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Long-term fate of non-neuronal cells in denervated nerve stumps

by

SIJUN YOU ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

Division of Neuroscience

Edmonton, Alberta

Fall 1995

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: SIJUN YOU

TITLE OF THESIS: LONG-TERM FATE OF NON-NEURONAL CELLS IN DENERVATED NERVE STUMPS

DEGREE: MASTER OF SCIENCE

YEAR THIS DEGREE GRANTED: 1995

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ABSTRACT

After peripheral nerve injury, non-neuronal cells including Schwann cells in the distal nerve stumps alter their gene expression in response to the injury. This study uses immunocytochemistry and *in situ* hybridization to examine the expression of low affinity NGF receptor (p75) in the long-term denervated (1-12 months) rat sciatic nerves and to test the possibility that the early upregulation of regeneration associated genes (RAGs) following nerve injury is not maintained when denervation is prolonged.

Quantitative analysis of *in situ* hybridization experiments and semi-quantitative analysis of its protein product revealed that the early rapid increase in expression of p75 mRNA and protein is not maintained in long-term denervated nerve stumps. After they reach their peak within 1 month, the mRNA levels decline exponentially to reach background levels at 4 months after nerve transection and protein declines with the same time constant to background levels by 6 months. Double immunocytochemistry for p75 and S100 revealed that p75 immunoreactivity is confined to the Schwann cells. The characteristic cytoplasmic marker of Schwann cells, S100, is also downregulated if denervation is prolonged for > 6 months. Electron microscopic evidence of surviving Schwann cells indicates that the decline in expression of RAGs in long-term denervated stumps is due to atrophy of non-neuronal cells.

ACKNOWLEDGEMENT

I would like firstly to express thanks to my Supervisor, Dr. Tessa Gordon, for her enthusiasm and guidance during the extended duration of my research. None of this work would have been possible if not for the technical help and companionship of everyone who works in Dr Gordon's laboratory. I would like to express my gratitude to Dr. Theodor Petrov and Mr. Neil Tyreman for their help and advice during the preparation of this manuscript. The technical expertise of Drs. Teresa Krukoff and Freda Miller is greatly appreciated.

Lastly, my strongest gratitude is extended to my parents and my family for their constant love and support.

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INTRODUCTION

1.1. Regeneration in the PNS

It has long been known that neurons in the peripheral nervous system (PNS) can regenerate following injury in contrast to neurons in the central nervous system (CNS) of higher vertebrates (Cajal, 1928; Guth, 1956; 1975; Aguayo, 1985). The peripheral nerve but not the central nerve environment provides a permissive growth environment for regeneration of both central and peripheral nerves (Cajal, 1928; Aguayo et al., 1981; Politis, et al., 1982; Scaravilli, 1984; Bunge and Hopkins, 1990; Dobretsov et al., 1994). CNS injured neurons are capable of extensive axonal growth through peripheral nerve grafts and of establishing synaptic connections (David and Aguayo, 1981; Benfey and Aguayo, 1982; Munz et al., 1985; Bray et al., 1987; Scalia and Roca, 1992). These results suggest that axonal regeneration does not depend on the location of the cell body but rather is a function of the environment that surrounds the injured axon. Successful nerve regeneration requires the survival of injured neurons and their synthesis of sufficient materials for axonal sprouting and elongation (Graftstein and Mcquarrie, 1978) as well as the appropriate trophic and substrate environment for the regenerating nerves provided by non-neuronal cells in the nerve stumps distal to

the injury (Cajal, 1928; Aguayo, 1985).

There are differences in the morphological and biochemical responses of CNS and PNS neurons to axonal injury (Barron, 1983; Misantone, et al. 1984; Schwartz, 1987; Tetzlaff, et al., 1991). Nevertheless, many in vivo and in vitro studies indicate that the difference in the non-neuronal cellular environment is a critical factor in differentiating the contrasting regenerative capacities of CNS and PNS neurons since replacement or alteration of environment of non-neuronal cells appears to be the major factor in influencing the regenerative capacity of central neurons (Aguayo, et al., 1981; Aguayo, 1985; Schwab, 1990; Guenard et al., 1994).

1.2. The purpose of this study

Despite the permissive growth environment in the PNS, functional recovery after surgical repair of injured peripheral nerves is often disappointing (Sunderland, 1978; Terzis and Smith, 1990). This is true particularly when nerves regenerate over long distances and/or long delays between injury and target reinnervation. When target reinnervation was experimentally delayed in rat hind limbs, prolonged sheath denervation severely reduced the number of motor nerves which regenerated successfully to reinnervate the denervated muscles (Fu and Gordon, 1995b).

