and Cl concentrations in Schwann cell cytoplasm; ▪ ↑in Ca concentrations in myelin ▪ accumulation of organelles at the transected end of the nerve	Olsson, 1967; Cravioto, 1969; Donat, Wisniewski, 1973; LoPachin et al., 1990
12-24 h	▪ degeneration of motor-end plates ▪ loss of action potentials ▪ calcium-dependent granular disintegration of axons ▪ fragmentation of myelin lamellae at nodes of Ranvier ▪ mitosis of endothelial cells	Miledi, Slater, 1970; Oaklander et al., 1987; Griffin, Hoffman, 1993; Liu et al., 1995;
24-48 h	▪ linear fragmentation of myelin	Schlaepfer, 1974
2-4 d	▪ infiltration of macrophages ▪ phagocytosis of myelin and axonal debris ▪ Schwann cell mitosis	Chumasov, Svetikova, 1991; Liu et al., 1995; Taskinen, Röyttä, 1997;

Table 2. Changes in the expression of Schwann cell genes in peripheral nerves.

Marker	During Development	Nonmyelinating S. C.	Myelinating S.C.	Wallerian Degeneration	References
S100	+	+	+	+	Jessen, Mirsky, 1991; Jessen et al., 1994
L1	+	+	-	+	Martini, Schachner, 1988a; Martini, Schachner, 1988b Jessen, Mirsky, 1991
NCAM	+ (PSA)	+	-	+	Martini, Schachner, 1988a; Martini, Schachner, 1988b; Jessen, Mirsky, 1991
P75 LNGFR	+	+	±	+	Jessen et al., 1994; Scherer et al., 1994; Zorick et al., 1996
GFAP	+	+	-	+	Jessen, Mirsky, 1991; Jessen, Mirsky, 1992
P_0	-	-	+	-	Poduslo, Windebank, 1985; Gupta et al., 1988; Trapp et al., 1988; Kuhn et al., 1993
MBP	-	-	+	-	Kuhn et al., 1993
PMP-22	-	-	+	-	Gupta et al., 1988; Trapp et al., 1988; Martini, Schachner, 1988b; Gupta et al., 1993
MAG	-	-	+	-	Martini, Schachner, 1988a; Gupta et al., 1993; Jessen, Mirsky, 1991
Neuregulin	-	-	-	+ (GGF)	Carroll et al., 1997
ErbB2	++	+	+	++	Kwon et al., 1997; Carroll et al., 1997
ErbB3	++	+	+	++	Carroll et al., 1997

(S.C.) Schwann cell; (PSA) polysialic acid; (GGF) glial growth factor;
(+) present; (-) absent; (±) barely detectable

Table 3. Changes in expression of trophic factors in peripheral nerves following nerve injury.

Factor	Normal Nerve	After Injury	References
NGF	±	++ (biphasic)	(Heumann et al., 1987b; Heumann et al., 1987a)
BDNF	±	++	(Miledi, Slater, 1970; Funakoshi et al., 1993)
NT-3	++	±, ++	(Funakoshi et al., 1993)
NT-4	+	±, ++	(Funakoshi et al., 1993)
IL-6	-	+ (transient)	(Bolin et al., 1995; Zhong, Heumann, 1995; Bourde et al., 1996)
CNTF	+++	-	(Sendtner et al., 1992; Dobrea et al., 1992; Rabinovsky et al., 1992; Seniuk et al., 1992)
LIF	±	++	(Curtis et al., 1994; Banner, Patterson, 1994)
TGF-β	+	++	(Scherer et al., 1993; Rufer et al., 1994)
GDNF	+	++	(Henderson et al., 1994; Trupp et al., 1995; Naveilhan et al., 1997)
IL-1	±	++	(Rotshenker et al., 1992)
IL-10	±	++ (biphasic)	(Be'eri et al., 1998)
TNF-α	±	+	(Stoll et al., 1993a)

CHAPTER 2: PURPOSE OF THE STUDY

Severing axons from the nerve cell bodies leads to the restructuring of the nerve suggesting a very intimate relationship between the axons and the associated Schwann cells and tightly regulated interactions between the different non-neuronal cells in the nerve.

During Wallerian degeneration Schwann cells dedifferentiate – they reject the myelin and dramatically reduce the synthesis of myelin proteins while the synthesis of proteins normally present in immature and nonmyelinating Schwann cells increases (Schlaepfer, 1974; Gupta et al., 1988; Kuhn et al., 1993; Gupta et al., 1993; Liu et al., 1995). Schwann cells later proliferate and form the bands of Büngner (Liu et al., 1995). Macrophages in the distal stump increase in number and later remove axonal and myelin debris (Brück, 1997). At the lesion site mast cells degranulate within 15 minutes after injury (Olsson, 1967) and in the more distal segments their numbers increase by 1 week (Enerbäck et al., 1965). These cells release histamine and other biogenic amines from their granules and alter the blood-nerve barrier permeability (Seitz et al., 1989) and are potential source of cytokines (Bullock, Johnson, Jr., 1996). Fibroblasts in the nerve respond to the injury by undergoing proliferation (Thomas, 1966) and synthesis of molecules such as NGF that can contribute to the regeneration of axons (Lindholm et al., 1987).

The present study focused on the changes undergone by Schwann cells and other non-neuronal cells during Wallerian degeneration. The sources of the signals that initially drive these changes may be divided into the following:

a.) loss of molecules released from axons,

b.) loss of contact with molecules in the axolemma, and

c.) exposure to molecules present in the blood and those secreted by hematogenous cells.

To tease out the different signals, the regulation of three Schwann cell genes have been investigated. The molecules studied are ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF) and monocyte chemoattractant protein-1 (MCP-1).

CNTF, chosen for its ability to support neuronal survival, is expressed postnatally in the PNS, is abundant in adult myelinating peripheral nerves and is markedly reduced following injury (Sendtner et al., 1992b; Seniuk et al., 1992). LIF is closely related to CNTF and has functions that overlap with those of CNTF (Gearing, 1993; Cheema et al., 1994b). Unlike CNTF, LIF is normally present in peripheral nerves at very low concentrations and is increased after axotomy (Curtis et al., 1994). The third molecule studied is not similar to CNTF and LIF in tertiary structure and function. MCP-1 is one of the molecular signals involved in the migration of monocytes to sites of injury (Leonard, Yoshimura, 1990; Springer, 1994). However, like LIF, MCP-1 is barely detectable in normal PNS and is induced following nerve injury (Toews et al., 1998).

A greater understanding of the different cellular and molecular interactions during Wallerian degeneration may provide insights that can enhance axonal regeneration both in the PNS and CNS.

CHAPTER 3:

CILIARY NEUROTROPHIC FACTOR mRNA EXPRESSION IN CULTURED SCHWANN CELLS

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Keywords: ciliary neurotrophic factor, Schwann cells

Ciliary neurotrophic factor (CNTF) belongs to a family of cytokines that similar in their tertiary structure (Bazan, 1991) employs gp130 as the signal transducing receptor (Ip, Yancopoulos, 1992; Zhang et al., 1994; Stahl, Yancopoulos, 1994). CNTF was first described based on its ability to support the survival of parasympathetic chick ciliary neurons (Adler et al., 1979). It is now known to support the survival of sensory (Skaper, Varon, 1986), sympathetic (Blottner et al., 1989), hippocampal (Ip et al., 1991) and motor neurons *in vitro* (Arakawa et al., 1990). Furthermore, CNTF drives the differentiation of sympathetic neurons in culture (Ernsberger et al., 1989; Saadat et al., 1989). Administration of CNTF also rescues degenerating motor neurons (Snider et al., 1990; Sendtner et al., 1992a; Mitsumoto et al., 1994) and substantia nigra dopaminergic neurons (Hagg, Varon, 1993).

In the peripheral nervous system, CNTF is expressed postnally in a subset of Schwann cells and is abundant in adult myelinated nerves (Dobrea et al., 1992). Following nerve injury, CNTF mRNA, protein and bioactivity are dramatically reduced (Rabinovsky et al., 1992; Sendtner et al., 1992b; Seniuk et al., 1992). Thus, CNTF appears to be distributed in the PNS in a similar fashion as that of myelin proteins. It is likely that myelin proteins and CNTF are regulated by similar mechanisms. To study these regulatory mechanisms, it is essential to establish Schwann cell cultures and determine whether CNTF synthesis is also reduced *in vitro*.

Schwann cell cultures were established from sciatic nerves of 5-day old rats using the modified method of Brockes et al (1979). Briefly, cells were dissociated from the nerves by sequential collagenase and trypsin digestion. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, New York, USA)

supplemented with 10% serum. During the first three days in culture, cells were treated with 10:M cytosine-∃-D-aribinofuranoside (Ara-C; Sigma-Aldrich Canada Ltd, Oakville, Ont., Canada) to remove proliferating cells. After the Ara-C treatment cells were trypsinized and replated onto polylysine coated coverslips and/or petri plates.

Immunocytochemical studies were done on freshly dissociated cells that had been plated for 5.5 hours using the antibodies against S100, the marker for glial cells, myelin basic proteins (MBP) and myelin-associated glycoprotein (MAG). The polyclonal antibody against S100 was purchased from Sigma-Aldrich Canada,Ltd (Oakville, Ont.), the polyclonal antibody against MBP was a gift from Dr. Peter Braun (McGill University, Montreal, Que.) and the monoclonal anti-MAG was a gift from Dr. Robert Dunn (McGill University, Montreal, Que.). Results showed that 65% of freshly dissociated cells from sciatic nerves of 5-day old rats were immunopositive for S100, 12% stained for MBP and 11% stained for MAG. When these cells were cultured for 24 hours, MBP- and MAG-immunopositive cells were no longer observed. These data confirm the previous observation that myelin protein synthesis is reduced *in vitro* (Morrison et al., 1991).

The synthesis of CNTF mRNA in cultured Schwann cells was determined by RNase protection assays. RNase protection assay was performed following the method of Seniuk et. al. (1992). Data showed that cells freshly dissociated from 5-day old rats synthesized CNTF mRNA but in 1-day and 5-day old Schwann cell cultures CNTF mRNA concentrations were markedly reduced (Fig. 1).

To confirm that CNTF synthesis in cultured Schwann cells was reduced, Western blot analysis was performed using a polyclonal antibody against the rat recombinant CNTF. CNTF-immunoreactive bands were visualized using ^{125}I-Protein-A (ICN,

California, USA) at 0.3 μCi/ml blocking solution. Results showed that CNTF-immunoreactivity was present in freshly dissociated cells and in 1-day old cultures but not in cells cultured for 5 days (Fig. 2). Thus, similar to myelin proteins CNTF synthesis in cultured Schwann cells also decreases. However, the amount of immunoreactive CNTF in freshly dissociated Schwann cells appears to be a disproportionately less than the amount of CNTF message present in these cells. As CNTF mRNA has been reported to have a half-life of 6 hours (Carroll et al., 1993), perhaps factors important for optimal post-transcriptional processing and/or translation of CNTF mRNA are present at very low concentrations in Schwann cells from neonatal rats.

These observations suggest that CNTF, like myelin proteins, is influenced by the presence of axons. However, the nature of this axonal signal remains to be determined. In the co-culture of embryonic rat DRG neurons and neonatal Schwann cells, CNTF mRNA and protein were produced after 10 days *in vitro* (Lee et al., 1995). Furthermore, in experiments where ensheathment of axons/neurites occur but myelination is inhibited CNTF protein synthesis was still observed (Lee et al., 1995). However, these experiments did not determine whether axonal contact per se or molecules secreted by neurons stimulate CNTF synthesis. We and others (Carroll et al., 1993) have attempted to induce CNTF production in cultured Schwann cells by adding soluble factors into the medium but failed. Schwann cells treated with forskolin (Sigma), which elevated the intracellular concentration of cAMP and has been shown to induce the expression of myelin proteins in the absence of serum, macrophage-conditioned medium or neuregulin (a gift from Dr. Salvatore Carbonetto) did not synthesize CNTF mRNA. To date, the signals that stimulates CNTF synthesis remains unknown.

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Figure 1. RNase protection assay of total RNA from freshly dissociated cells from Schwann cells (SC) cultured for 0-5 days using a ^{32}P-labelled CNTF probe. Sciatic nerves (SN) from 22-day old rats were used as positive control.

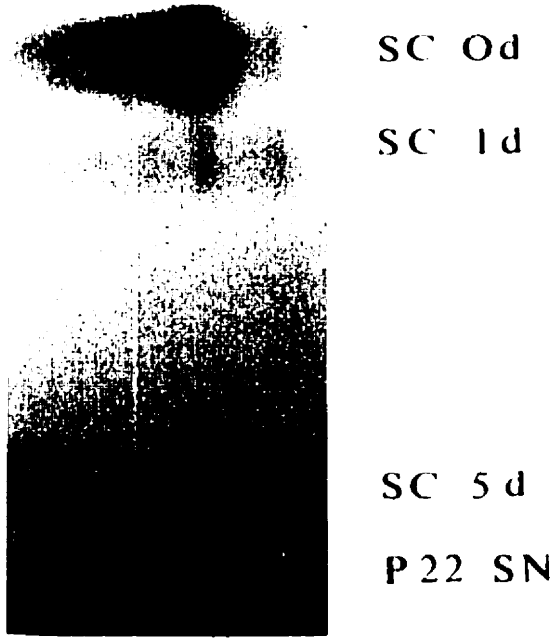
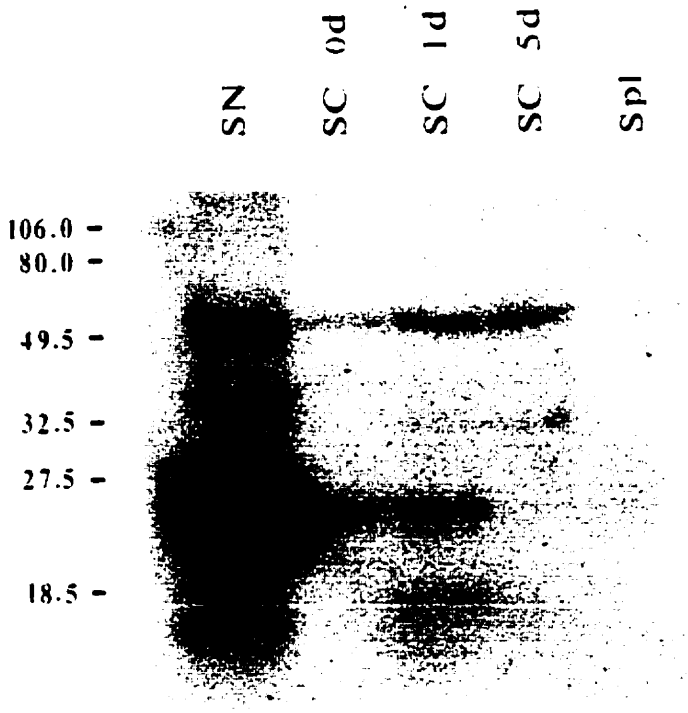

SC Od
SC 1d
SC 5d
P22 SN

Figure 2. Western blot analysis of proteins extracted from Schwann cells (SC) cultured for 0-5 days using a polyclonal antibody against the rat recombinant CNTF (1:500) and ^{125}I-Protein-A for detection. Sciatic nerve (SN) and spleen (Spl) homogenates were used as positive and negative controls, respectively.


SN
SC 0d
SC 1d
SC 5d
Spl
106.0
80.0
49.5
32.5
27.5
18.5

CHAPTER 4:

DELAY OF CNTF DOWN-REGULATION FOLLOWING PERIPHERAL NERVE INJURY IN C57BL/WLD MICE

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ABSTRACT

In peripheral nerves, ciliary neurotrophic factor (CNTF) is localized to a subset of Schwann cells and is decreased in synthesis during Wallerian degeneration. This pattern of expression is similar to that of myelin protein genes. In the present study, C57BL/Wld mice, which exhibit delayed Wallerian degeneration, were used to determine the role of axonal contact on the regulation of CNTF synthesis. Western blot analysis showed that CNTF-immunoreactivity in Wld nerves remained almost normal even 10 days after ligation when it was almost undetectable in control mice. RT-PCR analysis revealed that 4 days after ligation, concentrations in Wld and control mice were comparably low. These observations suggest that maintenance of axonal contact in the absence of axonal transport from the cell body delays the down-regulation of CNTF mRNA normally seen after injury. Also, during Wallerian degeneration in Wld mice, the decrease of CNTF protein is delayed for many days longer than the decrease in CNTF mRNA.

Keywords: CNTF, P_0, C57BL/Wld mice, Wallerian degeneration

INTRODUCTION

Ciliary neurotrophic factor (CNTF), a 20-24 kDa protein named for its ability to promote the survival of parasympathetic ciliary neurons from embryonic chicks, is now known to have potent effects on several classes of neurons and glial cells. In intact peripheral nerves, CNTF is synthesized in a subpopulation of Schwann cells from the first post natal week throughout adulthood. Following nerve injury, CNTF mRNA and protein are markedly reduced during Wallerian degeneration and gradually increase to normal levels upon axonal regeneration and remyelination (Friedman et al., 1992; Sendtner et al., 1992; Seniuk et al., 1992).

The expression of myelin protein genes involves a subset of Schwann cells, rapidly rises during the first week of postnatal life, persists in mature animals, declines after nerve injury, and is restored to normal levels following nerve regeneration (Trapp et al., 1988; Gupta et al., 1993). The similar patterns of synthesis of CNTF and myelin proteins have led to the hypothesis that CNTF and myelin proteins are regulated by common mechanisms.

Axons have long been known to modulate the expression of myelin proteins although the molecular nature of the putative axonal signal is unknown. The influence of axonal contact in the maintenance of expression of P_0, the major myelin protein of Schwann cells, has been demonstrated in the mutant mouse strain C57BL/Wld, previously known as C57BL/Ola. After nerve injury these mice have delayed breakdown of axons and myelin and subnormal recruitment of myelomonocytic cells to the distal stump (Lunn et al., 1989). This primary axonal defect is associated with an autosomal

dominant mutation (Perry et al., 1990b) in Wallerian degeneration (*Wld*) on chromosome 4 (Lyon et al., 1993). After nerve injury in these mice, P_0 synthesis declines at an abnormally slow rate (Thomson et al., 1991). The present study in WLd mice was undertaken to ascertain whether axonal contact regulates CNTF expression.

MATERIALS AND METHODS

Surgery

C57BL/Wld, C57BL/6J and CD1 mice were obtained from a colony at Queen's University; C57BL/6J and CD1 mice were used as controls. Six animals from each mouse strain were used for every experiment. Each experiment was repeated twice. Mice were anesthetized with ether delivered via a nose-cone. The left sciatic nerve was ligated in the mid thigh with a 4/0 braided nylon thread. Following sacrifice 4, 7, and 10 days after lesion, nerve segments more than 3 mm distal to the lesion site (distal stumps) and the contralateral nerves were collected, immediately frozen on solid CO_2 and processed for protein extraction or the preparation of total RNA.

Immunoblotting

For the extraction of proteins, frozen sciatic nerves were pulverized and homogenized in 20 mM Tris-Cl, pH 7.5, buffer containing 1 % Triton X-100, 150 mM Na Cl, 1 mM EDTA, and 1 mM PMSF. The total protein content in the homogenates was determined by the dye-binding method of Bradford (Bio-Rad Protein Assay Kit II) (Bradford, 1976). Sciatic nerve homogenates (20-30 μg of protein) were separated by SDS poluacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane for immunoblotting. The membranes were blocked with 5 % bovine serum albumin in Tris saline solution and incubated with either a polyclonal antibody against the recombinant rat CNTF protein raised in rabbit at a concentration of 1:500 for 3 hours at room temperature or a polyclonal antibody against P_0 protein raised in rabbit at a

concentration of 1:2000 overnight at 4°C. Immunoreactive bands were detected using ^{125}I-protein-A (ICN) at a concentration of 0.3 μCi/ml blocking solution for 1 hour at room temperature. Autoradiograms were exposed from 6 to 24 hours.