Poor regeneration after prolonged denervation has been attributed to deterioration of distal nerves (Gutmann and Young, 1944; Politis et al., 1982a,b; Fu

and Gordon, 1995b) and denervated target organs (Gutmann, 1948; Irinchev et al., 1990) although the nature of this deterioration is not well understood. The experimental evidence indicates that the deterioration of the distal stumps rather than the inability of denervated muscles to recover from denervation atrophy may account for the reduced regenerative capacity (Fu and Gordon, 1995b). One possibility is that the non-neuronal cells within the distal stumps cannot maintain their expression of regenerating associated genes (RAGs). These genes include nerve growth factors and their receptors, [eg. nerve growth factor (NGF), brain-derived growth factor (BDGF) and their low affinity receptor (P75)], cell adhesion molecules (CAMs) (eg. N-CAM and L1) and extracellular matrix (ECM) components (eg. laminin and tenascin) which are upregulated after nerve denervation and downregulated when regeneration occurs (Richardson and Ebendal, 1982; Hansson et al., 1986; Heumann et al., 1987a; Raivich and Kreutzberg, 1987; Martin and Timpl, 1987; Martini and Schachner, 1988; Kanje et al., 1989; Acheson et al., 1991a; Chiquet and Wehrle-Haller, 1994; Lettle et al., 1995).

To test the possibility that the expression of RAGs cannot be maintained in the distal non-neuronal cells after long-term denervation, the present work uses immunocytochemistry and *in situ* hybridization to determine whether expression of p75 is maintained by non-neuronal cells when regeneration is prevented. We have chosen to study the expression and location of p75 protein in denervated nerve stumps as an indicator of response of non-neuronal cells to long-term denervation. The objective is to elucidate the cellular basis for poor regeneration

in long-term denervated nerve stumps.

P75 was chosen for several reasons. It is upregulated after denervation and downregulated when nerve regenerates (Taniuchi et al., 1988; Jessen et al., 1994). P75 is a sensitive indicator of the response of Schwann cells to axon injury (Kerkhoff et al., 1991; Robertson et al., 1995). In vitro experiments have shown that p75 binding of NGF promotes the migration of Schwann cells (Anton et al., 1994). Schwann cell migration in vivo plays an important role in nerve regeneration since the regeneration is impaired if Schwann cells are prevented from dividing and migrating following nerve injury (Hall, 1989). P75 is a receptor which can sequester all members of the neurotrophin family, including NGF, BDNF and neurotrophin-3 (NT-3), and thereby may provide access of the neurotrophins to regenerating axons (Ebendal, 1992). P75 may be required for the initiation of myelination of regenerated axons (Fan and Gelman, 1992). Since Schwann cells express both p75 receptor and neurotrophins, p75 may also be involved in autocrine regulation of the functions of Schwann cells (Schecterson and Bothwell, 1992).

This study will determine whether the high level of p75 expression in the distal nerve stump at one week after nerve injury is maintained when regeneration is prevented for periods of up to 1 year.

1.3. Background

1.3.1. Poor functional recovery after prolonged injury

Clinical evidence showed that the functional recovery of proximal muscles is often better and more complete than those muscles in the distal part of the limb after nerve injury (Sunderland, 1978). The explanation is that the regenerating axons which reach the distal muscles have a greater distance to cover, so that their reinnevation is greatly delayed. The reasons for poor functional recovery after delayed repair are still not well understood. Three anatomical structures must be considered as potentially contributing to poor functional recovery: axotomized neurons, denervated nerve sheaths and denervated muscles.

1.3.1.1. Axotomized neurons

After peripheral nerve injury, the injured neurons undergo a variety of anatomical changes and modifications in gene expression and cellular metabolism in response the injury (Fawcett and Keynes, 1990). They down-regulate the expression of neurofilament protein which accounts, at least in part, for the decline in nerve fiber diameter and conduction velocity of proximal stump axons (Hoffman et al., 1984, 1987; Tetzlaff et al., 1988, 1991; Gordon et al., 1991) and neurotransmitters (Grafstein and McQuarrie, 1978) and up-regulate growth associated proteins (GAPs), such as GAP-43, and cytoskeletal proteins including tubulin and actin (Hoffman et al., 1989; Miller et al., 1989; Tetzlaff et al., 1991). Thus molecular changes in neurons appear to revert the neuron from a transmitting to a growing mode (Grafstein and McQuarrie, 1978; Gordon, 1983).

Although regenerating axons have the capacity to send axonal sprouts into

the distal nerve stump after prolonged axotomy (Holmes and Young, 1942), recent experiments which independently controlled the duration of axotomy of the neurons, distal nerve sheath and muscle denervation, showed that number of regenerating nerves which successfully reinnervate the muscles decreases with the duration of axotomy to level off at 30% by 6 months (Raji, 1994; Fu and Gordon, 1995a). In these experiments, long-term axotomized posterior tibial nerves regenerated into a freshly denervated common peroneal (CP) nerve or nerve graft to reinnervate the freshly denervated tibialis anterior (TA) muscle (Raji, 1994; Fu and Gordon, 1995a).

Thus, long-term axotomy is an important contributing factor to poor functional recovery. The reason for the reduction in the number of regenerating axons in prolonged axotomized neurons is not well understood. It may due to a decrease in the expression of GAPs and cytoskeletal proteins such as tubulin in axotomized neurons after prolonged axotomy. Experiments are currently in process in our laboratory to investigate this possibility (You unpublished; Cassar et al., 1993; Cassar and Tetzlaff, 1993).

1.3.1.2. Denervated nerve sheaths and muscles

When freshly cut posterior nerves were sutured to the CP nerve to reinnervate long-term denervated TA muscles, the number of regenerating tibial axons which succeeded in making functional nerve-muscle connections fell to less than 10% of that when the posterior tibial axons regenerated through a fresh CP

nerve stump (Fu and Gordon, 1995a,b). Although neurites can grow and branch directly on denervated muscle in vitro (Covault et al., 1987), the number of regenerating axons are dramatically reduced when regenerating nerves are forced to grow into muscle outside of the intramuscular nerve sheaths (Fu and Gordon, 1995b). These data indicate that the nerve sheaths are a superior substrate and provide more ideal trophic support for regenerating nerves and that deterioration of the nerve sheaths is a major factor which limits the functional recovery after prolonged injuries (Fu and Gorden, 1995b).

1.3.2. Early response of non-neural cells in the denervated nerve sheaths

1.3.2.1. Macrophages

Following peripheral nerve injury, the distal nerve stumps undergo Wallerian degeneration which involves macrophage invasion, axonal fragmentation, removal of axon and myelin debris, Schwann cell and other non-neuronal cell proliferation. Several studies indicate that macrophage invasion is important for the removal of axon and myelin debris and nerve regeneration (Perry et al, 1987; Stoll et al., 1989a). If macrophage invasion is slow or the number of invading macrophages is low during the acute stage of degeneration, as seen in the CNS after injury, or the slowed degeneration of the distal stumps of cut peripheral nerves in C57BL/Ola mice, the removal of myelin debris and the degeneration are delayed with the result that regeneration is impaired (Perry et al., 1987; Stoll et al., 1989b; Hall, 1989; Brown et al., 1992). It has been suggested that the dramatic difference

in the rate of clearance of debris from CNS as compared to the PNS may account for the difference in the success of regeneration since peripheral nerve regeneration does not occur when myelinated axons are present in the distal stump (Perry et al., 1993; Brown et al., 1994). The poor regeneration in the CNS may be related to the slow removal of myelin debris which present myelin protein inhibitors for axonal elongation (Schwab, 1990; Perry et al., 1993).

Macrophages mediated phagocytosis of myelin debris involves a galactose-specific lectin MAC-2. In vivo, both macrophages and Schwann cells in a degenerating nerve display MAC-2 on their surface and in their cytoplasm. There is a strong positive correlation between levels of MAC-2 expression and the extent of myelin destruction by phagocytosis (Reichert et al., 1994). In vivo, the failure to degenerate was associated with deficient MAC-2 production. In vitro, degeneration was associated with MAC-2 production (Reichert et al., 1994).

Macrophages not only phagocytose myelin debris during degeneration, but also secrete a variety of factors which are potentially important for regeneration. In C57BL/ola mice, recruitment of macrophages was much lower, except at the actual lesion site; levels of mRNA for both NGF and its low affinity receptor, p75, were raised only slightly above normal as compared with the normal mice. Sensory axon regeneration is substantially impaired since this regeneration is much more dependent on NGF (Brown et al., 1991; 1994). There is evidence that macrophages affect the synthesis of NGF by secreting interleukin-1 (Lindholm et al., 1987; Guenard et al., 1991; Rotshenker et al., 1992).

Macrophages may stimulate Schwann cell proliferation. The period of recruitment of the macrophages in the peripheral nerve is before and during the period of maximal proliferation of the Schwann cells (Perry et al., 1987). In vitro experiments suggest that macrophages which have digested myelin membrane fragments may release a soluble Schwann cell mitogen and, therefore, stimulate Schwann cell division (Baichwal et al., 1988; Alheim et al., 1991). In addition, exposure of the axon membrane by removing myelin is a crucial mitogenic signal in addition to the by release of growth factors from the exposed axon, including transforming growth factor (TGF) and fibroblast growth factor (FGF), which stimulate Schwann cell proliferation (Bunge et al., 1990; Divis and Stroobant, 1990; Elde et al., 1991; Bolin and Shooter, 1993; Bunge, 1993; Einheber et al., 1995).

Macrophages may also participate in lipid transport during regeneration and remyelination by expressing apolipoprotein E (Apo-E) (Ignatius et al., 1986; Boyles et al., 1989). After nerve injury, macrophages produce large quantities of Apo-E distal to the injury site. During regeneration, the axon tips and Schwann cells contain highly concentrated low density lipoprotein (LDL) receptors. The Apo-E binds to these receptors and may play a key role in myelin-cholesterol reutilization during nerve repair (Muller et al., 1987; Boyles et al., 1989).