RT-PCR and Southern Blotting

The total RNA was extracted from frozen sciatic nerves using the method of Chomczynski and Sacchi (Chomczynski, Sacchi, 1987) and reverse transcribed using pd(N)6 random hexamers as primers and Moloney murine leukemia virus reverse transcriptase. CNTF cDNA was amplified by polymerase chain reaction for 18 cycles of 95°C for 45 sec, 55°C for 45 sec and 72°C for 2 min using 5'-GAATTCGGATCCATGGCTTTCGCAGAGCAA-3' (NT 1069-1086, Genbank accession number U05342, with added BamH1 site) as a sense primer and 5'-CTACATTTGCTTGGCCCC-3' (complimentary sequence to nt 1648-1665, Genbank accession number U05342) as antisense primer. As controls, parallel assays were performed for myelin protein P_0 using 5'-ATACGGATCCATTGTGGTTTACACGGAC-3' (nt 362-379 of exon 2 (You et al., 1991) with added BamH1 site) and 5'-AAGTGGATCCCTATTTCTTATCCTTGCG-3' (complimentary sequence to nt 357-374 of exon 6 (You et al., 1991) with added BamH1 site) as sense and antisense primers, respectively; and for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using 5'-TGAAGGTCGGTGTCAACGGATTTGGC-3' (nt 35-60 (Tso et al., 1985)) and 5'-CATGTAGGCCATGAGGTCCCACCAC-3' (complimentary sequence to nt 994-1017 (Tso et al., 1985)) as sense and antisense primers, respectively. The amplified products were separated by agarose gel electrophoresis and transferred onto a nylon hybridization

membrane (Hybond-N$^+$, Amersham). Bound DNA was fixed on the membranes by UV cross-linking at 0.12 joules. Southern blotting was performed using ^{32}P labelled antisense oligonucleotide probes for CNTF (complimentary sequence to nt 1132-1171) (Genbank accession number U05342), P_0 (complimentary sequence to nt 795-845 of exon 4) (You et al., 1991), and GAPDH (complimentary sequence to nt 710-750) (Tso et al., 1985). CNTF and P_0 mRNA levels were normalized to GAPDH mRNA levels. To compare the concentrations of CNTF and P_0 mRNA in the normal and axotomized nerves, cDNA reverse transcribed from the total RNA from intact nerves was diluted serially prior to the amplification. Autoradiograms were exposed overnight and scanned by densitometry. The concentration of message in the ligated nerves was determined as a percentage of that in normal contralateral nerves.

RESULTS

In control mice, CNTF-immunoreactivity was reduced 4 days after ligation and was almost undetectable by 10 days post-surgery (Fig. 1a). In injured Wld nerves, CNTF-immunoreactivity remained at near normal levels even at 10 days post-lesion (Fig. 1b). Autoradiograms exposed for a shorter time revealed that immunoreactivity observed in injured Wld nerves was not due to oversaturation of the signal. In control mice, all axons have degenerated by 10 days following axotomy, but in Wld mice, many axons survive (Lunn et al., 1989). Thus, it appears that axonal contact even in the absence of transport from the nerve cell body prevents the reduction of CNTF protein that occurs after nerve injury.

Ten days after ligation, P_0-immunoreactivity was no longer detected by immunoblotting in the distal nerve stump of control mice while it was still present at reduced concentrations in Wld mice (Fig. 2). In the region of the ligation site, P_0-immunoreactivity was similar to that in normal nerves. Previous investigations using immunohistochemical techniques have reported that myelin proteins are still present in lesioned Wld nerves 1 week after injury but not in lesioned nerves from control mice (Lunn et al., 1989).

The polyclonal antibody used for immunoblotting recognized proteins with molecular weights higher than that of CNTF (Fig. 1). These bands were reduced during Wallerian degeneration in both Wld and control mice. However, in autoradiograms with longer exposure times of homogenates from rat spleen, which was used as negative control, these bands were also observed. These proteins still have not been identified. The

polyclonal antibody used to detect myelin P_0 recognized other proteins which were present both in the uninjured and ligated nerves (Fig. 2). The identity of these proteins remains unknown.

To confirm the finding that axonal contact maintains CNTF synthesis in axotomized Wld nerves, CNTF mRNA concentrations were measured. Four days after ligation, CNTF mRNA concentrations in the distal nerve segments of Wld mice were 83% of normal whereas concentration in CD1 mice had decreased to 23% of normal (Fig. 3, Table 1). Ten days after axotomy, the concentrationsof CNTF mRNA in the injured nerves of Wld were comparable to those of control mice. In comparison, P_0 mRNA concentrations in nerves ligated 4 days before sacrifice were 36% of normal in Wld mice and 7% of normal in control mice. These observations of P_0 mRNA concentrations in axotomized Wld nerves are consistent with previous evidence by Northern blot analysis that concentrations in the distal nerve segments, 5 days after transection, decreased to 41% of normal in Wld mice and almost nil in control mice (Thomson et al., 1991).

DISCUSSION

Axonal transport is not necessary for CNTF induction

Induction of CNTF mRNA and protein by axonal contact has been demonstrated in cocultures of rat DRG neurons and Schwann cells (Lee et al., 1995). However, it has not been determined if axonal/neurite contact is sufficient to induce CNTF synthesis or a rapidly transported signal from the cell body is required. Our results suggest that the putative signal must be a slowly turned-over component of the axolemma, since 4 days after ligation, CNTF mRNA concentrations remained high in injured Wld nerves where some axons persist, even though they were isolated from molecules recently synthesized in the nerve cell body. The rapid decline of CNTF in axotomized nerves of control mice is presumably due to the rapid degeneration of the axolemma, rather than to loss of a rapidly-transported, rapidly turning-over signal from the cell body. The fact that the same inference was made in the study of the myelin protein P_0 (Thomson et al., 1991) strengthens the suggestion that these two Schwann cell proteins are regulated by similar intercellular mechanisms.

In control mice, CNTF mRNA concentration in nerves, ligated 10 days before sacrifice, remained at 28% of normal whereas, in rats, the concentrations of CNTF mRNA, determined by RNase protection assay, decreased to less than 10% of normal by 3 days after transection (Seniuk et al., 1992) and only increased once regeneration and remyelination had commenced. In the present investigation, the mRNA levels were determined semi-quantitatively by RT-PCR. It remains to be proved that rats mice differ significantly in this regard.

Persistence of CNTF protein in Wld mice

Although concentrations of CNTF mRNA were low in both Wld and control mice by 10 days after axotomy, CNTF-immunoreactivity was only slightly reduced in Wld nerves, but barely detectable in control nerves. This finding suggests that the CNTF protein is more stable in Wld mice than in normal mice. Three explanations can be forwarded for the relative stability of CNTF protein in Wld mice.

First, proteolytic processes may be altered within Schwann cells. In Wld mice the degradation of neurofilaments by calcium-activated neutral proteases (calpains) is subnormal (Glass et al., 1994). Calpain, which is present in virtually every eukaryotic cell, is present in both cytocol and plasma membrane. Membrane bound calpain in the brain and peripheral nerves is closely associated with the myelin sheath and is thought to be involved in normal turn-over of myelin proteins (Chakrabarti et al., 1993; Li, Banik, 1995). However, the primary abnormalities observed in Wld mice have been ascribed to the axon (Glass et al., 1993; Perry et al., 1990a) and it is not known whether the subnormal activity of calpain extends to non-neuronal cells of Wld mice.

An alternative explanation for the relative persistence of CNTF in Wld mice involves the subcellular localization of CNTF within Schwann cells. In normal sciatic nerve, CNTF-immunoreacitivity has been detected in the vicinity of the myelin sheath of Schwann cells (McMahon, Kett-White, 1991; Rende et al., 1992) whereas in injured nerve, it is present in patches surrounded by Schwann cell outer membrane and globules of disintegrating myelin (McMahon, Kett-White, 1991). Thus, it is possible that CNTF is targeted to the submembranous region of the cytoplasm and is removed with myelin debris by macrophages. Accordingly, the limited phagocytosis by macrophages in injured

nerves of Wld mice would account for the delayed removal of CNTF and myelin proteins.

A third possible explanation of the persistence of CNTF protein in injured nerves of Wld mice despite the reduced mRNA concentration is the decreased rate of release. CNTF does not possess a signal peptide and is thought to be released in similar manner as basic fibroblast growth factor (bFGF). Release of CNTF from its cell of synthesis has been studied in astrocytes which, unlike Schwann cells, constitutively synthesize CNTF in culture (Kamiguchi et al., 1995). Treatment with cytokines such as epidermal growth factor, IL-1β and tumor necrosis factor-α (TNF-α) appear to increase the release of CNTF into the culture medium without increasing the amount of protein within the cell. In Wld mice, infiltration of myelomonocytic cells in the distal nerve stump is delayed. In the absence of macrophage cytokines, a slow secretory rate could contribute to the persistence of CNTF protein in injured Wld nerves even though CNTF mRNA is already reduced.

Does CNTF have a function in myelination?

The similar pattern of expression of CNTF and P_0 suggest either that parallel regulatory mechanisms are involved or that CNTF, together with other molecules, is involved in regulation of P_0 expression. Although the molecular nature of the axonal signal modulating myelin P_0 synthesis has not been identified, the transcription factor krox-20 has been implicated in myelination. The presence of krox-20 in Schwann cells also requires axonal contact and spatial and temporal distributions of krox-20 are very similar to those of P_0 (Murphy et al., 1996; Zorick et al., 1996). There are two phases of

krox-20 induction in the developing peripheral nervous system, first 15.5 days of gestation during the transition from precursor to Schwann cells and, second, at one day after birth when the Schwann cells start expressing the myelin genes. Recently, it has been shown that the early induction of krox-20 in Schwann cells is influenced by diffusible factors from the neural tube (Murphy et al., 1996). Treatment with neu differentiation factor-β2 or the combination of FGF2 and either CNTF or LIF can also induce krox-20 expression. Less is known about the second phase of krox-20 induction, at the time the Schwann cells have committed to myelinate. The absence of any abnormality in myelination in mice with null deletion of the *CNTF* gene does not support the hypothesis that CNTF is important in inducing myelin protein genes in Schwann cells. However, it is possible that other molecules, for example LIF or cardiotrophin, compensate for the lack of CNTF in these mice.

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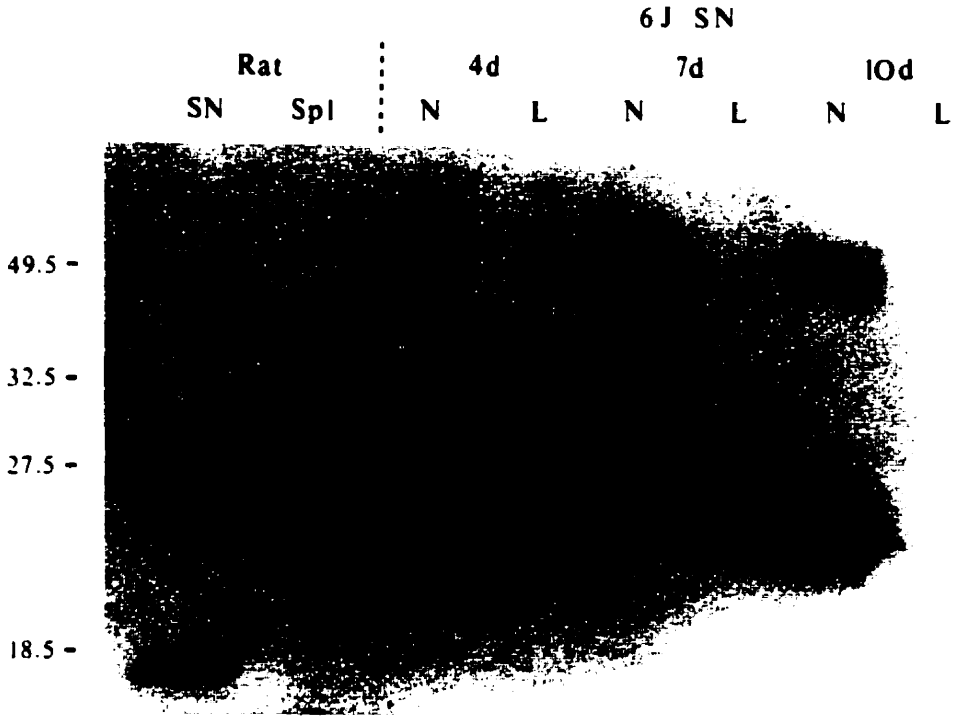
Table 1. CNTF and P_0 mRNA concentrations in injured sciatic nerves of CD1 and Wld mice 4 and 10 days after ligation.

	CNTF		P_0
	4 d	10 d	4 d
CD1	22.9 ± 2.7 %	27.9 ± 2.2 %	7.0 ± 0.7 %
Wld	82.9 ± 2.7 %	20.2 ± 2.7 %	36.2 ± 1.0 %

Results are expressed as percentage of message in normal contralateral nerve.

Figure 1. Immunoblot analysis of homogenates (30 μg protein) from normal contralateral (N) and ligated (L) sciatic nerves of C57BL/6J (a) and Wld (b) mice sacrificed 4, 7, and 10 days after ligation. CNTF was detected using a polyclonal antibody against the recombinant rat CNTF (1:500) and ^{125}I-Protein-A (0.3 μCi/ml). CNTF-immunoreactivity is observed at 24 kDa. Rat sciatic nerve (SN) and spleen (Spl) were used as positive and negative controls, respectively.

A


6J SN
Rat
4d
7d
10d
SN
Spl
N
L
N
L
N
L
49.5
32.5
27.5
18.5

B

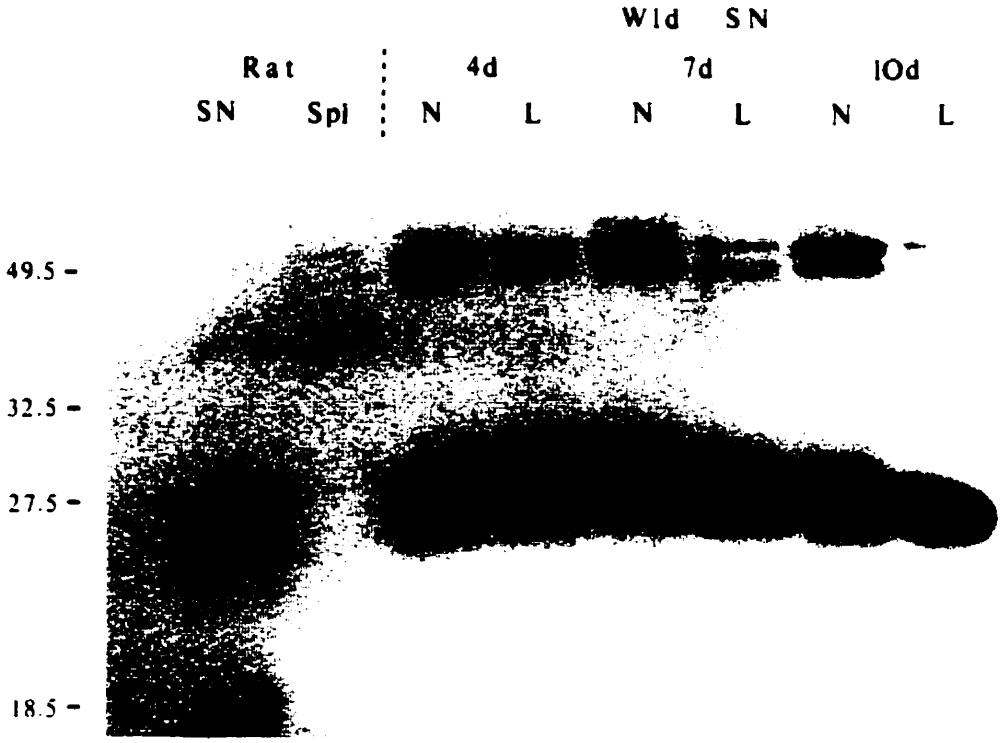

W1d SN
Rat
4d
7d
10d
SN
Spl
N
L
N
L
N
L
49.5
32.5
27.5
18.5

Figure 2. Immunoblot analysis of homogenates (20 μg) from normal contralateral (N) and ligated (ligature site Lt and distal stump Ld) sciatic nerves of C57BL/6J and Wld mice sacrificed 10 days after surgery. P_0 was detected using a polyclonal antibody at a concentration of 1:2000 and ^{125}I-Protein-A (0.3 μCi/ml). P_0-immunoreactivity is observed at 30 kDa.

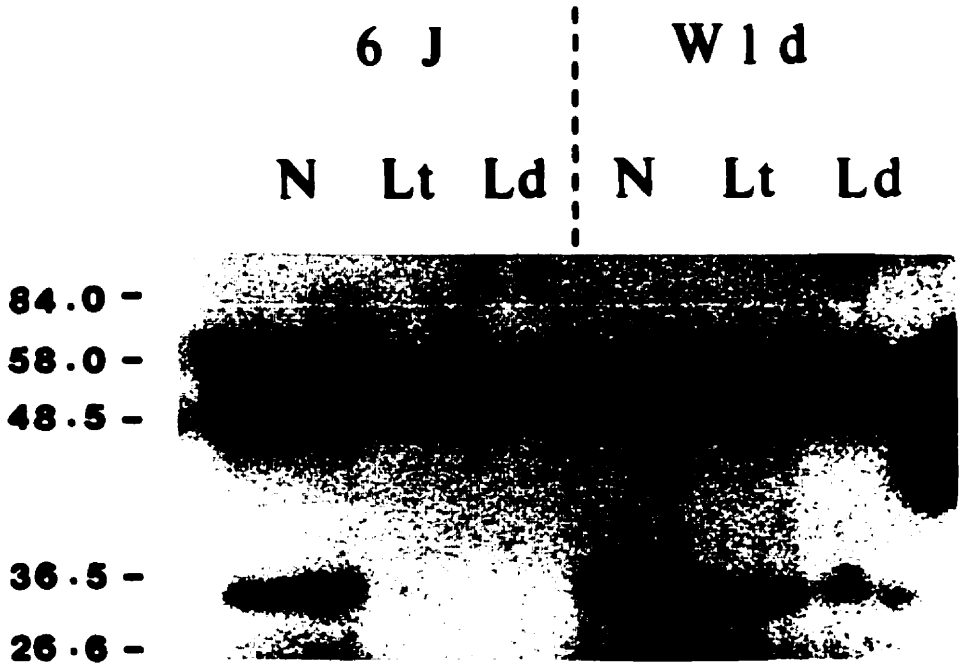
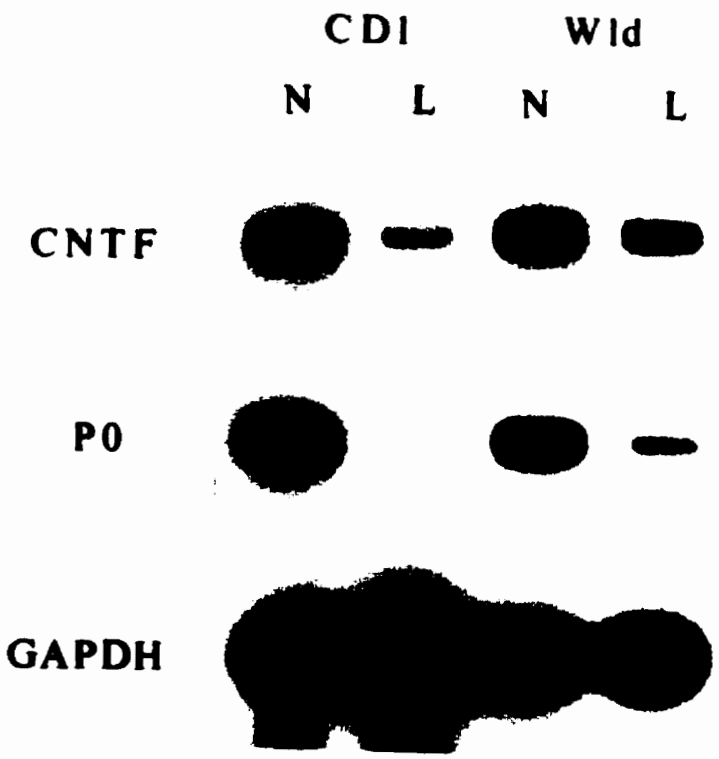

6 J
W l d
N Lt Ld N Lt Ld
84.0 -
58.0 -
48.5 -
36.5 -
26.6 -

Figure 3. RT-PCR analysis of total RNA extracted from normal contralateral (N) and ligated (L) sciatic nerves of CD1 and Wld mice four days after ligation.


CDI
Wld
N
L
N
L
CNTF
P0
GAPDH

CHAPTER 5:

LEUKEMIA INHIBITORY FACTOR EXPRESSION IN LESIONED SCIATIC NERVE

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ABSTRACT

Leukemia inhibitory factor (LIF) promotes the cholinergic differentiation of some sympathetic neurons and rescues axotomized motor neurons. In peripheral nerves, LIF is expressed normally at very low concentrations and is increased following nerve injury. This study determined the spatio-temporal distribution of LIF mRNA in injured peripheral nerves and the regulation of LIF mRNA in Schwann cell cultures. Data showed that LIF mRNA is induced at the lesion site within 6 hours after sciatic nerve transection but only after 24 hours in the more distal segments. This pattern of distribution of LIF mRNA is similar to that of NGF mRNA. To determine the mechanisms involved in the regulation of LIF, Schwann cell and fibroblast cultures were established and treated with IL-1β, a potent inducer of NGF synthesis. Results showed that IL-1β treatment led to an increase in LIF mRNA concentrations in fibroblasts but not in Schwann cells. Thus, the regulation of LIF mRNA appears to be similar to that of NGF mRNA.