Moreover, there is evidence that macrophages play a role in adhesive processes in the injured spinal cord, and thus may also affect axonal regrowth after spinal cord injury (Frisen et al., 1994).

1.3.2.2. Schwann cells

The majority of non-neuronal cells in the PNS are Schwann cells which surround the axons and form the myelin sheath. In normal adult sciatic nerve of rats, Schwann cells comprise 70%-80% of the total number of non-neuronal cells (Salonen et al., 1988). Schwann cells carry out different functions at various stages of their development (Bunge, 1993). For instance, during development, Schwann cells proliferate and express growth factors including NGF, BDNF, platelet derived growth factor (PDGF), and insulin-like growth factors (IGF) (Richardson and Ebendal, 1982; Hansson et al., 1986; Heumann et al., 1987a; Raivich and Kreutzberg, 1987; Kanje et al., 1989; Acheson et al., 1991a). They also express CAMs including N-CAM and L1 (Nieke and Schachner, 1985; Martini and Schachner, 1986; Mirsky et al., 1986), as well as ECM proteins including laminin and tenascin (Martin and Timpl, 1987; Chiquet and Wehrle-Haller, 1994; Lettle et al., 1995) to support nerve growth. When nerves reach their targets, the Schwann cells are induced to form the myelin sheaths around the larger axons (Mirsky and Jessen, 1990) and become quiescent once the myelination has been established (Brown and Asbury, 1981).

Following nerve injury, Schwann cells dedifferentiate, proliferate and upregulate the expression of the growth associated proteins but downregulate the expression of proteins associated with myelin, including Po and myelin basic protein (MBP), in response to the injury (Pellegrino and Spencer, 1985; Martini and Schachner, 1986; Salonen et al., 1988; Welcher et al., 1991; Toma et al., 1992;

Reynolds and Woolf, 1993; Wu et al., 1994). These molecular changes have been associated with regeneration because their expressions are closly associated with nerve degeneration and regeneration. Numerous studies using grafts or artificial chambers or tubes demonstrate that the Schwann cells are a critical cellular component for nerve regeneration (Fawcett and Keynes, 1990). If the Schwann cells in the graft were previously killed by a repeated freezing/thawing process (acellular graft), the regeneration was impaired (Hall, 1986; Berry et al., 1988; Nadim et al.,1990; Paino and Bunge, 1991; Dezawa and Nagano, 1993). However, if the artificial chamber or tube was implanted with Schwann cells or the nerve extracts, the axons grew longer and the rate of growth was faster enhancing regeneration (Politis et al., 1982a; Jenq and Coggeshall, 1986; Guenard et al., 1992; Maeda, et al., 1993; Kim et al., 1994).

1.3.2.3. Fibroblasts

Following nerve injury, fibroblasts in the distal nerve stumps also proliferate and synthesize growth factors, such as NGF (Heumann et al., 1987a; Salonen et al., 1988; Hengerer et al., 1990; Matsuoka et al., 1991; Liu et al., 1995). It has been shown that fibroblasts promote Schwann cell basal lamina deposition and elongation in the absence of neurons in vitro. Therefore, it is proposed that fibroblast stimulation of Schwann cell ECM deposition occurs in normal development in the presence of axons and in their absence after peripheral nerve injury (Obremski et al., 1993). Fibroblasts also plays a role in restructuring of the

distal nerve stumps after nerve injury. Two weeks after nerve injury, it was seen that the proliferating endoneurial fibroblast-like cells reformed endoneurium in the distal nerve stumps (Popovic et al., 1994). In an autografting study, new perineural tissue was formed by endoneurial fibroblasts from the inside of the funiculus. When nerve regeneration proceeded, the fibroblasts encircled the regenerating axons and the Schwann cells to form new compartments resulting in a large number of minifascicles of regenerating nerve (Hirasawa et al., 1994).

1.3.3. Changes in expression of growth associated molecules in denervated nerve sheaths

1.3.3.1. Growth factors and their receptors

Within three days after nerve injury, Schwann cells and/or fibroblasts proliferate and upregulate several growth factors (Richardson and Ebendal, 1982; Hansson et al., 1986; Heumann et al., 1987a; Raivich and Kreutzberg, 1987; Kanje et al., 1989; Acheson et al., 1991a; Funakoshi et al., 1993; Schumacher et al., 1993; Glazner, et al., 1994; Liu et al., 1995). The activated Schwann cells also upregulate growth factor receptors including low affinity NGF receptor (p75), epidermal growth factor receptors (EGFR) and receptors for IGF-I (Taniuchi et al., 1988; Toma et al., 1992; Jung-Testas et al., 1994; Liu et al., 1995), as well as downregulate the ciliary neurotrophic factor (CNTF) (Rabinovsky et al., 1992; Sendtner et al., 1992a; Smith et al., 1993).