Keywords: leukemia inhibitory factor; sciatic nerve; Wallerian degeneration; IL-1

INTRODUCTION

Cholinergic neuronal differentiation factor was first described as a 45 kDa glycoprotein present in heart cell conditioned medium that influences the neurotransmitter phenotype of cultured rat sympathetic neurons (Patterson, Chun, 1977; Fukada, 1985). Cloning and sequencing of this molecule revealed that it is identical to the previously characterized leukemia inhibitory factor (LIF), which was initially identified as a molecule secreted by murine Krebs II ascites tumor cells that promotes the terminal differentiation of the murine leukemic cell line M1 (Hilton et al., 1988). LIF is now known to be involved in bone metabolism (Mori et al., 1989), fat metabolism (Abe et al., 1986; Reid et al., 1990), maintenance of embryonic stem cells (Williams et al., 1988; Smith et al., 1988), and induction of acute phase proteins (Baumann, Wong, 1989). In addition, in the nervous system, LIF has been shown *in vitro* to stimulate the differentiation of spinal cord neurons from their neural tube precursors (Richards et al., 1992) and the generation of sensory neurons from neural crest cells (Murphy et al., 1991). *In vivo* LIF rescues motor neurons from cell death following peripheral nerve injury (Cheema et al., 1994).

LIF belongs to a family of cytokines that share similar tertiary structures (Bazan, 1991). Ciliary neurotrophic factor (CNTF) and LIF have overlapping functions in the PNS as CNTF employs the functional LIF receptor complex, which is comprised of gp130 and LIF receptor-β, in addition to CNTF receptor-α for signal transduction (Ip et al., 1992; Ip et al., 1993). In contrast to the distribution of CNTF, which is abundant in adult, myelinated peripheral nerves and is markedly reduced after nerve injury (Seniuk et

al., 1992; Sendtner et al., 1992), LIF mRNA is barely detectable in normal peripheral nerves and increases following nerve injury (Curtis et al., 1994).

This study was undertaken to determine the spatial and temporal distribution of LIF in the nerve after injury and to investigate the mechanisms involved in the regulation of LIF mRNA in injured peripheral nerves.

MATERIALS AND METHODS

Animal Manipulations. Female Sprague-Dawley rats were purchased from Charles River Laboratory, Quebec, Canada. The right sciatic nerve of deeply anesthetized animals was transected near the origin. Upon sacrifice, 3 hours to 8 days following surgery, the contralateral and degenerating sciatic nerves were collected. The injured nerves were divided into 3 segments: 0-5 mm (distal 1), 5-25 mm (distal 2) and 25-45 mm (distal 3) from the transected end. The nerve segments were then frozen in liquid nitrogen.

Cell Cultures. Schwann cells were established from sciatic nerves of 2-day old rats (Charles River Laboratory, Quebec) following the method of Brockes et al (Brockes et al., 1979). Briefly, cells were dissociated by sequential trypsin (Life Technologies, New York, USA) and collagenase (Boehringer-Mannhein,) digestions. Cells were grown in DMEM (Life Technologies, New York, USA) supplemented with10% serum. Cultures treated with 10 μM cytosine -β-D-arabinofuranoside (Ara-C; Sigma-Aldrich Canada Ltd., Oakville, Ont., Canada) for the first 3 days to eliminate fast dividing cells, which are presumably fibroblasts. Cells were trypsinized and replated onto polylysine coated petri dishes. Cells were allowed to recover for 24 hours. Schwann cell cultures, which are 90-95% S100 immunopositive, were treated with 1 ng/ml interleukin-1β (IL-1β; R & D Systems, Minnesota, USA) for 3 hours. At the end of the treatment period, cells were harvested and processed for total RNA extraction.

Fibroblasts from sciatic nerves of 2-day old rats were established by growing the freshly dissociated cells in the absence of Ara-C during the first 3 days. Once cells

became confluent the cultures were then passaged. More than 95% of the cells obtained after 15 passages were immmunopositive for Thy1.1, a marker for fibroblasts, and were used in the experiments. Fibroblast cultures were maintained in DMEM and supplemented with 10% serum. These cells were treated with 1 ng/ml IL-1β for three hours. At the end of the treatment period, cells were harvested and processed for total RNA extraction.

RNase Protection Assay. Frozen sciatic nerves were pulverized and homogenized in a solution containing guanidinium isothiocyanate as total RNA was extracted using the method of Chomczynski and Sacchi (Chomczynski, Sacchi, 1987). RNase protection assay was performed on total RNA extracted either from cells or from sciatic nerves as described by Seniuk et al (Seniuk et al., 1992).

Reverse Transcriptase- Polymerase Chain Reaction (RT-PCR) and Southern Blotting. One microgram of total RNA was reverse transcribed using the pd(N)6 random hexamers as primers and Moloney murine leukemia virus reverse transcriptase. The amplification of LIF cDNA by polymerase chain reaction for 21 cycles of 95°C for 45 seconds, 55°C for 45 seconds and 72°C for 2 minutes was performed using the 5'-CAACTGGCTCAACTCAAC-3' (nt 151-168; (Yamamori et al., 1989)) and 5'-CTAGAAAGGCCTGGACCAC-3' (complimentary sequence to nt 592-609; (Yamamori et al., 1989)) as sense and antisense primers, respectively. The cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed using 5'-TGAAGGTCGGTGTCAACGGATTTGGC-3' (nt 35-60; (Tso et al., 1985)) and 5'-

CATGTAGGCCATGAGGTCCCACCAC-3' (complimentary sequence to nt 994-1017; (Tso et al., 1985) as sense and antisense primers, respectively. The amplified products were separated by 1% agarose gel electrophoresis and transferred onto a nylon hybridization membrane (Hybond-N+, Amersham, Arlington Heights, IL, USA). Southern blotting was done using antisense oligonucleotide probes labelled with ^{32}P. The probe used for LIF was the complementary sequence to nt 451-500 (Yamamori et al., 1989) and the GAPDH probe was complimentary sequence to nt 710-750 (Tso et al., 1985). Autoradiograms were exposed overnight.

RESULTS

The temporal distribution of LIF was determined in the nerves after sciatic nerve transection. Results from RNase protection assays showed that LIF mRNA concentrations in the transected nerves started to increase 6 hours after injury (Fig. 1). No detectable LIF mRNA was observed in dorsal root ganglia associated with nerves that had been transected 1 to 8 days earlier (data not shown).

To determine the spatial distribution of the LIF mRNA in injured nerves, upon sacrifice, at several time points after transection, nerves were divided into three segments (Fig. 2). The first 5-mm fragment distal to the lesion (distal 1 or the lesion site), the first 2-cm fragment distal to the site of lesion (distal 2) and the next 2-cm segment of the nerve (distal 3) were collected. Data showed that 6 hours after transection LIF mRNA is induced at the site of lesion but not in the more distal segments (Fig. 3). This pattern of expression was maintained for at least 18 hours (Fig. 4A). By 24 hours after injury, LIF mRNA was induced in the entire distal stump. The expression of LIF mRNA in the distal stump remained elevated at 8 days post-injury (Fig. 4B).

This biphasic pattern of LIF mRNA expression in the injured nerve is similar to that of nerve growth factor (NGF) mRNA. These two molecules could be induced in the nerve by similar mechanisms. One of the molecules that induce NGF synthesis in fibroblasts but not in Schwann cells is IL-1. It is possible that IL-1 regulates also the expression of LIF mRNA. Thus, Schwann cells and nerve fibroblasts were cultured and treated with 1 ng/ml of IL-1β. Results showed that IL-1β induced the expression of MCP-1 mRNA in fibroblasts (Fig. 5). Schwann cells, which constitutively expressed

MCP-1 mRNA did not respond to IL-1β. Thus, LIF mRNA is regulated in a similar manner as NGF mRNA *in vitro*.

The lack of response of Schwann cells to IL-1∃ stimulation may be attributed to either the absence of the components for IL-1 signalling in Schwann cells or the excessive production of IL-1 receptor antagonist (IL-1ra). RT-PCR and Southern blot analysis showed that IL-1 receptor 1 mRNA and IL-1 receptor accessory protein mRNA were expressed by cultured Schwann cells (data not shown). Thus, it is possible that in our Schwann cell cultures IL-1ra might be produced in concentrations sufficient to block the amount of IL-1β present in the medium.

DISCUSSION

Our results showed no increase in LIF mRNA concentrations in the ipsilateral DRG while a strong induction of LIF mRNA was observed in the transected nerve. In contrast, a previous study showed that in organotypic cultures of DRG LIF mRNA concentration increased after 24 hours of incubation (Banner, Patterson, 1994). Hence, it is likely that signals from the blood or the resident immune cells participate in the regulation of LIF mRNA. Alternatively, *in vivo* an inhibitory signal may prevent the induction of LIF mRNA in the DRG after sciatic nerve transection.

In transected peripheral nerves, LIF mRNA synthesis occurs in a biphasic manner – an early expression that occurs within 6 hours at the lesion site and a later expression in the entire distal stump 24 hours after axotomy. The signal for the early induction of LIF mRNA at the lesion site could be derived from cells or molecules present in the blood and/or the degranulation of mast cells at the site of lesion (Olsson, 1967; Olsson, 1965; Johnson et al., 1988; Dietsch, Hinrichs, 1991; Purcell, Atterwill, 1995).

The later induction of LIF mRNA in the distal nerve segments is consistent with the idea that an inhibitory signal is lost when axonal transport from the nerve cell body is interrupted. In addition, cytokines from the blood, resident mast cells, or macrophages are possible sources of positive signals for the induction of LIF mRNA in the transected nerve.

The biphasic expression of LIF mRNA in injured peripheral nerves is reminiscent of that of NGF mRNA. This correlation suggests that LIF mRNA synthesis may be regulated by cellular mechanisms similar to those that regulate the synthesis of NGF

mRNA (Heumann et al., 1987b; Heumann et al., 1987a), in particular by macrophage-derived cytokines (Heumann et al., 1987b; Lindholm et al., 1987).

IL-1β, TNF-α and TGF-β have been shown to stimulate the production of LIF mRNA in other cell types (Aloisi et al., 1994; Lotz et al., 1992; Arici et al., 1995; Jansen et al., 1996). This study verified previous finding that LIF mRNA is constitutively expressed in cultured Schwann cells (Curtis et al., 1994). Furthermore, results showed that IL-1 stimulates LIF mRNA production in nerve fibroblasts but not in Schwann cells. These observations support the hypothesis that some of the molecular regulatory mechanisms involved in the synthesis of LIF and NGF in injured nerves are similar.

In summary, the results of this investigation showed that LIF mRNA is expressed in the transected nerve in a biphasic manner and its synthesis may be regulated by mechanisms similar to that of NGF mRNA synthesis.

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Figure 1. RNase protection assay of total RNA from the contraletral nerve and the distal stump 3 hours, 6 hours and 1 day after sciatic nerve transection. Total RNA from Schwann cells was used as positive control. Cyclophilin (Cyclo) probe was used to determine the relative amounts of total RNA used in the assay.

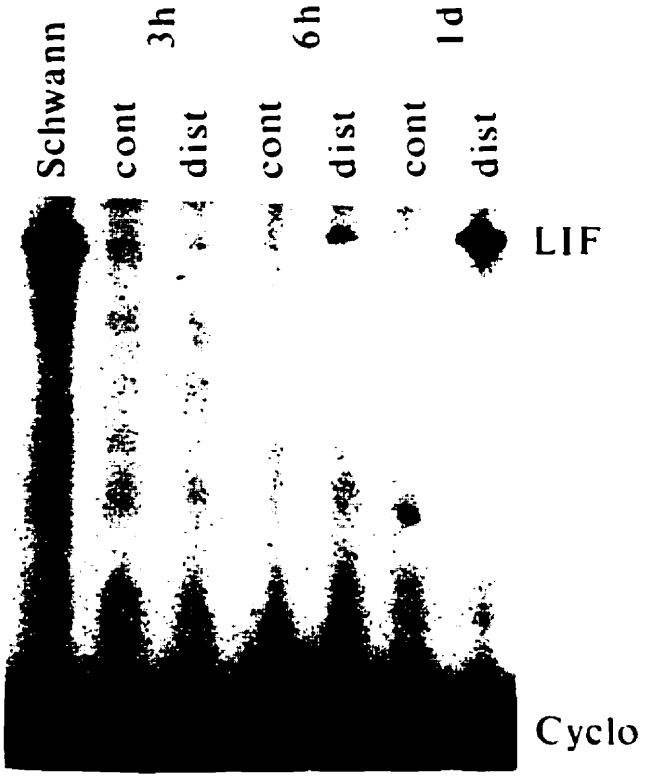

Schwann
cont
3h
dist
cont
6h
dist
cont
1d
dist
LIF
Cyclo

Figure 2. Schematic diagram of the distal stump after it was divided to separate the lesion site (dist 1) from the more distal segments (dist 2 and 3).

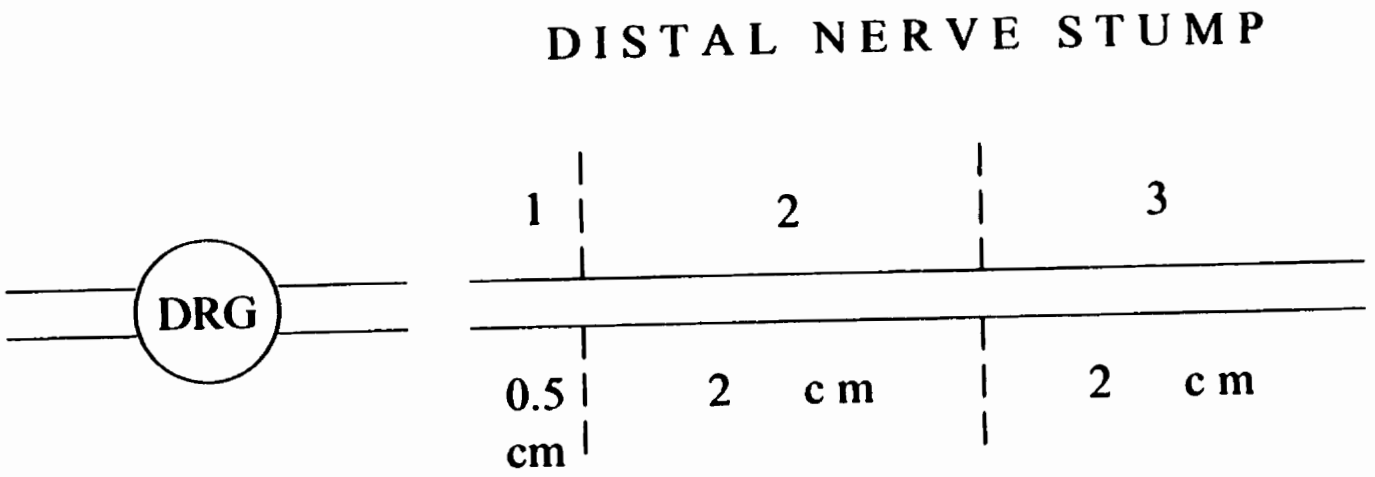

DISTAL NERVE STUMP
DRG
1
2
3
0.5 cm
2 cm
2 cm

Figure 3. LIF mRNA distribution in the sciatic nerve 6 hours after transection as determined by RNase protection assay. Total RNA from Schwann cells was used as a positive control. cont – contralateral nerve; dist 1, 2, and 3 – distal nerve segments 1 (lesion site), 2, and 3; Cyclo - cyclophilin.

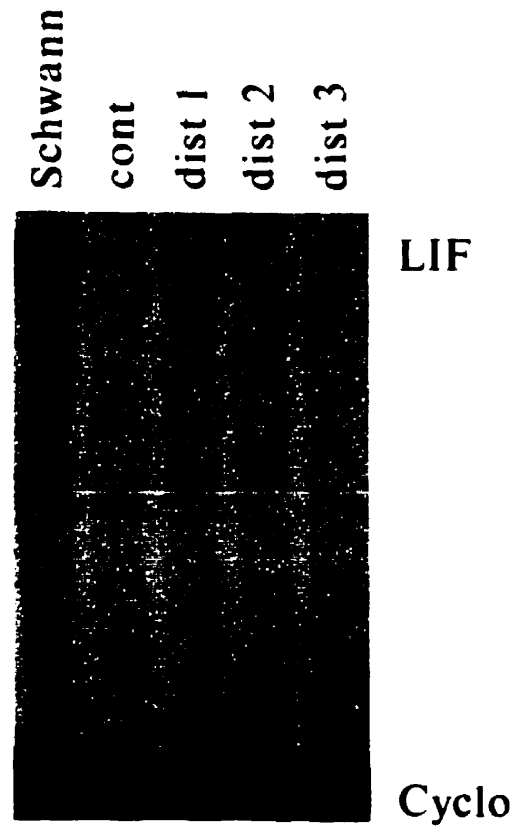
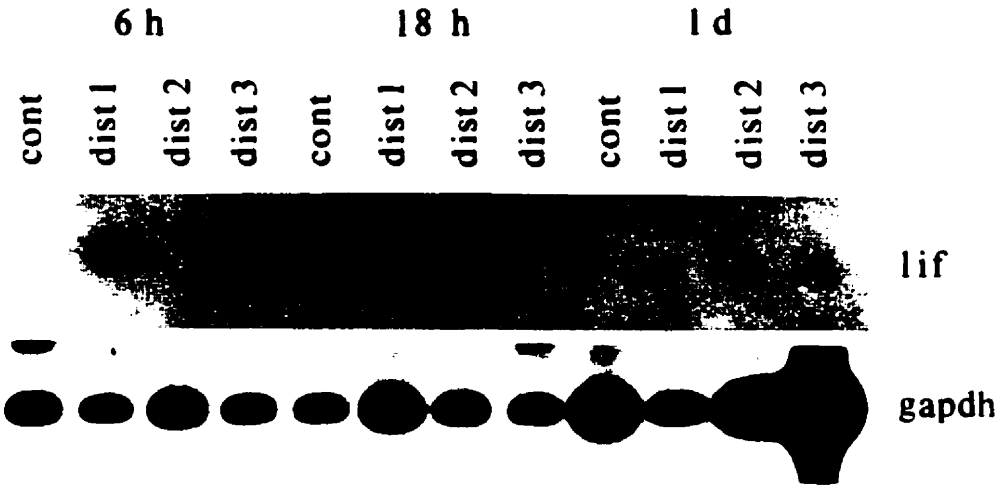

Schwann
cont
dist 1
dist 2
dist 3
LIF
Cyclo

Figure 4. RT-PCR and Southern blot analysis for LIF and GAPDH were performed using the total RNA from the different nerve segments 3 hours to 1 day after transection (A) and 2 to 4 days post-injury (B). cont – contralateral nerve; dist 1, 2, and 3 – distal nerve segments 1 (lesion site), 2, and 3.

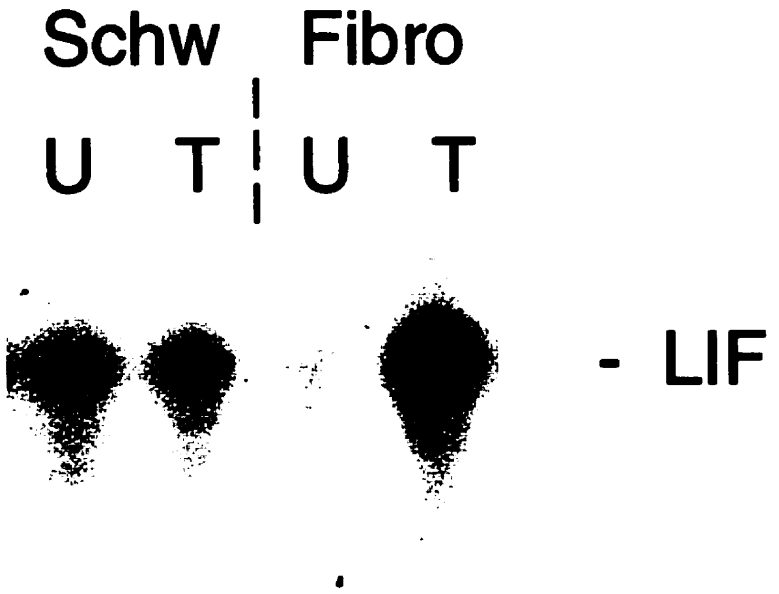
A


6 h
18 h
1 d
cont
dist 1
dist 2
dist 3
cont
dist 1
dist 2
dist 3
cont
dist 1
dist 2
dist 3
lif
gapdh

B


2 d
4 d
cont
dist 1
dist 2
dist 3
cont
dist 1
dist 2
dist 3
LIF
GAPDH

Figure 5. RNase protection assay of total RNA isolated from Schwann cell (Schw) and fibroblasts (Fibro) cultures treated with 1 ng/ml IL-1β (T) for 3 hours. U – untreated cultures; Cyclo – cyclophilin.


Schw
Fibro
U
T
U
T
- LIF


- Cyclo

CHAPTER 6:

TUMOR NECROSIS FACTOR-ALPHA INDUCES MONOCYTE CHEMOATTRACTANT PROTEIN-1 mRNA IN A SCHWANN CELL LINE

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Keywords: Schwann cells; monocyte chemoattractant protein-1; tumor necrosis factor

Schwann cells synthesize neurotrophic factors (1) as well as myelin proteins and some molecules with immunological functions (2). Recently, a mRNA for a small basic protein which specifically attracts and activates monocytes to sites of inflammation, monocyte chemoattractant protein-1 (MCP-1) (3), has been detected in peripheral nerves during Wallerian degeneration (4) and after demyelination following tellurium intoxication (5). Schwann cells (4) and other cell types (6, 7) stimulated by tumor necrosis factor-α (TNF-α) synthesize MCP-1 mRNA. Knowledge of the regulation of MCP-1 in Schwann cells would provide insight on the recruitment of monocytes to the endoneurium during Wallerian degeneration.

This study using the rat Schwann cell line SCL 4.1/F7 was designed to investigate the mechanism of regulation of MCP-1 mRNA by TNF-α. SCL 4.1/F7 cells (European Collection for Animal Cell Culture, UK) were grown in DMEM supplemented with 10% fetal calf serum and treated for 3 hours with various concentrations of TNF-α (R & D, Minnesota, USA). At the end of treatment period, cells were washed with DEPC-treated PBS and total RNA was extracted using Trizol Reagent (Life Technologies, New York, USA). RNase protection assays (8) were performed with ^{32}P-labelled MCP-1 cRNA probe and cyclophilin mRNA as a control gene. After scanning of the autoradiograms, the MCP-1 mRNA signal was expressed as a percentage of the signal for cyclophilin mRNA.

Data from Rnase protection assays revealed that MCP-1 mRNA is present at low concentration in untreated SCL 4.1/F7 cells and increases markedly in concentration after stimulation by TNF-α (Fig. 1).

Binding of TNF-α to its cell surface receptor, TNF receptor 1 (p55) or TNF receptor 2 (p75) may lead to either the activation of NF-κB as a consequence of the phosphorylation and subsequent degradation of IκB or the activation of the c-Jun N-terminal kinase (JNK) pathway (9). The two pathways bifurcate at the level of adaptor

protein, TNF receptor-associated factor 2 (TRAF 2). The induction of MCP-1 mRNA by TNF-α in murine fibroblast cells involves activation of the NF-κB signaling pathway (6).

Curcumin (diferuloyl methane) is a naturally occurring polyphenolic phytochemical with anti-inflammatory and anti-oxidant properties. In earlier studies curcumin was shown to suppress the induction of MCP-1 in bone marrow stromal cells by IL-1β and TNF-α (7) and inhibit the activity of NF-κB in T cell lines through direct modification of p50 and blocking the degradation of IκB (10). To ascertain whether the induction of MCP-1 mRNA in Schwann cells by TNF-α depends upon the generation of oxygen anions, SCL 4.1/F7 cells were incubated for 3 hours with TNF-α (5 ng/ml) in the absence or presence of varying concentrations of curcumin. As shown in Fig. 2 curcumin treatment decreases the TNF-α-induced MCP-1 mRNA expression.

In a Schwann cell line SCL 4.1/F7, TNF-α stimulates the transcription of MCP-1 mRNA through a pathway that is inhibited by curcumin.

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Figure 1. SCL 4.1/F7 cells were treated for three hours with TNF-α with concentrations ranging from 0 to 10 ng/ml. At the end of the treatment period, cells were washed with PBS, and total RNA was extracted and used for RNase protection assays. Concentrations of MCP-1 mRNA are expressed as percentage of cyclophilin mRNA concentration.

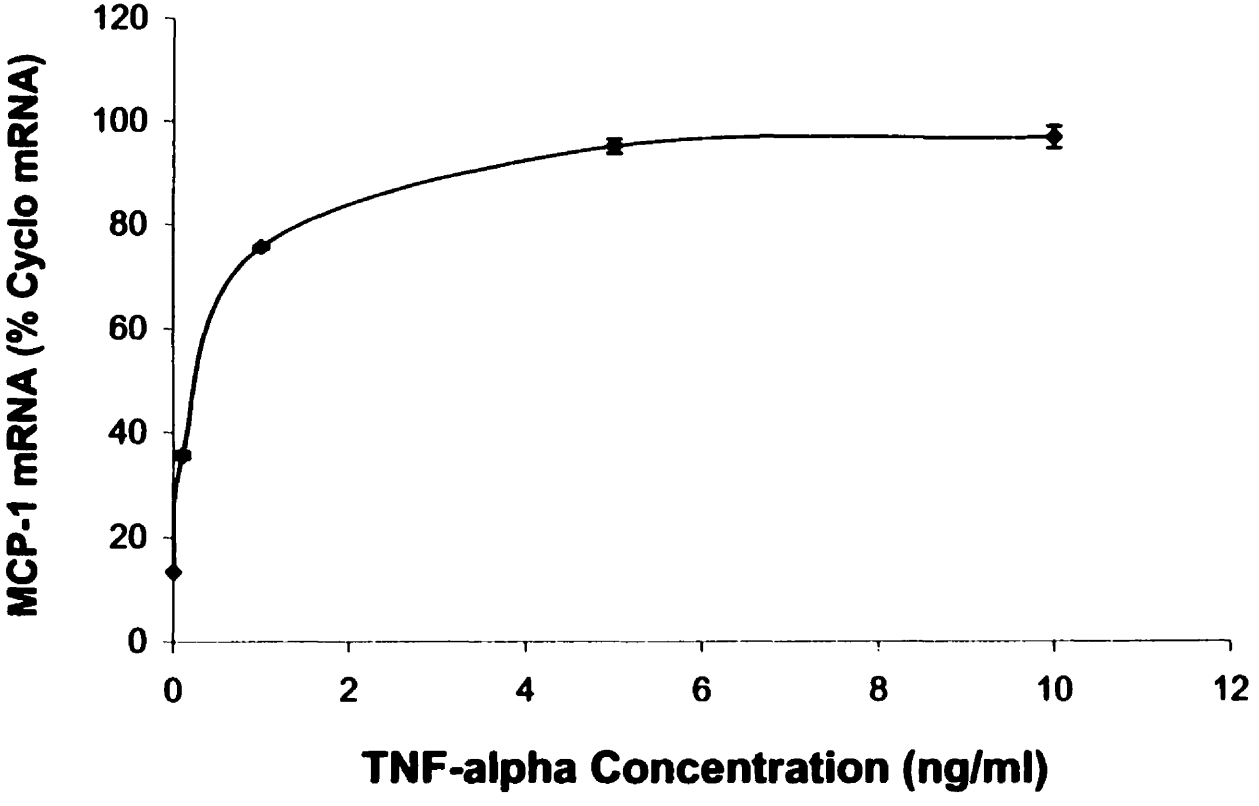
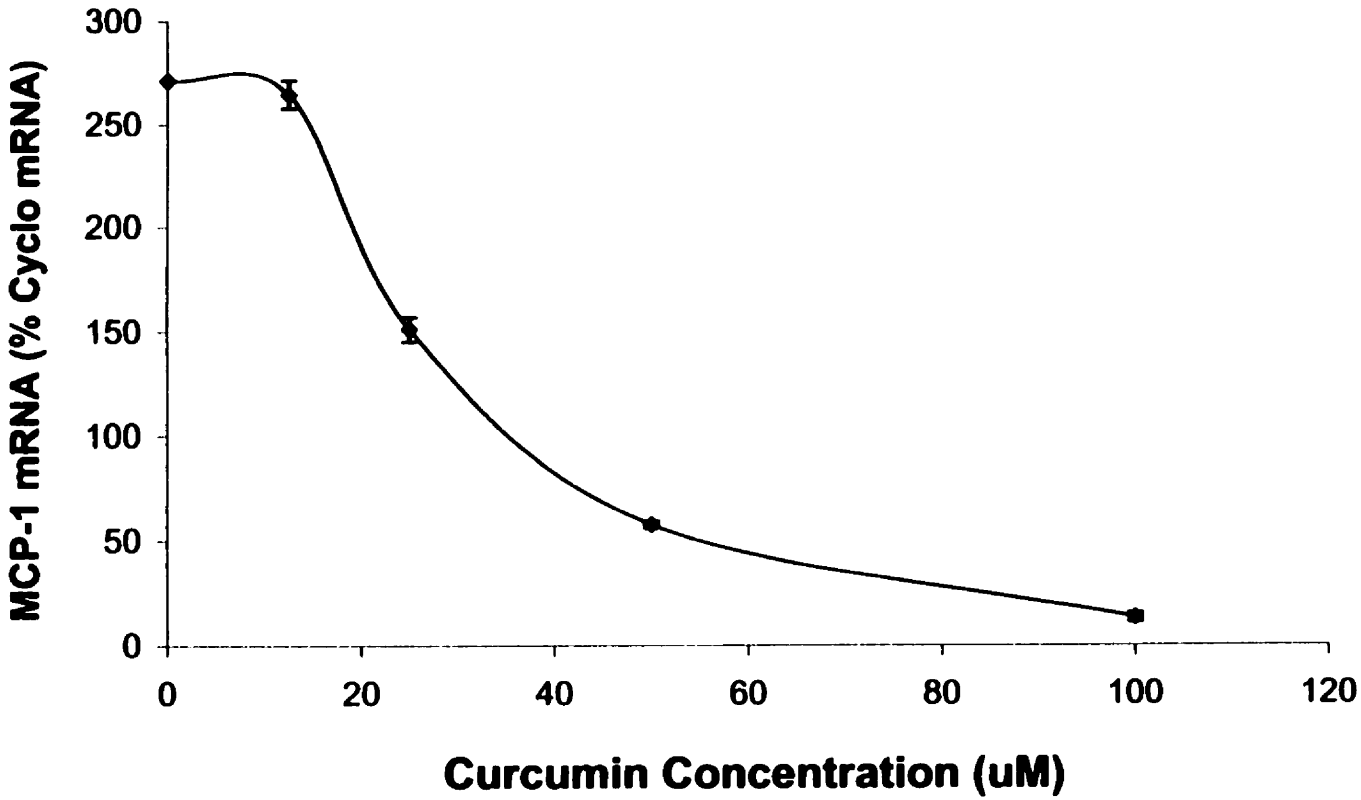

MCP-1 mRNA (% Cyclo mRNA)
120
100
80
60
40
20
0
0
2
4
6
8
10
12
TNF-alpha Concentration (ng/ml)

Figure 2. SCL 4.1/F7 cells were treated for 3 hours with 5 ng/ml TNF-α in the presence of varying concentrations of curcumin (0-100 μM). RNase protection assays were performed on total RNA from these treated cells. MCP-1 mRNA concentrations are expressed as percentage of cyclophilin mRNA concentrations.


MCP-1 mRNA (% Cyclo mRNA)
300
250
200
150
100
50
0
0
20
40
60
80
100
120
Curcumin Concentration (uM)

CHAPTER 7:

ANTI-OXIDANTS INHIBIT THE EXPRESSION OF MONOCYTE CHEMOATTRACTANT PROTEIN-1 IN PERIPHERAL NERVOUS TISSUE

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Keywords: MCP-1; Wallerian degeneration; Schwann cells, TNF-α;

TNF receptor null mice; Wld mice; curcumin; quercetin; anti-oxidant.

ABSTRACT

The signal and the source of the signal for monocyte/macrophage entry into the injured peripheral nervous tissue are currently not clear. This study was undertaken to determine the distribution of the chemokine monocyte chemoattractant protein-1 (MCP-1) and investigate the mechanisms that regulate MCP-1 expression in the injured peripheral nervous tissue. Results from RNase protection assays showed that MCP-1 mRNA concentrations increased in the ipsilateral dorsal root ganglia within 1 day after nerve transection and persisted for at least 16 days. In the nerve, MCP-1 mRNA concentrations increased at the lesion site within 3 hours after injury and in the more distal segments by 24 hours. MCP-1 mRNA expression was also observed in Schwann cells treated by TNF-α but not in cells treated with IL-1β, TGF-β, IL-6 or NGF. This induction by TNF-α of MCP-1 mRNA synthesis in Schwann cells was inhibited by anti-oxidants. However, in mice that lack TNF receptor types I and II, the increase in MCP-1 mRNA concentrations in the distal stump 2 to 8 days following nerve transection was comparable to that in control mice. Thus, TNF-α does not appear to be necessary for the induction of MCP-1 in injured peripheral nerves. However, the possible contribution of TNF-α in the stimulation of MCP-1 synthesis in the distal stump has not been excluded.

The importance of MCP-1 in the recruitment of monocytes into the injured peripheral nervous tissue was studied in C57BL/Wld (Wld) mice, which exhibit delayed axonal degeneration. Results showed that MCP-1 mRNA expression in the distal stump of Wld mice was similar to that of control mice. Thus, MCP-1 expression is not sufficient for the infiltration of monocytes/macrophages into the injured nerves of the Wld mice.

INTRODUCTION

Macrophages participate in the repair of damaged tissue. In the distal nerve stump, macrophages remove myelin and axonal debris (Stoll et al., 1989), modulate Schwann cell proliferation and differentiation (Baichwal et al., 1988) and salvage lipids for reutilization during regeneration (Goodrum, 1991; Goodrum, Bouldin, 1996) and induce synthesis of neurotrophic agents (Lindholm et al., 1987; Heumann et al., 1987b). Macrophages that surround axotomized dorsal root ganglion DRG neurons (Lu, Richardson, 1993) also may contribute to axonal regrowth.

The macrophages that constitute 2-9% (Arvidson, 1977; Oldfors, 1980) of the population of nonneuronal cells in the peripheral nervous system (PNS) are constantly renewed from the blood (Vass et al., 1993). Following injury, in contrast to events in other damaged tissue, the accumulation of macrophages in the injured peripheral nerve is not preceded by the infiltration of polymorphonuclear neutrophils (Perry, Brown, 1992). The increase in number of macrophages in both the DRG and nerve is believed to reflect an increase influx of monocytes. In general, migration of leukocytes depends on the interactions of selectins with their mucin ligands, chemoattractants with their receptors and integrins with members of Ig family (Springer, 1994). Chemoattractants are important for activation of leukointegrins and in directing the migration of leukocytes (Baggiolini, 1998). Chemokines, small cytokines with molecular weights ranging from 8 to 12 kD, are cell-type specific chemoattractants that help to determine the cellular composition of inflammatory infiltrates (Rollins, 1997). Monocyte chemoattractant protein-1 (MCP-1) is a member of the CC chemokine family that specifically attracts and activates monocytes to sites of inflammation (Leonard, Yoshimura, 1990). In the PNS, MCP-1 mRNA expression has been observed in the nerve after tellurium intoxication, nerve crush and transection (Toews et al., 1998).

The present study was designed to i) investigate the spatio-temporal distribution of MCP-1 mRNA in sciatic nerve following nerve transection, ii) ascertain its presence in the DRG after nerve injury and iii) determine some of the factors which regulate MCP-1 expression in the PNS.

MATERIALS AND METHODS

Animal Manipulations

Adult, female Sprague-Dawley rats were purchased from Charles River Laboratory (New York, USA). The mouse strain deficient in *c-kit* and its control strain were purchased from Jackson Laboratories (Maine, USA) and the mice lacking the two TNF-∀ receptors were gifts from Horst Bleuthmann of Hoffman-LaRoche (Basel, Switzerland). The C57BL/Wld (Wld) mice were gifts from Dr. M.A. Bisby and the age-matched controls, C57BL/6 (B6) mice, were purchased from Jackson Laboratories (Maine, USA). The right sciatic nerves of deeply anesthetized animals were transected at the sciatic notch. Upon sacrifice, from 3 hours to 16 days after surgery, sciatic nerves and dorsal root ganglia (DRG) were collected and frozen. Three segments were separated from the distal segment of each rat sciatic nerve, 0-5 mm (tip), 5-25 mm (distal 1) and 25-45 mm (distal 2) from the site of transection. For mouse nerve the first 3-4 mm (tip) segment was separated from the rest of the distal segment. In each experiment, 6 to 8 rats or 8 to 10 mice were used. Each experiment was repeated 3 times. The animal protocols were approved by McGill University Animal Care Committee.

Cell Culture

Schwann cell cultures were established from sciatic nerves of 2-day old Sprague-Dawley rats by a modification of the method of Brockes et. al. (Brockes et al., 1979).

Cultures grown in DMEM supplemented with 10% fetal calf serum were treated with 10μM cytosine arabinofuranoside (Ara-C) for 3 days. Cells were then trypsinized and replated on polylysine coated 6-well plates. Twenty-four hours later, cultures were treated for 3 hours with interleukin-1β (IL-1β) (1 ng/ml), tumor necrosis factor-α (TNF-α) (5 ng/ml), transforming growth factor-β1 (TGF-β1) (5 ng/ml), interleukin-6 (IL-6) (10 ng/ml) and lipopolysaccharide (LPS) (500 ng/ml). To determine whether anti-oxidants mediate the stimulation of MCP-1 mRNA synthesis by TNF-α, primary cultures of Schwann cells and the rat Schwann cell line SCL4.1/F7 (European Collection for Animal Cell Cultures, UK) were treated with TNF-α (5 ng/ml) in the presence or absence of either curcumin (50 μM) or quercetin (100 μM). Each *in vitro* experiment was repeated 3 times. The cytokines were obtained from R & D Systems (Minnesota, USA); Ara-C, LPS, quercetin and curcumin were from Sigma-Aldrich Canada Ltd (Oakville, Ont., Canada) and the Dulbecco's modified Eagle's medium (DMEM) was from Life Technologies (New York, USA).

RNA Extraction and RNase Protection Assay

For extraction of RNA frozen samples were pulverized in liquid nitrogen, homogenized in Trizol Reagent (Life Technologies, New York, USA), extracted in phenol-chloroform and subsequently precipitated in isopropanol. Samples were washed in 75% ethanol, dried and resuspended in DEPC-treated water.

Three micrograms of total RNA were used for RNase protection assay (Seniuk et al., 1992). The probe for MCP-1 protecting a 359-bp fragment of the rat MCP-1, generated by linearization of the pGEM7Z-rMCP-1#8 by PvuII and transcription with SP6 RNA polymerase. Cyclophilin probe was used as a control for the quantity and quality of RNA. Data were quantified by scanning the density and area of bands on autoradiograms with the SciScan 5000. Message for MCP-1 was normalized to that for cyclophilin mRNA.

Electrophoretic Mobility Shift Assay

SCL4.1/F7 cells were treated with TNF-α (5 ng/ml) for for 15 min., 30 min., 1 hr. and 2 hrs. At the time of harvest, cells were washed and scraped into 1.5 ml cold phosphate buffered saline and processed for the isolation of nuclear proteins using the rapid micropreparation technique of Andrews and Faller (Andrews, Faller, 1991). Protein content in the extracts was determined by the dye-binding method of Bradford (Bio-Rad Protein Assay Kit II; (Bradford, 1976)).

The activation of the nuclear factor NF-κB in the different samples electrophoretic mobility shift assay as described by St-Denis et. al. (St-Denis et al., 1998)using the consensus oligonucleotide for NF-κB/c-Rel binding site (Lenardo, Baltimore, 1989) from Santa Cruz Biotechnology, Inc. (California, USA).

Histological Examination of Nerves

The contralateral nerve and the distal stump were collected from Wld and B6 mice 10 days after transection. The nerves were fixed with a mixture of aldehydes, processed and embedded in plastic. One- micron cross-sections were stained with toluidine blue and examined under light microscope.

RESULTS

MCP-1 mRNA synthesis in dorsal root ganglia

By RNase protection assay, MCP-1 mRNA was detected at low concentration in DRG collected from unoperated animals (Fig. 1). Concentrations of MCP-1 mRNA in lumbar DRG taken from sham operated animals and in cervical DRGs from nerve-injured

animals were comparable to that in DRG from unoperated rats. An increase in MCP-1 mRNA concentration in ipsilateral lumbar DRG was observed within 24 hours after nerve transection and persisted for at least 16 days.

MCP-1 mRNA synthesis in sciatic nerves

To study the spatial distribution of MCP-1 mRNA in the injured nerve (Fig. 2), the first 5-mm fragment distal to the lesion (tip), the first 2-cm fragment distal to the "tip" (distal 1) and the next segment of the nerve (distal 2) were collected separately. Results from RNase protection assays show that the concentration of MCP-1 mRNA at the tip or the site of lesion increased within 3 hours and peaked at 6 hours after axotomy (Fig. 3a). Twenty-four hours after injury, the concentrations of MCP-1 mRNA decreased at the tip but started to increase in distal segments 1 and 2. The signal for MCP-1 mRNA remained elevated in the distal stump for at least 8 days (Fig. 3b). As MCP-1 mRNA expression in the ipsilateral DRG persisted for at least 16 days, the concentration of MCP-1 mRNA in the distal nerve segment 16 days after transection was also determined. Result showed that MCP-1 mRNA concentration remained elevated in the distal stump 16 days following injury (Fig. 4). The induction of MCP-1 mRNA in injured nerve is biphasic.