NGF NGF was the first factor found to be required for neuronal survival. It supports the survival and differentiation of sympathetic and primary sensory neurons (Levi-Montalcini and Angeletti, 1968; Thoenen and Barde, 1980; Yip et al., 1984; Ernsberger and Rohrer, 1988; Jiang and Smith, 1993; Valmier et al., 1993; Elkabes et al., 1994; LoPresti and Scott, 1994). It is thought that NGF is normally produced in the target tissues and is retrogradely transported to cell body where it exerts its action (Raivich and Kreutzberg, 1993; Campenot, 1994). However, recent evidence indicates that neurons also express the NGF (Koliatsos et al., 1993; Zhang et al., 1994). Experiments using compartmented cultures of rat sympathetic neurons have shown that neurite outgrowth is a local response of neurites to NGF (Campenot, 1994).

After peripheral nerve injury, the Schwann cells in the distal nerve stumps upregulate the expression of NGF (Heumann et al., 1987a,b). This increase in NGF is mediated by the expression of the immediate early genes, c-fos and c-jun (Hengerer et al., 1990; Onteniente et al., 1994). Experiments showed that lesion of the sciatic nerve caused a rapid increase in c-fos and c-jun mRNA that was followed by an increase in NGF mRNA about two hours later (Hengerer et al., 1990). In an experiment where fibroblasts of transgenic mice, carrying an exogenous c-fos gene under the control of a metallothionein promoter, showed a rapid increase in exogenous c-fos mRNA by CdCl2. This was followed immediately by an increase in endogenous c-jun mRNA and after a slight delay by an increase in NGF mRNA (Hengerer et al., 1990). The role of the endogenous

NGF in nerve regeneration is not clear. In the C57BL/Ola mouse, which has low levels of NGF synthesis during nerve degeneration, the regeneration of sensory neurons is much retarded (Brown et al., 1991). However, antibodies to NGF do not interfere with the speed of regeneration of injured sensory axons (Diamond et al., 1992).

The positive effect of NGF on nerve growth may derive from its ability to induce the expression of other molecules. In vitro, NGF was shown to increase L1 expression in Schwann cells (Seilheimer and Schachner, 1987). L1 appears on peripheral nerve Schwann cells after axotomy and during nerve regeneration, and plays a role in nerve growth and myelination (see cell adhesion molecules). In cultured adult rat dorsal root ganglion, it was shown that sensory neurons require NGF for synthesis of neuropeptides (Mulderry, 1994). Interestingly, NGF can induce interleukin-1 (IL-1) expression in PC12 pheochromocytoma cells. IL-1 can act as a growth factor for the promotion of glial cell proliferation and, even importantly, IL-1 itself induces the expression of NGF at peripheral nerve injury (Alheim et al., 1991).

BDNF BDNF is a neurotrophic factor which is closely related to NGF. It belongs to a gene family of neurotrophins which also includes neurotrophin 3 (NT-3) and neurotrophin 4/5 (NT-4/5). These factors support the survival of different subpopulations of neurons and have distinct patterns of expression and function during development and nerve regeneration (Ebendal, 1992; LoPresti and Scott,

1994).

In vitro BDNF promotes the maturation and survival of some subtypes of peripheral sensory neurons (Davies et al., 1986; Wright et al., 1992; LoPresti and Scott, 1994). There is good evidence that BDNF prevents the death of spinal cord motoneurons, either during normal development or after axotomy (Oppenheim et al., 1992; Sendtner et al., 1992b; Yan et al., 1992; Koliatsos et al., 1993; Clatterbuck et al., 1994; Friedman, et al., 1995).

After nerve injury, Schwann cell synthesis of BDNF in the distal nerve stumps is increased (Meyer et al., 1992; Funakoshi et al., 1993) and BDNF is believed to be taken up by axons and retrogradely transported to spinal motor neurons where it prevents the retrograde degeneration of axotomized motor neurons (DiStefano et al., 1992; Koliatsos et al., 1993).

Although the synthesis of both NGF and BDNF in Schwann cells increases after nerve injury, their regulation differs (Meyer et al., 1992). After nerve injury, there is a marked and rapid increase in NGF mRNA in the non-neuronal cells in the distal stump of the damaged nerve. The elevation of NGF mRNA levels is related to the immigration of activated macrophages, IL-1 being the most essential mediator of this effect (Lindholm et al., 1987; Guenard et al., 1991; Rotshenker et al., 1992). In contrast, the upregulation of BDNF by transecting of the rat sciatic nerve is much slower. There is a continuous slow increase in BDNF mRNA starting 3 days post-lesion and reaching maximal levels 3-4 week later (Meyer et al., 1992).

CNTF and LIF CNTF and leukaemia inhibitory factor (LIF) are a group of growth factors structurally distinct from the neurotrophins. They are ligands for a multicomponent receptor which is shared with the haemopoetic cytokines (Ip et al., 1992).

Although CNTF was originally identified by its ability to support the survival of chick ciliary ganglionic neurones, it has now been shown to act as both a trophic survival factor and as a differentiation factor (Reynolds and Woolf, 1993). In the PNS, CNTF is localized to myelinating Schwann cells in the adult (Dobrea et al., 1992; Friedman et al., 1992; Sendtner et al., 1994). In vitro, CNTF has been shown to promote survival and differentiation of developing motoneurons (Arakawa et al., 1990; Magal et al., 1991; Thaler et al., 1994). In vivo, infusion of CNTF prevents axotomy-induced cell death of neonatal facial motoneurons for at least 7 days (Sendtner et al., 1990). Inactivation of CNTF by gene targeting experiments results in progressive atrophy and degeneration of motoneurons (Thoenen et al., 1993). This suggests that CNTF plays an essential role as a maintenance factor for motoneurons postnatally.

Following nerve injury, CNTF expression is dramatically decreased in the Schwann cells 7 days after injury (Rabinovsky et al., 1992; Sendtner et al., 1992a; Smith et al., 1993). Retrograde axonal transport of CNTF is increased after nerve injury (Curtis et al., 1993), suggesting that increased transport from intact Schwann cells may have an important role in the survival of neurons after injury and response of neuronal cell bodies during regeneration (Curtis et al., 1993; Thoenen

et al., 1993). Moreover, CNTF increases or induces the expression of p75 receptor in neurons in the CNS of adult rats (Hagg et al., 1992).

LIF is a member of large family of hematopoietic factors. It is found to have function not only in hematopoietic cells but also in the nervous system. Recent studies have shown that LIF can regulate differentiation and survival of both NGF-dependent and NGF-independent sensory neurons (Hendry et al.,1992; Murphy et al., 1993; Thaler et al., 1994). In the DRG of the neonatal rat, LIF also prevents the death of axotomized sensory neurons (Cheema et al., 1994). Experiments in which ^{125}I-LIF was injected into the footpads and gastrocnemius muscles of newborn and adult mice showed that LIF can specifically bind to and be transported by sensory neurons of DRG and sympathetic neurons (Hendry et al.,1992; Ure and Campenot, 1994). Following peripheral nerve injury, retrograde axonal transport of LIF is increased correlating with increased LIF expression in distal nerve (Hendry et al.,1992; Curtis et al., 1994).

Other growth factors The PDGFs are a group of three dimeric proteins including two homodimers of PDGF-AA and PDGF-BB, and one heterodimer of PDGF-AB (Heldin, 1992). They act on two relatively selective receptors: α-type receptor which binds PDGF-AA and PDGF-AB, and ß-type receptor which binds PDGF-BB (Heldin et al., 1988). PDGFs are powerful fibroblast mitogens (Heldin, 1992), however, PDGF-BB and PDGF-AB also have been shown to stimulate Schwann cell

proliferation (Hardy et al., 1992; Schubert, 1992; Watabe et al., 1994). In the neonatal rats, Schwann cells express high levels of PDGF, which subsequently decline during the first postnatal weeks (Eccleston et al., 1993). After nerve injury, Schwann cells upregulate the expression of PDGF (Raivich and Kreutzberg, 1987). These data suggested that PDGF might play a role in the development of the PNS and in nerve regeneration.

IGFs including IGF-1 and IGF-2 stimulate neurite formation and regeneration of sensory nerve axons in vitro (Ishii et al., 1989; Edbladh et al., 1994). Following sciatic nerve injury, IGFs promote neurite outgrowth in vivo (Kanje et al., 1989; Near et al., 1992; Glazner et al., 1994; Ishii and Lupien, 1995). Schwann cells of regenerating nerves show IGF-1 immunoreactivity, and local application of antibodies against IGF-1 and/or IGF-2 strongly inhibits axonal regeneration (Hannson et al., 1986; Kanje et al., 1989; Near et al., 1992). These data suggested that IGFs are important regulatory factors in nerve regeneration. In addition, IGFs also promote the proliferation of Schwann cells and are involved in motor axon sprouting in skeletal muscle (Sjoberg and Kanje, 1989; Caroni and Grandes, 1990).

Nerve Growth Factor Receptors The neurotrophins act via their receptors. There are two types of neurotrophin receptors (Chao, 1992; Ebendal, 1992). One is the family of high affinity NGF receptors, also called trk receptors, including trkA, TrkB and TrkC. The other is the low affinity NGF receptor, also called the p75 receptor.