TNF-α induces MCP-1 mRNA expression in cultured Schwann cells

In many cell types, the proinflammatory molecules, IL-1β (Villiger et al., 1992) and TNF-α (Hayashi et al., 1995; Ping et al., 1996), are potent inducers of MCP-1. To determine the factors which regulate the synthesis of MCP-1 *in vitro*, Schwann cells were treated for 3 hours with IL-1β, TNF-α, TGF-β1, IL-6 or LPS. By RNase protection assays, MCP-1 mRNA concentrations were shown to increase dramatically following treatment with TNF-α but not with IL-1β, TGF-β1, or IL-6 (Fig. 5a). A slight increase in MCP-1 mRNA concentration was seen following LPS stimulation.

To ascertain the time course of MCP-1 mRNA induction in Schwann cells following TNF-α stimulation, cells were treated for 30 minutes, 1 hour, 3 hours and 5 hours. Data from RNase protection assays showed that TNF-α treatment increased the concentration of MCP-1 mRNA as early 1 hour after stimulation (Fig. 5b).

The induction of MCP-1 mRNA by TNF-∀ is mediated by oxygen radicals

One possible signalling pathway for the induction of MCP-1 mRNA by TNF-α involves the reactive oxygen species (ROS). The expression of MCP-1 in synovial cells (Sato et al., 1996; Sato et al., 1997) and bone marrow stromal cells (Xu et al., 1997) in response to TNF-α can be attenuated by anti-oxidants. In human aortic smooth muscle cells, induction of MCP-1 by PDGF is mediated by the generation of superoxide anion ($O_2^{\cdot-}$) and the subsequent activation of NF-κB (Marumo et al., 1997).

In the rat Schwann cell line SCL 4.1/F7, TNF-α induced the synthesis of MCP-1 mRNA in a dose-dependent fashion and this induction was inhibited by curcumin. In the present study, we investigated whether the effect of TNF-∀ in primary cultures of Schwann cells will also be inhibited by anti-oxidants. Schwann cells were treated for 3 hours with TNF-α in the absence or presence of quercetin or curcumin. Data from RNase protection assays revealed that these compounds inhibited the induction of MCP-1 mRNA in response to TNF-α stimulation (Fig. 6a). Thus, TNF-α induces the expression of MCP-1 mRNA in Schwann cells through a pathway that involves the generation of oxygen radicals.

The involvement of oxygen radicals in the induction of MCP-1 mRNA in degenerating nerves is difficult to investigate in an *in vivo* paradigm, thus, the effect of anti-oxidants on MCP-1 mRNA expression in organ cultures of sciatic nerves was determined. RNase protection assays showed that in organ cultures of sciatic nerves incubated for 3 hours in a medium containing serum, the induction of MCP-1 mRNA was

not abrogated by the addition of anti-oxidants (Fig. 6b). However, in cultures of sciatic nerves incubated for 3 hours in a serum-free medium, the addition of quercetin reduced the induction of MCP-1 mRNA. Curcumin was not as effective in reducing the concentrations of MCP-1 mRNA induced in organ cultures of sciatic nerves. These observations suggest that the early induction of MCP-1 mRNA in the nerve is mediated, at least in part, by oxygen radicals.

TNF-α exerts its effects binding to its receptors and activating either one of two pathways – one pathway involves the activation of NF-κB and the other involves the activation of c-Jun N-terminal kinase (JNK) (Henkel et al., 1993; Liu et al., 1996). In a number of cell types TNF-α induces the production of MCP-1 through a pathway that activates nuclear factor NF-κB (Ueda et al., 1997; Ping et al., 1999). The *in vivo* footprinting of the murine and human MCP-1 promoter revealed that there are at least 2 sites for 6B binding (Ping et al., 1996; Ueda et al., 1997). Hence, it is likely that in Schwann cells TNF-α mediates its effect through the activation of NF-κB.

The Schwann cell line SCL4.1/F7 has been shown previously to express MCP-1 mRNA in response to TNF-α treatment in a dose-dependent manner. This induction of MCP-1 mRNA by TNF-α was inhibited by the anti-oxidant curcumin in a dose-dependent fashion. Using nuclear extracts of SCL4.1/F7 cells treated with TNF-α for varying lengths of incubation time, eletrophoretic mobility shift assays for NF-κB were performed. Results showed that NF-κB binding to the labelled consensus oligonucleotide was observed using the nuclear extracts of untreated and TNF-α treated SCL4.1/F7 cells (Fig. 7a). It appears that at least one of the DNA binding subunits of NF-κB is constitutively activated in SCL4.1/F7 cells.

To further verify whether NF-κB activation is important in the expression of MCP-1 mRNA in Schwann cells, cultures were treated for 3 hours with either TNF-α or NGF, which had been shown to stimulate the nuclear translocation of the p65 subunit of NF-κB in rat Schwann cells (Carter et al., 1996). Results from RNase protection assays

showed that NGF does not stimulate the expression of MCP-1 mRNA in Schwann cells (Fig. 7b).

Presence of mature mast cells are not necessary for the early expression MCP-1 mRNA in the nerve

Mast cells have been shown to degranulate within 15 minutes following nerve injury (Olsson, 1967) and certain molecules known to be capable of stimulating the synthesis of MCP-1. In fact, mast cells are able to produce MCP-1 in response to stem cell factor (SCF) stimulation (Baghestanian et al., 1997). To determine whether mast cell degranulation is important in the induction of MCP-1 mRNA at the lesion site, sciatic nerve transection was also performed on mice which are deficient in *c-kit*, the receptor for SCF, which is necessary for maturation of mast cells (Galli et al., 1993). RNase protection assays show that within 6 hours after injury, the mRNA for JE, the mouse homologue for MCP-1, was induced in injured sciatic nerves of *c-kit* mice (Fig. 8) with increase in concentration comparable to that of the control strain.

TNF-α is not necessary for MCP-1 expression in injured sciatic nerves

To determine the importance of TNF-α in the regulation of MCP-1 synthesis in the PNS *in vivo*, the right sciatic nerve was transected on mice with null mutation of TNF receptor types 1 and 2. Data from RNase protection assays show that the concentration of MCP-1 mRNA was increased at the lesion site within 6 hours of nerve transection and elevated at the distal stump at least during the following 2-4 days (Fig. 9). These responses in the mice with deleted TNF-α receptors mice were not observed to be different than in wild type mice.

Mouse JE, the homologue of MCP-1, is expressed in transected Wld nerves

The mutant strain C57BL/Wld (Wld) is characterized to have delayed Wallerian degeneration and is associated with retarded breakdown of myelin and subnormal recruitment of macrophages (Lunn et al., 1989). Transection of the right sciatic nerve was performed on C57BL/6 (B6) and C57Bl/Wld (Wld) mice. Results from RNase protection assays showed that the message for JE increased at the lesion site within 6 hours after injury and by 4 days JE mRNA was also increased in the distal nerve segments (Fig. 10a). Similar to the JE mRNA expression in B6 nerves, JE mRNA concentrations increased within 6 hours at the site of lesion and by 4 days the increase in JE mRNA concentrations was also observed in the distal nerve stumps of Wld mice (Fig. 10b). Histological examination of the nerves transected 10 days earlier showed that in B6 mice the myelin has degenerated while in Wld mice many of the fibers still have intact myelin (Fig. 11). Thus, it appears that expression of JE mRNA in transected nerves is not enough to recruit macrophages.

DISCUSSION

MCP-1 mRNA is induced quickly in the injured DRG

Previously, many of the macrophages in DRG associated with injured nerves were shown to surround healthy neurons and appear before neuronal death occurs (Lu, Richardson, 1993). The present finding that MCP-1 mRNA expression increases in the injured DRG before the start of macrophage infiltration (Lu, Richardson, 1993)is

consistent with the hypothesis that MCP-1 contributes to the recruitment of monocytes to the DRG. However, the possibility that some resident macrophages dedifferentiate and proliferate during Wallerian degeneration has not been excluded.

The localization of MCP-1 mRNA in the DRG has not been ascertained. However, the non-neuronal cells are the most likely sources of MCP-1 in the ganglia. If so, some of the neuronal cell body responses may provide a direct or an indirect stimulus for MCP-1 synthesis.

Biphasic expression of MCP-1 in the injured sciatic nerve

The distribution of MCP-1 mRNA in injured sciatic nerve, an early induction within 3 hours at the site of injury and a later induction beginning 24 hours after injury in distal segments is similar to that for NGF (Heumann et al., 1987b; Heumann et al., 1987a) and LIF (unpublished data) mRNAs.

The initial increase in MCP-1 mRNA concentration is attributable to the exposure of the nerve to molecules rapidly released from the axons, components of the blood or those secreted by the non-neuronal cells. The murine gene JE, which encodes the mouse homologue of MCP-1, was first identified as a platelet-derived growth factor (PDGF)-inducible early response gene (Cochran et al., 1983). Activated platelets are known to release PDGF and other molecules which induce MCP-1 expression (Deuel, Kawahara, 1991). The hypothesis that mast cells, which degranulate rapidly after injury (Olsson, 1967), contribute to the early induction of MCP-1 in injured nerve is not supported by our observations in *c-kit* mice. The contribution of mast cells in the regulation of MCP-1 mRNA synthesis in the distal nerve stump remains to be ascertained.

Factors contributing to the sustained expression of MCP-1 in the distal stump might include the diffusion of molecules from the site of injury, activation of resident macrophages and/or dedifferentiation of Schwann cells following loss of axonal contact. Activated macrophages are good sources of reactive oxygen species (MacMicking et al.,

1997) and cytokines (Brück, 1997), such as TNF-α, which are capable of stimulating the synthesis of MCP-1. However, results in this study showed that in Wld mice wherein delayed Wallerian degeneration is associated with subnormal recruitment of macrophages (Lunn et al., 1989), the expression of MCP-1 mRNA is similar to that of control mice. Thus, macrophages in degenerating nerves do not appear to be the only signal for MCP-1 synthesis.

TNF-α stimulates the expression of MCP-1 mRNA in cultured Schwann cells but is not crucial for MCP-1 mRNA induction in injured peripheral nerves

Five possible cellular sources of MCP-1 in the PNS are blood cells, mast cells, resident macrophages, fibroblasts and Schwann cells. In this study, the production of MCP-1 mRNA by Schwann cells was investigated. Present observations show that primary Schwann cell cultures increase the synthesis of MCP-1 mRNA in response to TNF-α and, to a lesser extent, LPS but not to IL-1β, TGF-β1 and IL-6. In many cell types, IL-1 and TNF-α are potent inducers of MCP-1 synthesis (Villiger et al., 1992; Hayashi et al., 1995; Ping et al., 1996; Ueda et al., 1997). However, in this study, IL-1β failed to stimulate the expression of MCP-1 mRNA in Schwann cells. To date Schwann cells have not clearly been shown to respond to IL-1 although IL-1 receptor 1 and IL-1 receptor accessory protein have been detected in cultured Schwann cells by RT-PCR and Southern blot analysis (unpublished data). Perhaps high concentrations of the IL-1 receptor antagonist are present in the cultures and thus blocking the binding of IL-1 to its receptor.

TNF-α stimulation of Schwann cells has been shown to inhibit proliferation, reduce the expression of myelin protein P_0 and glial fibrillary acidic protein (GFAP) and decrease gap junctional conductance (Chandross et al., 1996). Binding of TNF-α to its

receptors may trigger the phosphorylation and subsequent degradation of I6B resulting in the activation of NF-κB (Henkel et al., 1993) and/or the activation of the activator protein-1 (AP-1) through the JNK pathway (Liu et al., 1996). The bifurcation of the two kinase cascades occurs at the level of TNF receptor-associated factor 2 (TRAF 2) (Song et al., 1997). In the present study, induction of MCP-1 mRNA in Schwann cells by TNF-α could be abrogated by anti-oxidants, curcumin and quercetin. These anti-oxidants have been shown to inhibit the action of TNF-α by blocking the activation of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (MEKK1) in the JNK pathway (Yokoo, Kitamura, 1997; Chen, Tan, 1998) and by preventing the phosphorylation and the subsequent degradation of IκB in the NF-κB pathway (Sato et al., 1997; Brennan, O'Neill, 1998). Curcumin also interferes with the binding of the p50 subunit of NF-κB to the DNA binding site (Brennan, O'Neill, 1998).

The results of this study showed that TNF-α stimulates the synthesis of MCP-1 mRNA in cultured Schwann cells through a pathway that is mediated by oxygen radicals. In addition, the induction of MCP-1 mRNA in Schwann cells does not appear to be mediated by NF-κB activation. However, the nucleotide sequence of the commercially obtained probe, which is the consensus binding site for NF-κB/c-Rel (Lenardo, Baltimore, 1989), used in the EMSAs is not identical to any of the known 6B binding sites in the human and murine MCP-1 promoter (Ueda et al., 1997; Ping et al., 1996; Ping et al., 1999). There are different proteins in the NF-κB/Rel family: p50, p52, p65/RelA, c-Rel and RelB (Feuillard et al., 1996; Ueda et al., 1997; Krushel et al., 1999). The functional nuclear factor NF-κB is a dimer of any of the subunits (Lenardo, Baltimore, 1989; Baeuerle, 1991). There are different isoforms of NF-κB in the nuclear extracts of TNF-α-treated cells and the p50/p65 heterodimer is the predominant isoform (Ueda et al.,

1997; Ping et al., 1996). Furthermore, the p65 subunit appears to be crucial for MCP-1 induction in TNF-α-treated cells (Ueda et al., 1997; Ping et al., 1999). However, NGF, which failed to induce the expression of MCP-1 mRNA in Schwann cells, had been previously shown to activate the nuclear translocation of NF-κB isoforms containing the p65 subunit in cultured Schwann cells (Carter et al., 1996). Thus, it is likely that the induction of MCP-1 mRNA in Schwann cells in response to TNF-α stimulation may be mediated by other NF-κB isoforms besides those that have the p65 subunit. Alternatively, TNF-α may mediate its effects in Schwann cells through the activation of the JNK pathway.

Although TNF-α stimulates the production of MCP-1 mRNA in cultured Schwann cells, the observations on MCP-1 mRNA concentrations in injured nerves of mice lacking both p55 and p75 receptors for TNF-α suggest that TNF-α is not necessary for the induction of MCP-1 in injured PNS tissue. These findings corroborate the observations that after injury the increase in macrophage numbers in p55-/p75 knock-out mice is comparable to that of the wild type while mice deficient in both IL-1R type 1 and p55 TNF receptor show reduced macrophage infiltration (Dailey et al., 1998). It is probable that the absence of TNF-α bioactivity is compensated by the actions of other cytokines, such as IL-1β(Villiger et al., 1992), TGF-β (Takeshita et al., 1995), PDGF (Cochran et al., 1983) and LIF (Musso et al., 1995). These findings, however, do not exclude the possibility that TNF-α contributes to the regulation of MCP-1 in the distal stump.

Expression of MCP-1 mRNA is not enough for the normal recruitment of macrophages

The results of this study showed that JE mRNA was induced in the distal stump of Wld mice 4 days after transection even though axonal degeneration in these mice is delayed. Hence, these observations suggest that the expression of MCP-1 in axotomized

nerves is not sufficient for normal monocyte infiltration. MCP-1 is not the only chemokine involved in the migration of monocytes (reviewed by (Springer, 1994; Springer, 1995; Rollins, 1997)). Also, in general, there are 2 other molecular interactions that are necessary for leukocyte migration. The patterns of distribution of selectins and leukointegrins that are important for monocyte migration have not been determined in Wld transected sciatic nerves.

The present findings are in contrast to the data in an earlier study where JE mRNA in Wld mice was not detected by *in situ* hybridization in the distal stump of Wld mice 7 days after injury (Carroll, Frohnert, 1998). These conflicting observations may be due to the difference in the sensitivity between the two mRNA detection methods - the RNase protection used in the present study and the *in situ* hybridization used in the previous study. In addition, the time points employed in the two investigations are different.

Summary

MCP-1 mRNA concentrations increase after nerve injury in both nerve and DRG well before increases in macrophage numbers are observed. The induction of MCP-1 mRNA in Schwann cells may be regulated by factors, such as TNF-α, which mediate their effects through a pathway that is inhibited by anti-oxidants. However, TNF-α is not crucial for the induction of MCP-1 in degenerating mouse sciatic nerves. In addition, in C57BL/Wld mice, which exhibit delayed axonal degeneration, MCP-1 mRNA concentrations in the distal nerve stump increase.

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Ref Type: Generic

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Figure 1. RNase protection assay of total RNA isolated from dorsal root ganglia (DRG) taken from normal, unoperated (N) and 2-day sham operated animals and those which had sciatic nerve transection (SN cut). Contralateral, left (L) and ipsilateral, right (R) DRG were taken 1 –16 days (d) after injury. MCP-1 – monocyte chemoattractant protein-1; Cyclo – cyclophilin.

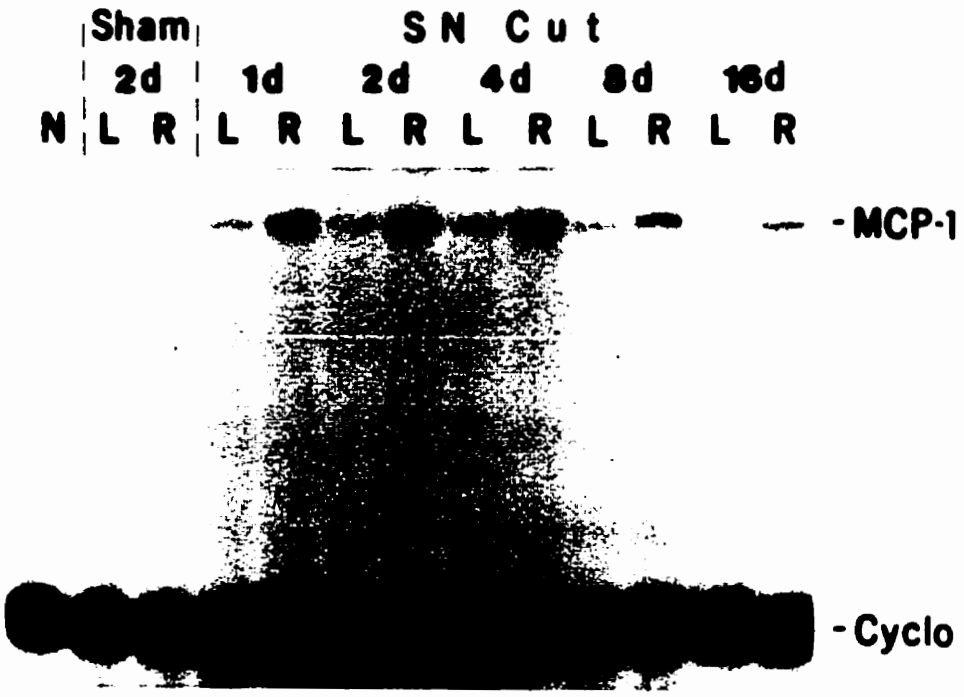

Sham
SN Cut
2d
1d
2d
4d
8d
16d
N
L
R
L
R
L
R
L
R
L
R
L
R
-MCP-1
-Cyclo

Figure 2. Schematic diagram of the transected sciatic nerve. DRG – dorsal root ganglion; distal – distal nerve stump.

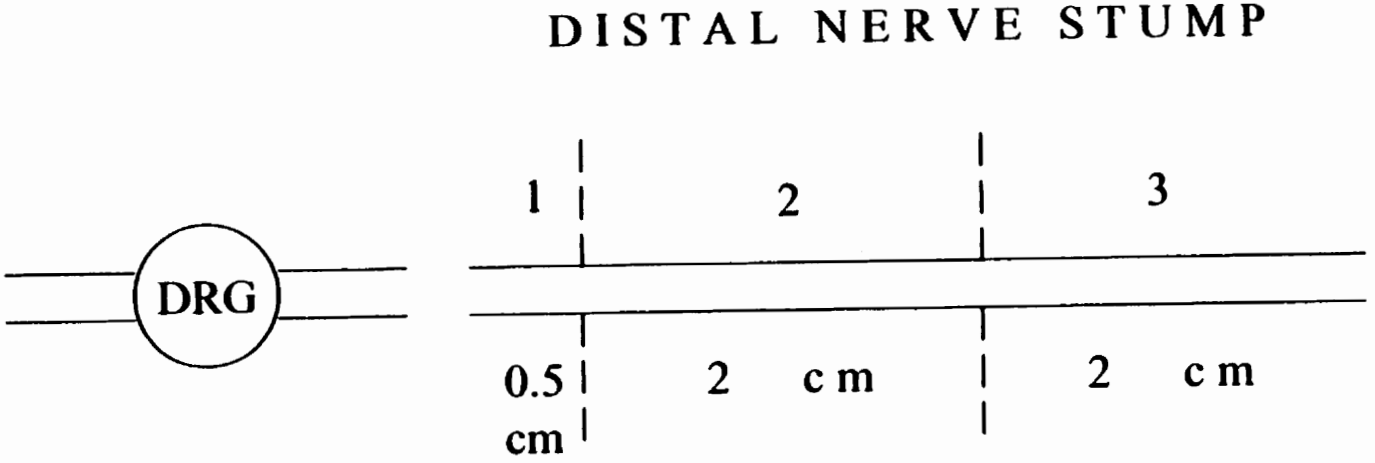
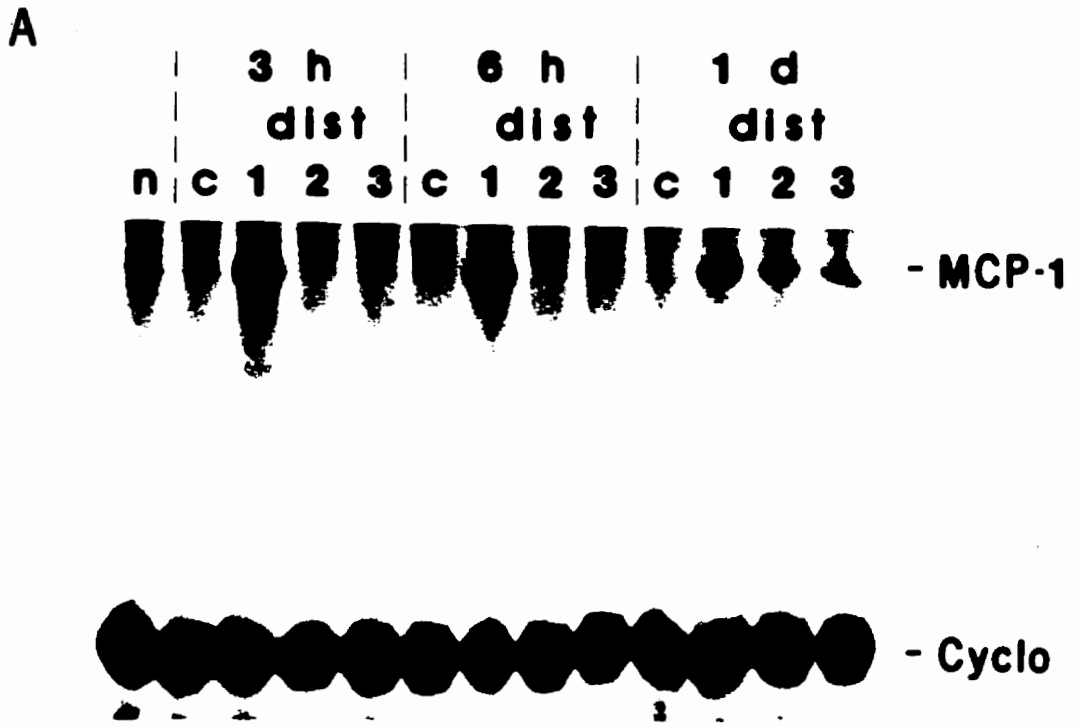

DISTAL NERVE STUMP
DRG
1
2
3
0.5 cm
2 cm
2 cm

Figure 3. RNase protection assay of total RNA isolated from sciatic nerves of normal, unoperated rats (n) and from those which had undergone sciatic nerve transection. **(A)** Rats were sacrificed 3 hours to1 day after transection and **(B)** 2 to 8 days post-injury. c – contralateral nerve; dist 1, 2 and 3 – distal nerve segment 1, 2 and 3; MCP-1 – monocyte chemoattractant protein-1; Cyclo – cyclophilin.


A
3 h
6 h
1 d
dist
dist
dist
n c 1 2 3 c 1 2 3 c 1 2 3
- MCP-1
- Cyclo

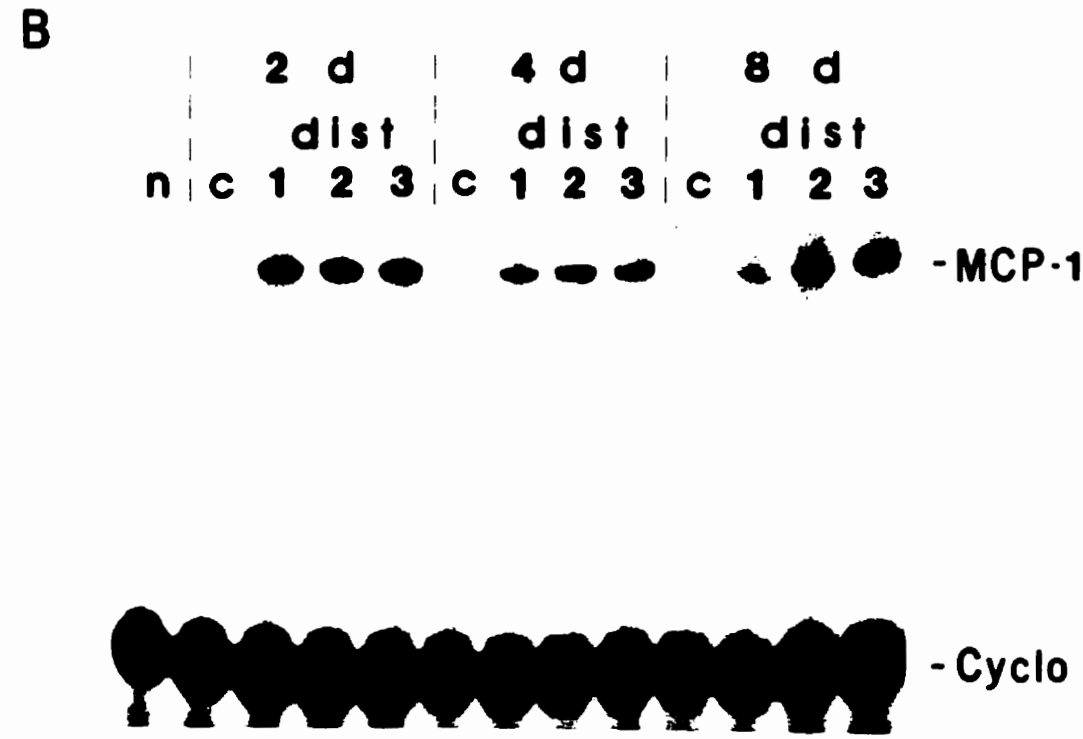

B
2 d
4 d
8 d
dist
dist
dist
n c 1 2 3 c 1 2 3 c 1 2 3
- MCP-1
- Cyclo

Figure 4. Concentrations of MCP-1 mRNA in the contralateral and transected sciatic nerves 16 days after injury as measured by RNase protection assay. 0 – sciatic nerves from unoperated rats. MCP-1 mRNA concentrations are expressed relative to the cyclophilin mRNA concentrations.

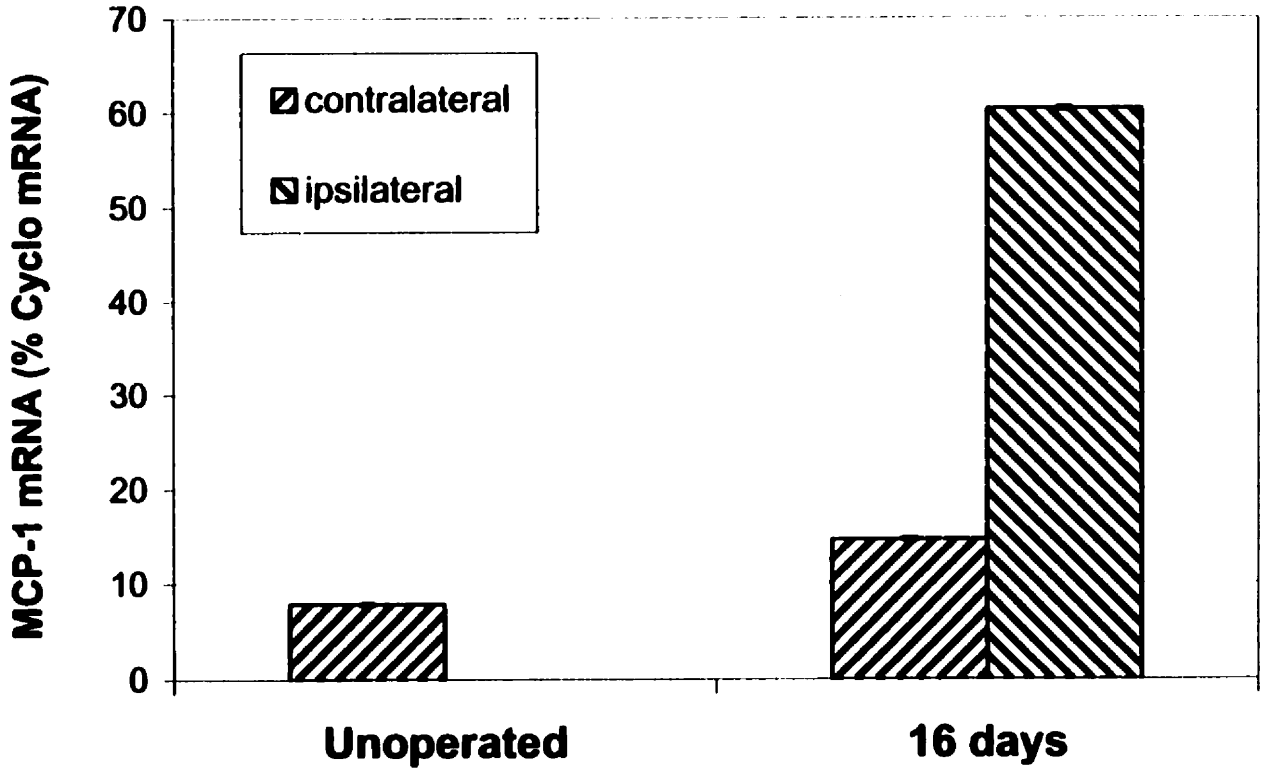
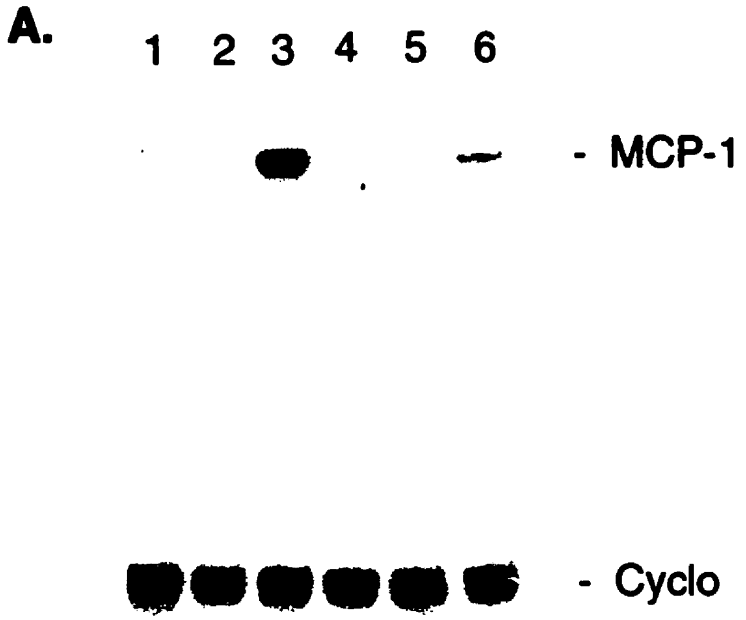

MCP-1 mRNA (% Cyclo mRNA)
70
60
50
40
30
20
10
0
contralateral
ipsilateral
Unoperated
16 days

Figure 5. Induction of MCP-1 mRNA in Schwann cells. **(A).** MCP-1 mRNA concentrations in Schwann cells treated for 3 hours with various cytokines were measured by RNase protection assay. Lane 1 – no treatment; lane 2 - IL-1β (1 ng/ml); lane 3 - TNF-α (5 ng/ml); lane 4 - TGF-β (5 ng/ml); lane 5 - IL-6 (10 ng/ml), and lane 6 - LPS (500 ng/ml). **(B).** RNase protection assay to measure MCP-1 mRNA concentrations in Schwann cells treated with 5 ng/ml TNF-α for 30 minutes (lane 2), 1 hour (lane 3), 3 hours (lane 4), and 5 hours (lane 5). Lane 1 – MCP-1 mRNA concentration in Schwann cells at the start of the treatment. Cyclophilin (Cyclo) probe was used as basis for comparing MCP-1 mRNAs in different samples.


A.
1 2 3 4 5 6
- MCP-1
- Cyclo

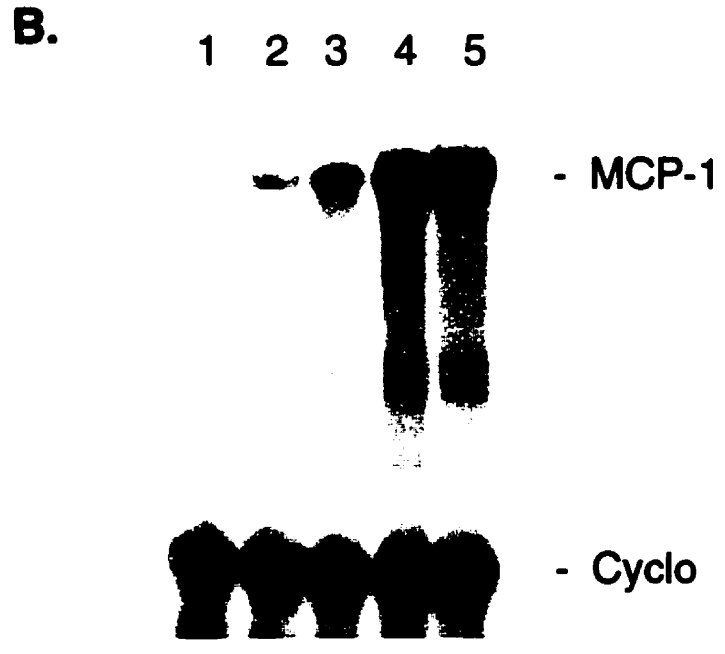

B.
1 2 3 4 5
- MCP-1
- Cyclo

Figure 6. Anti-oxidants inhibit the MCP-1 mRNA induction. **(A).** MCP-1 mRNA concentrations in the Schwann cells treated for 3 hours with 5 ng/ml TNF-α in the presence of either 50 μM curcumin or 100 μM quercetin. **(B).** Organotypic cultures of sciatic nerves incubated in either serum-containing or serum-free medium were treated with either curcumin or quercetin for 3 hours. Cyclophilin mRNA (Cyclo) was used to measure the relative amounts of total RNA used in RNase protection assay.

A.

TNF-alpha	-	+	+	+
Quercetin	-	-	+	-
Curcumin	-	-	-	+

- MCP-1

- Cyclo

B.

Serum	+	+	+	-	-	-
Quercetin	-	+	-	-	+	-
Curcumin	-	-	+	-	-	+

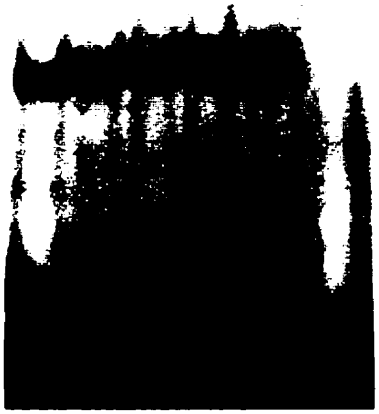
- MCP-1

- Cyclo

Figure 7. (A). Electrophoretic mobility shift assay for NF-κB of nuclear extracts from SCL 4.1/F7 cells treated with 5 ng/ml TNF-α. Lane 1 – start of treatment; lane 2 – 15 minutes; lane 3 – 30 minutes; lane 4 – 1 hour; lane 5 – 2 hours; lane 6 – addition of 50x excess of cold oligonucleotide probe to cells treated for 2 hours. **(B).** RNase protection assay of total RNA from untreated Schwann cells (U) and cultures treated with either 10 ng/ml NGF (N) or 5 ng/ml TNF-α (T).

A.

1 2 3 4 5 6



B.

U N T

- MCP-1

- Cyclo

Figure 8. Murine JE mRNA (mJE) concentrations in contralateral (L) and distal sciatic nerve stump (R) of control mice and those which are deficient in *c-kit* gene 6 hours after transection as measured by RNase protection assay.

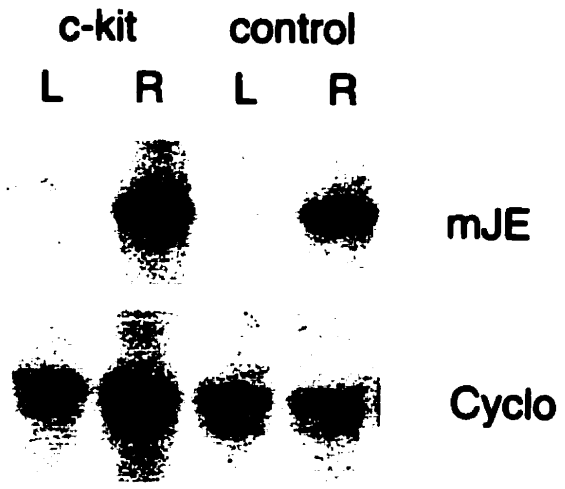

c-kit
control
L
R
L
R
mJE
Cyclo

Figure 9. Murine JE mRNA (mJE) concentrations in B6x129F1 mice and those that are deficient in the genes encoding the two TNF-α receptors (TNFRKO) 6 hours to 4 days after right sciatic nerve transection as determined by Rnase protection assay. C – contralateral nerve; T – tip, lesion site; D – distal stump; Cyclo – cyclophilin.

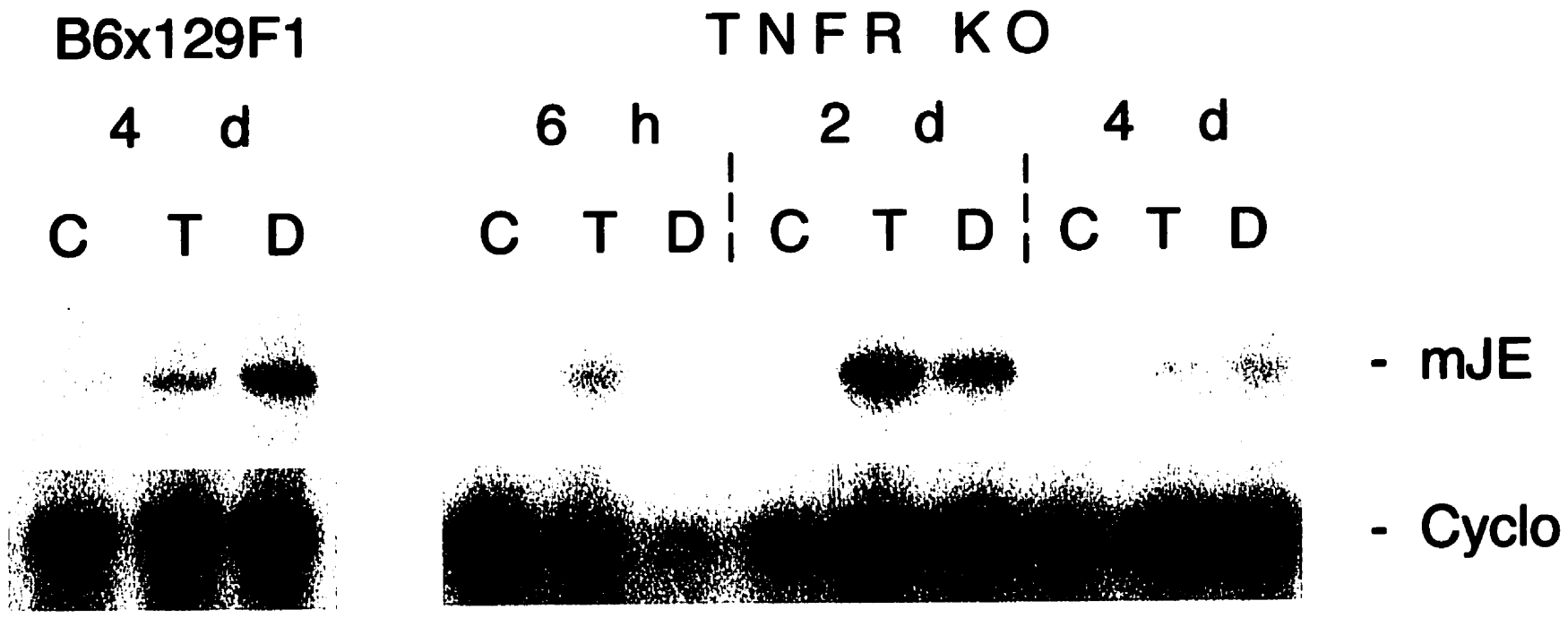
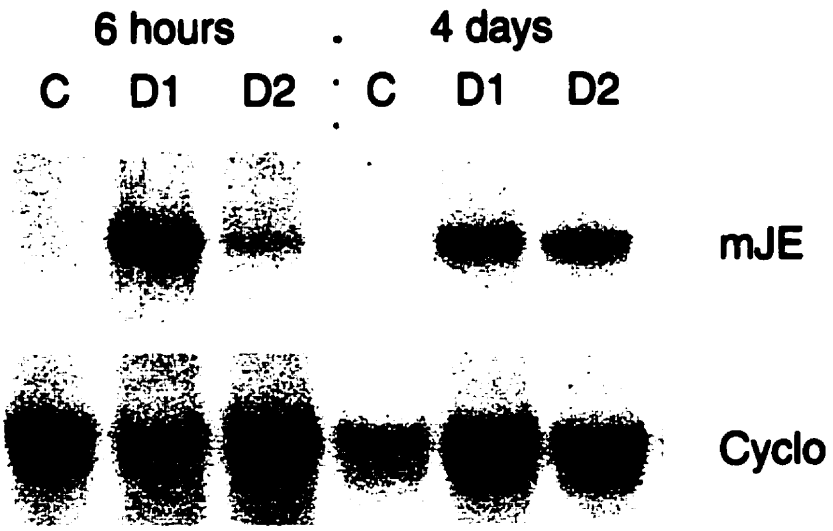

B6x129F1
4 d
C T D
TNFR KO
6 h
2 d
4 d
C T D C T D C T D
- mJE
- Cyclo

Figure 10. Concentrations of murine JE in C57BL/6 (A) and C57BL/Wld (B) mice 6 hours and 4 days after right sciatic nerve transection as measured by Rnase protection assay. C – contralateral nerve; D1 – distal 1 or the lesion site; D2 – distal stump; Cyclo – cyclophilin.