Trks It is known that trk function is essential for neurotrophin signal transduction and individual trk receptor subtypes bind preferentially with different neurotrophins. For instance, NGF binds to the trkA. BDNF and NT-3 preferentially bind to and activate trkB and trkC, respectively (Ebendal, 1992). In addition, trkB is also shown to bind NT-4/NT-5 (Berkemeier et al., 1991; Klein et al., 1992). Neurotrophin binding to the trk receptors results in activation of intrinsic tyrosine kinase activity involving the phosphorylation of intracellular proteins on tyrosine residues. These signals are then propagated to other messengers, including PLC-τ1, SHC and PI-3 kinase. These proteins are involved in stimulating the formation of various second messenger molecules and activating the Ras signal transduction pathway (Kaplan and Stephens, 1994).

P75 The p75 receptor binds all the members of neurotrophins (Chao, 1992; Ebendal, 1992). This transmembrane receptor does not possess a tyrosine kinase domain (Johnson et al., 1986; Radeke et al., 1987; Chao and Hempstead, 1995). The precise roles of the p75 receptor are still not clear. Membrane fusion and gene transfer experiments showed that high-affinity NGF binding requires coexpression of both trk and p75 receptors (Hempstead et al., 1989; 1990; 1991). Other experimental results indicated the trk receptor can function independently of p75 receptor (Weskamp and Reichardt, 1991; Ibanez et al., 1992). Therefore, p75 may either act as a common subunit in a neurotrophin receptor complex with trk family members, or the p75 and the trk receptors act by independent mechanisms to

mediate biological actions of each neurotrophin (Chao, 1994). Recently, in vitro experiment showed that p75 receptor mediated different functions depending on the state of sensory neuron development: it is required, probably with trkA, to mediate neuronal survival in embryonic day 12-15 mice in the presence of NGF, but it acts as a constitutive death signal in the embryonic day 19 - postnatal day 2 in the absence of NGF since the lowering the level of p75 expression with antisense oligonucleotides increases the survival of sensory neurons in this period (Barrett and Bartlett, 1994). In the p75-deficient trigeminal sensory neurons in embryos with a null mutation in the p75 gene, P75 enhances the sensitivity of NGF-dependent cutaneous sensory neurons to NGF (Davies et al., 1994). This may also explain, at least in part, the cutaneous sensory abnormalities of mice homozygous for the p75 mutation (Lee, et al., 1992).

After peripheral nerve injury, the Schwann cells in the distal stumps upregulate the expression of p75 receptor (Taniuchi et al., 1986, 1988; Stoll et al., 1993). Although the function of the p75 receptor in the denervated distal nerve sheaths is not clear, it is known that all the Schwann cells which are associated with injured axons, regardless of their NGF dependency, express p75 receptor (Johnson et al., 1988; Taniuchi, et al., 1988). Since this transmembrane glycoprotein does not possess a tyrosine kinase domain (Johnson et al.,1986; Radeke et al.,1987) it is thought that this receptor is not involved in signal transduction. Since p75 receptor binds all neurotrophins including NGF and BDNF and the neurotrophins can be retrogradely transported to cell bodies after nerve

injury (Ebendal 1992; Raivich et al.,1991; Koliatsos et al., 1993), the presence of p75 receptor suggests a role in supporting regeneration of both sensory and motor neurons. For example, it has been proposed that p75 on Schwann cells binds to and thus concentrates NGF (provided in part by Schwann cells) and may thereby promote regeneration of NGF-dependent neurons (Johnson et al.,1988; Taniuchi et al.,1988). This notion is supported by results obtained using in vitro co-culture of olfactory epithelium explants with adult rat olfactory bulb containing both p75 positive cells and p75 negative cells. The neurites from the explants grow preferentially over p75 positive cells (Ramon et al., 1993).

Recent data indicate that BDNF which also binds to p75 has survival-promoting effects on motor neurons in vivo (Yan et al., 1992; Koliatsos et al., 1993; Friedman et al.,1995). After nerve injury, BDNF is increased in Schwann cells (Acheson 1991; Meyer et al., 1992). It is believed to be taken up by axons and transported retrogradely to spinal motor neurons which express the trkB receptor, a receptor involved in BDNF signal transduction (Koliatsos et al., 1993). BDNF thus may prevent the retrograde degeneration of axotomized neonatal motor neurons and also provides neurotrophic influences on injured adult motor neurons (Koliatsos et al., 1993; Friedman et al., 1995). Therefore, the elevated p75 on the degenerated Schwann cells' surfaces may also serve as a growth factor (such as BDNF) laden substratum to aid regenerating NGF-independent neurons, such as motoneurons.

In addition, it has been suggested by Feinstein and Larhammar (1991) that

the helix C of the p75 receptor may interact with G-protein and thus may facilitate binding to a cytoplasmic protein. This motif (helix C) was identified in the cytoplasmic domains of a number of other growth factor receptors, suggesting an important role in signal transduction. Therefore, the p75 receptor expressed on Schwann cells may also be involved in autocrine regulation of the functions of Schwann cells (Schecterson and Bothwell, 1992) since Schwann cells express both neurotrophins and p75 receptor.