A. C57BL/6



B. C57BL/Wld

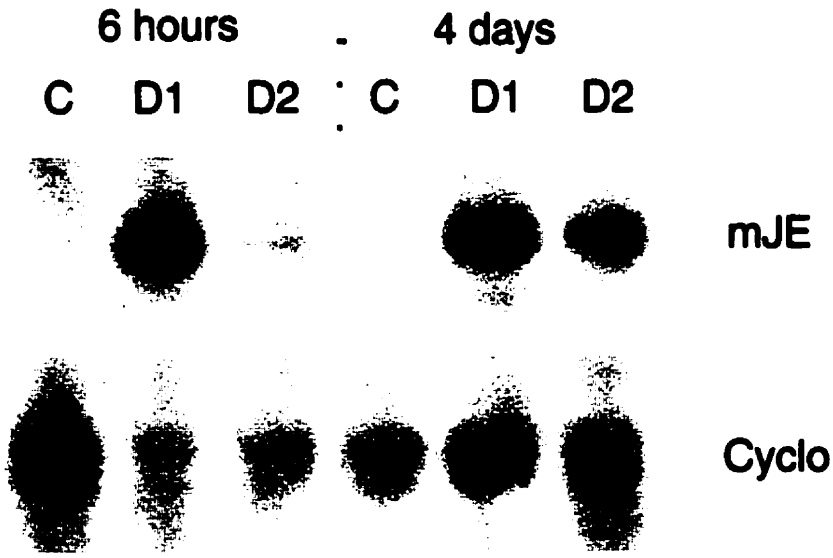
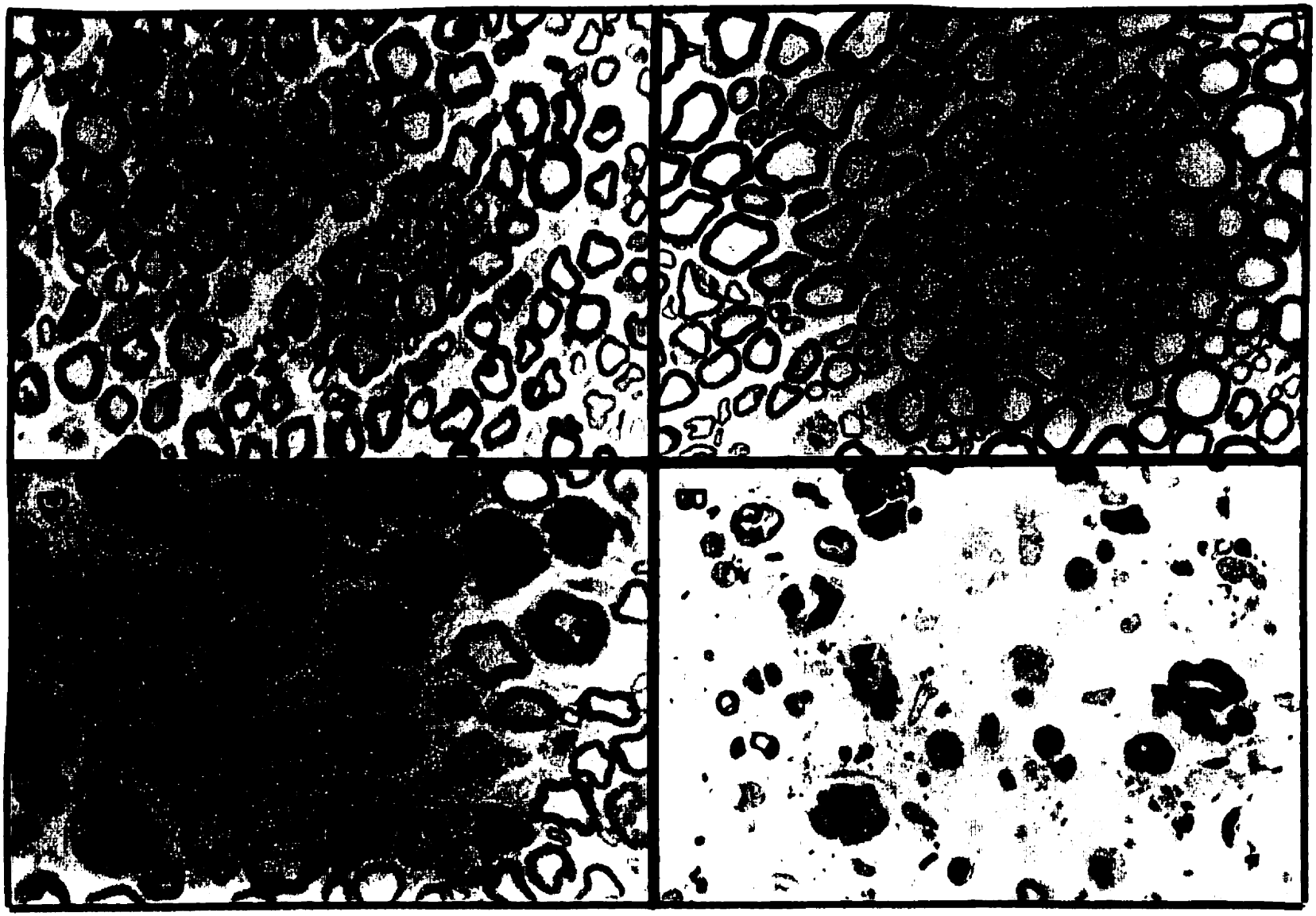


Figure 11. Semi-thin sections of sciatic nerves from contralateral nerves (A, C) and distal stumps (B, D) of C57BL/6 (A, B) and C57BL/Wld mice (C, D) 10 days following sciatic nerve transection..



CHAPTER 8: SUMMARY OF RESULTS

This and other studies of the events following the disintegration of axons after peripheral nerve injury have revealed an intimate relationship that occurs between axons and the associated Schwann cells and between the different non-neuronal cells. The expression of many of the Schwann genes is influenced by the presence of axons. The myelin proteins are the classical axonally-regulated molecules made by Schwann cells. Another signal that might influence Schwann cell responses to injury is the exposure to the different components of the blood. Molecules secreted by hematogenous cells have been shown to influence the changes in gene expression of fibroblasts (Heumann et al., 1987a; 1987b). To determine the importance of these different signals, the present study focused on three genes expressed by Schwann cells and other non-neuronal cells, ciliary neurotrophic and leukemia inhibitory factors and monocyte chemoattractant protein-1, and investigated the factors that regulate their expression in peripheral nerves.

CILIARY NEUROTROPHIC FACTOR

Ciliary neurotrophic factor (CNTF) is abundantly expressed in normal peripheral nerves. Early studies have shown that CNTF mRNA and protein are reduced in the nerve after injury (Friedman et al., 1992; Sendtner et al., 1992b; Seniuk et al., 1992). These findings have led to the hypothesis that similar factors regulate myelin protein and CNTF synthesis.

To investigate the factors that influence CNTF expression, Schwann cell cultures were established from sciatic nerves of neonatal rats. Immunocytochemical studies on

cells dissociated from 5-day old rats showed that about 65% of the cells were positive for S100, a marker for glial cells and 11-12% stained for myelin proteins. Twenty-four hours after plating these cells did not stain for myelin proteins. Western blot analysis showed that freshly dissociated cells were immunopositive for CNTF but 5-day old cultures had no detectable CNTF protein. Rnase protection assay showed that the freshly dissociated cells expressed CNTF mRNA. The concentration of CNTF mRNA decreased in cells plated overnight. These results indicate that CNTF is also down-regulated *in vitro* supporting the hypothesis that CNTF and myelin proteins are regulated in a similar manner.

One factor that regulates myelin protein synthesis is the presence of axonal contact and another possible factor is the interruption of axonal transport. To distinguish between these two possibilities, sciatic nerve ligation was performed on the C57BL/Wld mouse strain, which is characterized by delayed Wallerian degeneration (Lunn et al., 1989). RT-PCR analysis of the total RNA from contralateral and axotomized Wld and CD1 nerves showed that at 4 days post-ligation CNTF mRNA declined to 23% of normal concentrations in control mice while it was reduced to 83% of normal concentrations in Wld mice. By 10 days CNTF mRNA concentration in Wld mice was already comparable to that of control mice. In comparison, P_0 mRNA concentrations 4 days after axotomy were reduced to 7% of normal in control mice while it was reduced to 36% of normal in Wld mice.

Western blot analysis showed that in ligated nerves of control mice CNTF-immunoreactivity (CNTF-IR) was reduced 4 days after surgery and was no longer detectable by 10 days. In Wld mice, CNTF-IR in ligated nerves remained at near normal

levels even at 10 days post-injury. In contrast, P_0-IR was greatly reduced in nerves 10 days after ligation.

In summary, after ligation of sciatic nerves of Wld mice, with consequent interruption of axonal transport from the nerve cell body but axonal contact is maintained, the reduction of CNTF mRNA concentrations was more delayed than in wild-type mice. Moreover, in Wld mice, the reduction in CNTF-immunoreactivity was even further delayed. This delay in protein degradation could be due to the known paucity of macrophages in the injured nerve or a possible subnormal activity of protease involved in CNTF degradation. CNTF and myelin proteins respond in similar fashion during Wallerian degeneration and in cultured Schwann cells. This concordance could reflect a common mechanism of induction or a dependence of one on the other.

LEUKEMIA INHIBITORY FACTOR

LIF is normally expressed in peripheral nerves at very low levels and is induced after injury (Curtis et al., 1994). The possible sources of LIF in the injured nerves include Schwann cells, fibroblasts, macrophages and mast cells. This study investigated the spatial and temporal distribution of LIF mRNA in injured sciatic nerves.

The message for LIF was determined in the different segments of the distal stump. Transection of the sciatic nerve led to the induction of LIF mRNA expression at the site of lesion within 6 hours but not in the distal stump. This pattern of expression persisted for at least 18 hours. LIF mRNA expression in the entire distal segment was observed by 24 hours and increased thereafter.

To study the regulation of LIF in injured nerves, Schwann cells were treated with various cytokines. However, none of the treatment regimes used resulted in the induction of LIF mRNA. In contrast, nerve fibroblasts expressed LIF mRNA when stimulated with IL-1β. Thus, for LIF as for NGF synthesis (Lindholm et al., 1987), fibroblasts but not Schwann cells respond to IL-1 treatment.

MONOCYTE CHEMOATTRACTANT PROTEIN-1

Regulation of MCP-1 in the PNS

This study determined the spatio-temporal distribution and the regulation of the mRNA for the chemokine monocyte chemoattractant protein-1 (MCP-1) in injured PNS.

RNase protection assays of DRG from the uninjured and injured side showed that MCP-1 mRNA was induced in the ipsilateral DRG within a day after nerve transection. Hence, MCP-1 mRNA appears to be synthesized in the DRG before the increase in macrophage numbers is observed (Lu, Richardson, 1993). These observations support the hypothesis that MCP-1 is involved in the recruitment of monocytes to injured PNS.

Analysis of the different segments of the distal stump by RNase protection assay showed that MCP-1 mRNA was induced at the site of lesion within 3 hours after transection. By 24 hours, the entire distal stump expressed MCP-1 mRNA. Thus, MCP-1 mRNA is expressed in the injured nerve well before the infiltration of blood-borne monocytes (Chumasov, Svetikova, 1991; Taskinen, Röyttä, 1997).

There are at least three possible cellular sources of MCP-1 in the nerve – fibroblasts, macrophages and Schwann cells. Expression of MCP-1 mRNA in fibroblasts, and macrophages had been documented in other systems (Ping et al., 1996; Liebler et al.,

1994; Weyrich et al., 1996; Gourmala et al., 1997; Ueda et al., 1997; Ping et al., 1999). In this study, the synthesis of MCP-1 by Schwann cells was investigated. RNase protection assay revealed that Schwann cells express low levels of MCP-1 mRNA and that treatment with LPS and TNF-α, but not IL-1β, TGF-β, IL-6 or NGF, increased MCP-1 mRNA concentrations. This induction of MCP-1 mRNA in Schwann cells was observed as early as 1 hour following TNF-α stimulation.

The anti-oxidants curcumin, quercetin, and N-acetyl-L-cysteine (NAC) suppress the expression of MCP-1 in response to cytokine stimulation in various cell types (Satriano et al., 1993; Sato et al., 1996; Sato et al., 1997; Xu et al., 1997). In primary cultures of Schwann cells and the rat Schwann cell line SCL14.1/F7, the effects of antioxidants on the expression of MCP-1 in response to stimulation by TNF-α, IL-1β and LPS were studied. Results showed that NAC had no effect on the induction of MCP-1 mRNA by TNF-α. Curcumin and quercetin were shown to inhibit MCP-1 mRNA expression in Schwann cells treated with TNF-α. These anti-oxidants inhibited MCP-1 mRNA induction in a dose response manner.

Results from Rnase protection assays showed that sciatic nerve fragments incubated for three hours in either serum-containing or defined medium synthesized MCP-1 mRNA and that quercetin reduced this induction of MCP-1 mRNA in organ cultures maintained in defined medium. These observations indicate that early synthesis of MCP-1 mRNA in the nerve is mediated by oxygen radicals.

TNF-α exerts its effect by transducing a signal through either the NF-κB or the JNK pathways (Song et al., 1997; Liu et al., 1996). Anti-oxidants are able to block both pathways (Sato et al., 1997; Chen, Tan, 1998). To determine whether TNF-α stimulates

MCP-1 mRNA synthesis through the NF-κB pathway, activation of NF-κB was ascertained by gel retardation assays. However, data showed that at least one protein in the nuclear extract of SCL4.1/F7 cell line constitutively binds to a consensus NF-κB DNA binding site. Also, treatment of Schwann cells with NGF, which is stimulates the nuclear translocation of the p65 subunit of the NF-κB complex (Carter et al., 1996), failed to induce MCP-1 mRNA. However, the activated NF-κB is a heterodimer of any of the members of the NF-κB/c-Rel family (Lenardo, Baltimore, 1989; Baeuerle, 1991). Hence, MCP-1 mRNA induction by TNF-α in Schwann cells may be mediated by members of the NF-κB/c-Rel family apart from p65. Alternatively, TNF-α mediates MCP-1 mRNA induction through the activation of the JNK pathway. The possibility that basal activation of transcription factors during the extraction of nuclear proteins could not be discounted.

To determine the possible contribution of mast cell products to the regulation of MCP-1 expression, right sciatic nerve transection was performed on mice with defective gene for *c-kit*, the receptor for the mast cell maturation factor, the stem cell factor (SCF) (Galli et al., 1993). Data showed that in the absence of c-kit and mature mast cells, MCP-1 mRNA concentration at the lesion site 6 hours after transection was comparable to that of control mice. Therefore, degranulation of mast cells at the lesion site is not essential to the early induction of JE, the mouse homologue of MCP-1, in injured nerves.

To investigate the physiological role of TNF-α in the induction of MCP-1 or JE mRNA in injured nerves, right sciatic nerve transection was performed on TNF receptor knock-out mice. Data showed that production of JE mRNA in the distal nerve stump was comparable to that of the control mice. Thus, TNF-α is not essential in the synthesis of

MCP-1 or JE in peripheral nerves after injury. This result is consistent with the finding that migration of monocytes and macrophages to the distal stump is hindered only when both IL-1 receptor and p55 TNF receptor have been deleted in mice (Dailey et al., 1998a).

Although MCP-1 mRNA distribution in degenerating nerves is similar to that of LIF mRNA, the mechanisms of regulation are shown to be different.

Function of MCP-1 in the PNS:

The delayed Wallerian degeneration in Wld mice is associated with delayed breakdown of myelin and subnormal recruitment of macrophages (Lunn et al., 1989). A previous study showed that JE mRNA was not detectable by *in situ* hybridization in Wld distal nerve stump 7 days after injury (Carroll, Frohnert, 1998). However, in the present study data from RNase protection assays showed that JE mRNA was induced in the distal stump within 2 days after transection and persisted for at least 4 days. The phenotype of the Wld mice used in this study was verified by histology with plastic embedded sections. These observations suggest that expression of MCP-1 mRNA is not the only signal necessary for the normal recruitment of myelomonocytic cells into the injured nerves.

CHAPTER 9: CONCLUSION

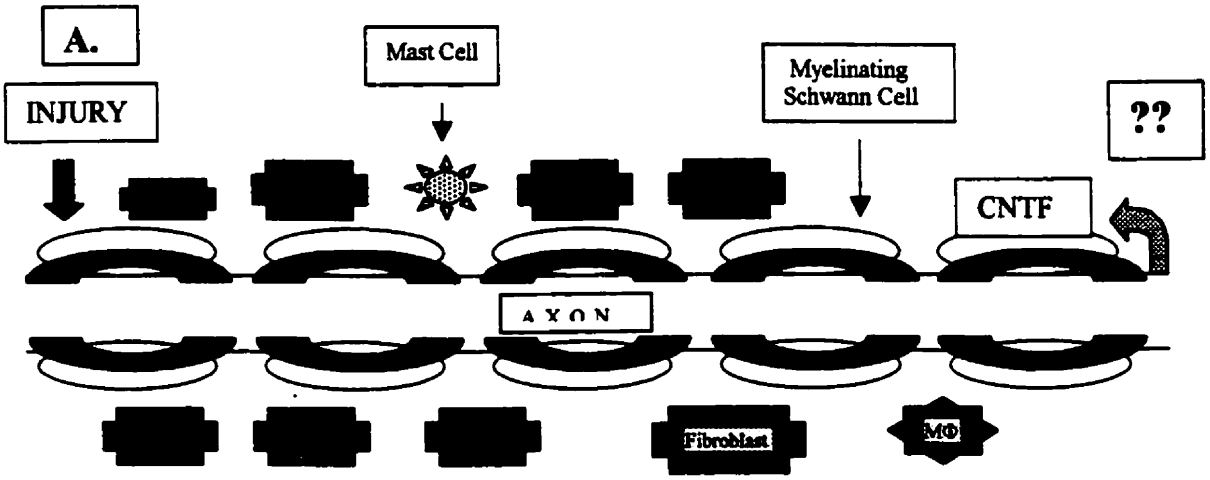
The results of this study suggest that the loss of axonal signals, particularly the molecules present in the axolemma, following peripheral nerve injury leads to the cessation of CNTF and myelin protein synthesis by Schwann cells. Thus, constant axonal signals are necessary to maintain the expression of these Schwann cell genes (Fig.1A).

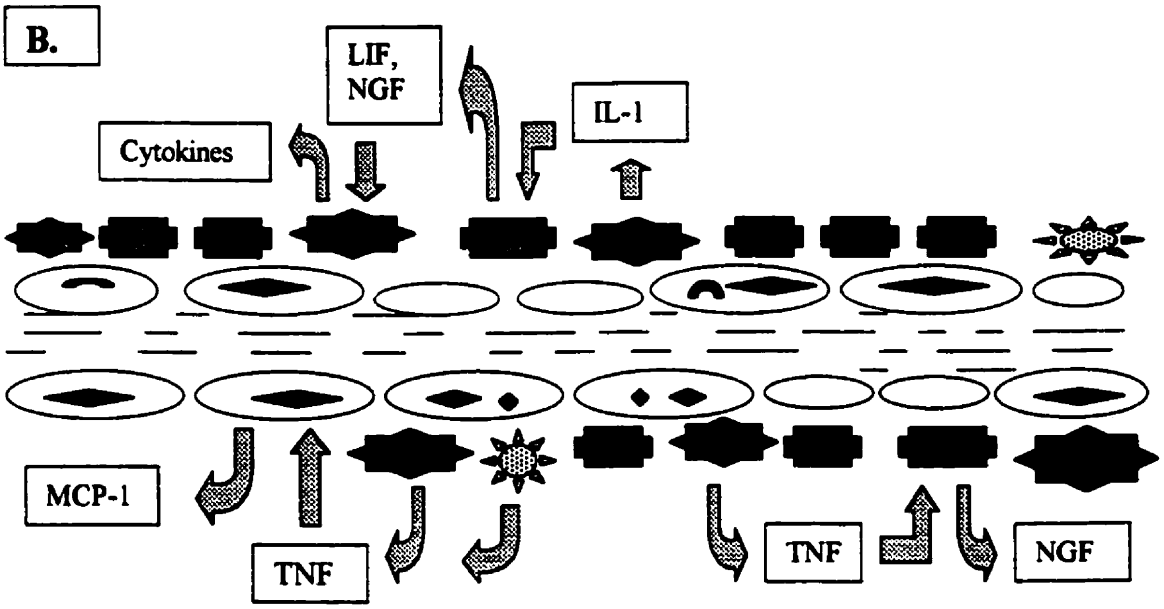
In addition to the loss of axonal signals, an injury results in the breakdown of the blood-nerve barrier exposing the intrinsic cells in the nerve to the various components of the blood. Cytokines, such as IL-1β or TNF-α, present in the plasma or secreted by hematogenous cells may stimulate the expression of LIF in fibroblasts and MCP-1 in Schwann cells and maybe in other non-neuronal cells (Fig. 1B).

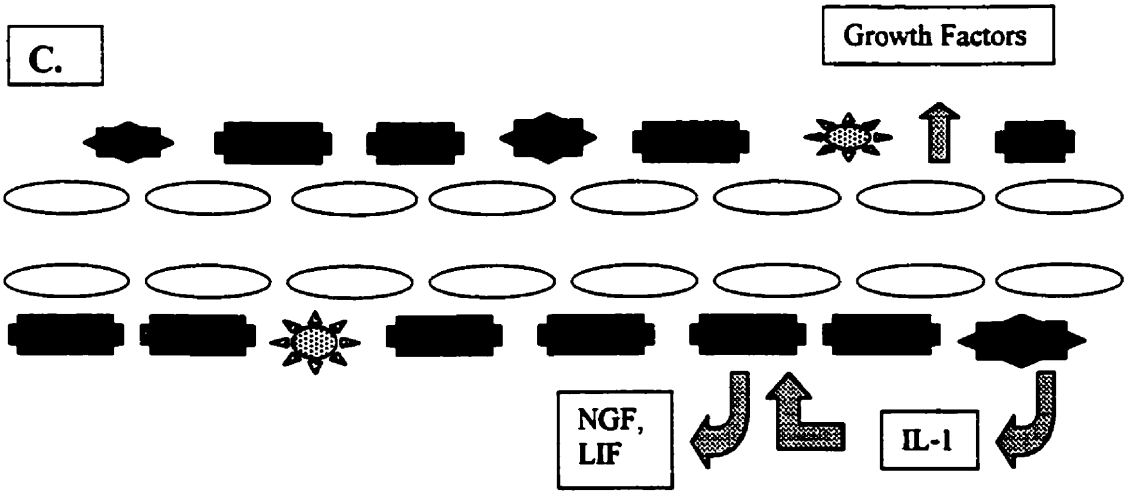
MCP-1 synthesized by Schwann cells may contribute to the signals necessary for the recruitment of monocytes into the injured nerve.

The physiological role of LIF in injured is still unclear. However, human monocytes treated with LIF synthesize IL-6 (Gruss et al., 1992) and chemokines such as IL-8 and MCP-1 (Musso et al., 1995). It is possible that the LIF synthesized in injured nerve contribute to the progression of the inflammatory reaction that is necessary for the removal of myelin and axonal debris and in the restructuring of the nerve. Figure 1C represents the state of the distal nerve stump wherein all the debris have been cleared, the Schwann cells have formed the bands of Büngner and may be producing some growth factors and the fibroblasts synthesize NGF (Heumann et al., 1987a; 1987b) and LIF in response to IL-1. This environment is now favorable for axonal regrowth or regeneration.

Figure 1. Schematic diagram of the events that occur in the nerve following injury. **(A.)** Diagram representing a myelinated nerve. Unknown axonal signals (**??**) influence the synthesis of CNTF in a subset of Schwann cells. The arrow (⬇) marks the site of injury. **(B.)** After injury, mast cells at the site of injury degranulates, the myelin breaks down and is later removed by macrophages (MΦ), Schwann cells and fibroblasts proliferate. IL-1 released from macrophages could stimulate the fibroblast to synthesize LIF and NGF. LIF can act on monocytes/macrophages to produce chemokines and maybe other cytokines. TNF-α from macrophages can stimulate Schwann cells to secrete MCP-1, which contributes to the recruitment of monocytes. It can also stimulate fibroblasts to synthesize NGF. **(C.)** This diagram represents a nerve wherein all axonal and myelin debris have been removed, Schwann have formed the bands of Büngner, the inflammatory cells start to be cleared from the distal stump and the nonneuronal cells are producing growth factors which promote axonal regeneration.


A.
INJURY
Mast Cell
Myelinating Schwann Cell
??
CNTF
AXON
Fibroblast
MΦ


B.
LIF, NGF
IL-1
Cytokines
MCP-1
TNF
TNF
NGF


C.
Growth Factors
NGF, LIF
IL-1

CHAPTER 10: FUTURE DIRECTIONS

The regulation of three genes studied – CNTF, LIF and MCP-1 – could be studied further and may provide a greater understanding of Wallerian degeneration.

Ciliary Neurotrophic Factor:

Analysis of CNTF promoter

The synthesis of molecules such as CNTF and myelin proteins (Gupta et al., 1993; Kuhn et al., 1993) depend on the contact between the neuron's axon and the Schwann cell. For several decades now attempts to identify the axonal component responsible for the regulation of myelin proteins have not been fruitful. Promoter analysis using transgenic mice or transfected cells has been the approach taken in last decade to further the study of the biology of myelin proteins. Thus, to better understand the mechanism of regulation of CNTF synthesis, it would be wise to determine the different *cis* elements present in the CNTF promoter. Furthermore, investigation of the post-transcriptional processing of the CNTF mRNA may be fruitful.

The sequences immediately upstream of the transcription start site of the human (Lam et al., 1991) and rat CNTF promoters (Carroll et al., 1993) have been reported. Sequence analysis showed that an AP-1 site and a G-rich region are present upstream of the transcription start site (Lam et al., 1991; Carroll et al., 1993).

Analysis of the regulatory regions of the human CNTF gene revealed that a 240 bp sequence upstream of the translation start codon is sufficient for transcription and an additional 4 kb upstream fragment is needed for expression in some Schwann cells (Stefanuto et al., 1995). For proper high level gene expression in sciatic nerves, a 2 kb downstream fragment is necessary in addition to the 2 upstream fragments (Stefanuto et al., 1995). To date these upstream and downstream fragments have not been sequenced and analyzed. To narrow down the specific regions within the 4 kb upstream and the 2 kb

downstream fragments that are necessary for robust Schwann cell-specific expression, Schwann cells will be transfected with several deletion constructs. Transgenic mice could also be generated to analyze the different regulatory regions of the CNTF promoter. Sequencing these upstream and downstream fragments could prove valuable. Once the sequences have been obtained, comparisons can be done between these CNTF regulatory regions with those of the different myelin protein genes and krox-20 gene.

Post-transcriptional regulation of CNTF mRNA

In chapter 3 of this thesis, the amount of CNTF protein in Schwann cells freshly dissociated from 5-day old rats appears to be disproportionately low compared to the amount of CNTF message present in these cells. As the half-life of CNTF mRNA is 6 hours (Carroll et al., 1993), it is possible that the CNTF expression is post-transcriptionally regulated. The CNTF protein does not have a consensus sequence for a signal peptide (Stöckli et al., 1989) and its subcellular localization is not yet known. It is likely that CNTF mRNA is transported to its subcellular localization before it is translated.

Several cell types employ RNA localization to achieve high local concentrations of proteins (Hazelrigg, 1998). β-actin mRNA has been observed to be rapidly localized into processes and growth cones of cerebrocortical neurons as RNA granules (Bassell et al., 1998). Granules containing MBP mRNA have been detected in the processes and myelin compartment of oligodendrocytes (Ainger et al., 1997; Boccaccio et al., 1999). In the 3' untranslated region (UTR) of MBP mRNA, a 21-nucleotide sequence has been identified as the RNA transport signal (RTS) and a region between nucleotides 1,130 and 1,473 is the RNA localization region (RLR) (Ainger et al., 1997). The RTS of the MBP mRNA is homologous to the 3' UTR of some mRNAs known to be transported including sequences in the 3' UTR of myelin associated/oligodendrocytic basic protein, the open reading frame (ORF) of glial fibrillary acidic protein and N-type calcium channel and the

5' UTR of nitric oxide synthase (Ainger et al., 1997). Homologous sequences are also found in mRNAs not known to be transported. Sequencing the 5' and 3' UTR of the CNTF mRNA and identifying sites where RNA binding proteins can bind may shed light on its post-transcriptional regulation and provide insights on its mode of transport and even its mechanism of release. In addition, microinjections of labelled CNTF mRNA into Schwann cells may be used to study the subcellular localization of the message.

Leukemia Inhibitory Factor:

Further analysis of LIF promoter

Murine LIF exist as a diffusible form as well as a matrix-bound form, which is generated by using an alternative promoter (Rathjen et al., 1990). The present study has not determined which type of LIF protein is synthesized by non-neuronal cells following nerve injury. *In situ* hybridization and RNase protection assays using probes that recognize exon 1 or the alternate exon 1 could be performed to determine which form of LIF is made in injured nerves. Analysis of the promoters and more distal regulatory elements may be helpful in determining the regulation of this gene.

The initial analysis of the murine LIF promoter showed that 72 bases upstream of the TATA-box are important for expression but a negative regulatory site is present between positions -360 and –249 (Stahl, Gough, 1993). The promoter of human LIF gene has 2 putative c-ets binding sites (Bamberger et al., 1997) and steroid-responsive elements within the 666 bp fragment in the 5'-flanking region (Bamberger et al., 1997, 1998). Cloning and sequencing a bigger fragment upstream of the transcription start site would reveal some of the *cis* elements present in the promoter and distal regulatory regions of the LIF gene.

Monocyte Chemoattractant Protein-1:

Dissecting the intracellular pathway in the induction of MCP-1 by TNF-α

The mechanism of regulation of MCP-1 in Schwann cells by TNF-α could be further studied by using inhibitors that specifically block the activation of either NF-κB or JNK.

The activation of JNK has been previously shown to be blocked by CEP-1347 (KT 7515), which is a semi-synthetic derivative of K-252a (Maroney et al., 1998). The effect of this inhibitor on the TNF-α-induced MCP-1 expression in Schwann cells could be investigated.

NF-κB-dependent gene expression is also regulated through the p38 pathway, which modulates the binding of NF-κB to the *cis* elements (Vanden Berghe et al., 1998). In human mesangial cells, the induction of MCP-1 by IL-1 is mediated by p38 MAP kinase (Rovin et al., 1999). An inhibitor of p38, SB 203580, inhibits the phosphorylation of TF IID (TBP or TATA-binding protein) and thus, blocks its activation and its interaction with p65 NF-κB (Carter et al., 1999). Hence, the importance of the NF-κB pathway in the induction of MCP-1 in Schwann cells could be examined using p38 inhibitors such as SB 203580 and compared to ERK inhibitor PD 0980589 (Vanden Berghe et al., 1998; Carter et al., 1999).

Post-transcriptional regulation of MCP-1

The NF-κB signaling pathway can also be inhibited by glucocorticoids by inducing the production of IκB (Auphan et al., 1995). However, in PDGF-treated rat smooth muscle cells, dexamethsone influenced the expression of MCP-1 by enhancing the degradation of the transcripts (Poon et al., 1999). The dexamethasone-sensitive site, a

224 nucleotide fragment, is located in the 5' end and not in the putative AU-rich region in the 3' untranslated end of the rat MCP-1 mRNA (Poon et al., 1999). Further analysis of the 5' and 3' untranslated regions of the MCP-1 mRNA might reveal more regulatory elements and identify the RNA-binding proteins involved in the regulation of MCP-1 expression.

Investigation on the role of proinflammatory cytokines in the regulation of MCP-1 in injured nerves

Data from the present study suggested that TNF-α is not the only factor involved in the regulation of MCP-1 in injured nerves. Perhaps IL-1 compensated for the absence of TNF-α bioactivity in mice lacking both TNF-α receptors. To determine whether the combination of IL-1 and TNF-α is physiologically important for the induction of MCP-1 in injured nerves, the expression of MCP-1 in injured nerves of mice lacking both IL-1R1 and p55 TNFR (Dailey et al., 1998a) should be investigated.

APPENDIX

Determination of RNA Concentrations

This entire thesis is based on the methodologies which determine the amount of messenger RNA present in the nerve samples as well as in cultured cells. Below are the protocols for total RNA extraction, reverse-transcription polymerase chain reaction (RT-PCR) and RNase protection assay.

Total RNA Extraction

The method was a modification of the protocol described by Chomczynski and Sacchi (1987).

1. Nerves were frozen in liquid nitrogen, pulverized with mortar and pestle and homogenized in a solution containing duanidinium isothiocyanate (Solution C). Cells in culture were washed with DEPC-treated phosphate-buffered saline, lysed with solution C and homogenized.
2. Three microliters of PolyI (10mg/ml) was added as carrier and added 1/10 x volume 2 M NaOAc, pH 4.0 and mixed well.
3. Added 1 x volume water-saturated phenol and mixed well.
4. Added 1/10 x volume $CHCl_3$:isoamyl alcohol (49:1), mixed well and incubated on ice for at least 15 minutes.
5. Spun at 14,000 x g for 10 minutes and collected the aqueous phase.
6. Extracted with Tris-saturated phenol:$CHCl_3$:isoamyl alcohol (25:24:1), spun and collected aqueous phase.

7. Extracted with $CHCl_3$:isoamyl alcohol (24:1), spun and collected aqueous phase.
8. Added 3 x volume 99% EtOH and incubated at -70°C for at least 2 hours.
9. Spun at 14,000 x g for 10 minutes and removed supernatant.
10. Added 300 µl 4M LiCl and incubated on ice for 30 minutes.
11. Spun, removed supernatant and resuspended pellet in 300 µl DEPC-treated water.
12. Added 30 µl 3M NaOAc, pH5.2 and 900 µl 99% EtOH.
13. Incubated at -70°C for at least 2 hours.
14. Spun, discarded supernatant and washed pellet twice with 75% EtOH and once with 99% EtOH.
15. Air dried pellet.
16. Resuspended in 10-20 µl DEPC-treated water.
17. Took 1 µl aliquot and diluted to 100 µl with DEPC-treated water.
18. Measured OD_{260} and OD_{280}.
19. Amount of RNA present in sample calculated as OD_{260} x 40 ug/ml x 100 (dilution factor) x 1 ml/1000 µl.
20. Ran 1 % formamide gel to determine the integrity of the RNA samples.

Reverse-transcriptase Polymerase Chain Reaction

For reverse transcription, 1µg total RNA was initially mixed with 1 µl $pd(N)_6$ (1 µg/µl) and the volume was brought to 15 µl with HPLC-grade water. Samples were then incubated at 65°C for 10 minutes then at 4°C for 10 minutes. The following were added to the samples: 6 µl Gibco's 5X First Strand Buffer, 7 µl 6.25 mM dNTPs, 1 µl 0.1 M

DTT, 1 μl RNase inhibitor and 1 μl MMLV-Reverse Transcriptase. The mixtures were incubated at 37°C for 2 hours then at 95°C for 5 minutes. Reverse transcribed samples were stored at -20°C.

For most of the mouse nerve samples, the total RNA extracted was almost always less than 1 μg. Thus, the RNA samples were divided into three and and one-third of each sample was used for reverse transcription. The rest of the samples were stored at -70°C for later use.

The amplification of a specific cDNA was carried out in 0.6-ml microcentrifuge tubes. The reaction mixture had the following recipe:

10 x PCR Buffer	5.0 μl
50 mM $MgCl_2$	1.5 μl
5 mM dNTPs	3.0 μl
10 μM 5' primer	5.0 μl
10 μM 3' primer	5.0 μl
HPLC water	20-29 μl
Taq polymerase	0.5 μl
cDNA	1-10 μl

Before each cDNA sample was added, 50 μl of mineral oil was layered on each mixture. The different primers used are described in chapters 4 and 5. Mouse CNTF cDNA was amplified for 18 cycles of 95°C for 45 sec, 55°C for 45 sec and 72°C for 2 min. Mouse P_0 and GAPDH cDNAs were amplified for 20 cycles. The rat LIF cDNA was amplified for 21 cycles. The linearity of the cycle numbers used was determined by amplifying the

positive control cDNA samples from 12 to 30 cycles. To visualize and to verify the identity of the amplified products, Southern blotting was performed.

To determine the amount of CNTF mRNA in the distal nerve stump relative to the concentrations present in the contralateral nerve, serial dilutions of the cDNA from the contralateral nerves were performed before the amplification step. cDNA from the contralateral nerves were diluted as follows: 1x→0.5x→0.25x→0.125→0.0625x.

Southern blotting

The PCR products were separated by 1 % agarose electrophoresis, transferred onto a nylon membrane (Hybond-N$^+$, Amersham) and the bound cDNA were fixed on the membrane by UV cross-linking at 0.12 joules. Membranes were incubated in prehybridization solution at 37°C for at least 2 hour. Once the prehybridization solution was discarded and replaced with hybridization solution, the ^{32}P labelled antisense oligonucleotide probe was then added. The probes for CNTF, P_0, GAPDH are described in chapter 4 while the probe for LIF is described in chapter 5. The membranes were hybridized with the labelled probe overnight (at most 18 hours) at 37°C. Blots were washed at least twice with 2 x SSC at room temperature for 45 minutes. After the removal of excess washing solution, blots were wrapped with Saran plastic wrap and exposed to a Kodak film overnight.

RNase Protection Assay

Prepartion of the cRNA probe:

Rat CNTF (Seniuk et al., 1992), LIF and MCP-1 and mouse JE cDNAs were obtained by RT-PCR using Schwann cell and sciatic nerve RNA as templates. Oligonucleotides (18-22 bases long) corresponding to each end of the mature proteins were used as primers. BamH1 restriction sites have been added to the primers. The amplified rat CNTF, LIF and MCP-1 cDNAs were ligated into the pGEM-7Zf(-) plasmid vector while the mouse JE cDNA was ligated into the pGEM-T plasmid vector. The 117-bp BamH1-PstI cyclophilin cDNA subcloned into the Bluescript $KS^{(+)}$ plasmid vector was a gift from Dr. George Kuchel. The CNTF-, LIF-, MCP-1-pGEM-7Zf(-) and JE-pGEM-T plasmids were linearized with PvuII while the cyclophilin-Bluescript $KS^{(+)}$ plasmid was linearized with EcoRI . Linearized plasmids were diluted to a concentration of 250 ng/μl and used as templates for cRNA synthesis.

For cRNA synthesis, the following reagents were mixed at room temperature: 5 μl 5 X Transcription Buffer, 2.5 μl BSA (1 mg/ml), 1 μl 0.75 M DTT, 1 μl 10 mM GTP, 1 μl ATP, 1μl UTP, 10 μl ^{32}P-CTP (400 mCi/mol), 1 μl RNase inhibitor, 0.5 μl DEPC water, 1 μl linearized plasmid, and 1 μl RNA polymerase. The mixture was incubated at 40°C for 2 hours. To remove the plasmid template, 10 units of RNase-free DNase I was added and the mixture was incubated at 37°C for 15 minutes. The probe was purified by phenol/chloroform extraction followed by the use of the Amicon Microcon-50 spin column and by ethanol precipitation. The pelleted probe was resuspended in 100 μl hybridization buffer.

Three micrograms of RNA sample and 10^5 cpm of probe were heated at 55°C and hybridized at 50°C overnight. RNase digestion was performed at 37°C for 1 hour using a combination of RNase T1 (300U/ml) and RNase A (10 μg/ml). Undigested hybridized RNAs were separated by 4 % acrylamide-urea electrophoresis. Gels were dried and exposed to a Kodak film overnight.

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