A more recent study has suggested that the elevated levels of NGF following nerve injury binds p75 on Schwann cell surfaces and promotes Schwann cell migration (Anton et al, 1994). Schwann cell migration may play a vital role in axonal growth and guidance since the regeneration of injured axons is markedly reduced when Schwann cells are prevented from dividing and migrating following nerve injury (Hall, 1989; Son and Thompson, 1995).

It has also been proposed that p75 is necessary for the initiation of myelination (Fan and Gelman, 1992), a process that occurs during reinnervation.

Epidermal growth factor receptor (EGFr) EGFr is a transmembrane protein. Not only is it bound by EGF but also by the other factors which belong to EGF family including transforming growth factor-α (TGF-α). Binding to the ligands results in receptor activation of tyrosine kinase (Carpent, 1987; Sasaoka et al., 1995). An experiment has shown that EGFr may play an important role in glial differentiation (Sang et al., 1995). Toma et al (1992) reported that following nerve injury, both

Schwann cells and fibroblast synthesize EGFr. The function of EGFr in the PNS is not clear, however, experiments have shown that in the CNS, EGF treatment of non-neuronal cells increases DNA synthesis (Huff et al., 1990).

1.3.3.2. Cell adhesion molecules

CAMs are a group of membrane glycoproteins which are involved in interaction between cells or cell and substrate by homophilic (such as N-CAM) and/or heterophilic (such as L1) mechanisms.

Expression of CAMs by Schwann cells are recognised to be important in regeneration. When regenerating axons grew into the distal stump of the injured nerve, regrowing axons made contacts with L1- and N-CAM- positive Schwann cells (Martini and Schachner, 1988). Interactions between CAMs on neurons and Schwann cells are critical because antibodies to CAMs influence the neurite outgrowth (Bixby et al., 1988; Martini, 1994). It has now been shown that growth of axons on Schwann cell surfaces can be ascribed to at least three classes of cell adhesion molecules: calcium-independent CAMs, such as N-CAM and L1, calcium-dependent CAMs, such as N-cadherin, and various integrins which interact with the many components in ECM (Bixby et al., 1988).

Calcium-independent CAMs N-CAM and L1/Ng-CAM belong to the calcium-independent adhesion molecule family. They are thought to mediate adhesion between neurons, neuron and glia, as well as glia and glia (Grumet et al., 1984;

Hoffman et al., 1986; Grumet, 1992; Martini et al., 1994). In the nervous system, L1 is generally found coexpressed with N-CAM and shares certain properties with it. L1 and N-CAM are involved in axonal fasciculation, initial axon-Schwann cell interaction, and onset of myelination. In contrast to L1, N-CAM may be further involved in the maintenance of compact myelin, axon-myelin apposition of larger diameter axons, and in influencing the branching pattern of the nerves when they innervate the muscles (Martini and Schachner, 1986; Landmesser, 1994).

Expression of N-CAM and L1 on Schwann cells and neurons has been associated with axonal growth during development and regeneration. In the development of the sciatic nerve, N-CAM and L1 were detectable on all axons and Schwann cells at embryonic day 17 and early postnatal animals. However, when myelination starts, both of these are dramatically down-regulated, but remain present on non-myelinated Schwann cells and the small axons ensheathed by non-myelinated Schwann cells. When the myelinating Schwann cells have made one and half turns around the axons, L1 becomes undetectable on both axons and Schwann cells, whereas N-CAM decreases to a very low level and becomes confined to the axon-Schwann cell interface (Nieke and Schachner, 1985; Martini and Schachner, 1986; Mirsky et al., 1986).

Following adult rat sciatic nerve injury, and an initial fall to 50%, N-CAM mRNA level increased to 3 times normal levels and L1 mRNA level to 2 times in the distal stump 3 weeks after injury (Tacke and Martini, 1990). The upregulation of N-CAM and L1 mRNAs in the distal nerve stump parallels the expression of N-CAM

and L1 proteins in Schwann cells of injured nerves (Nieke and Schachner, 1985; Martini and Schachner, 1988). In vitro experiments have shown that cultured Schwann cells localize L1 and N-CAM selectively at cell contact sites and may thereby stabilize their attachment to the neighbouring cellular partners (Martini et al., 1994).

The re-appearance of N-CAM and L1 on denervated Schwann cells is possibly a prerequisite for significant axonal regrowth after nerve injury (Legenaur and Lemmon, 1987). In experiments where purified Schwann cells and neurons were co-cultured, L1 antibodies blocked neurite outgrowth and Schwann cell myelination (Bixby et al., 1988; Wood et al., 1990). In contrast to L1-specific antibodies, N-CAM antibodies showed only a partial blockade of Schwann cell myelination (Seilheimer et al., 1989). Applying specific antibodies against N-CAM to the injured sciatic nerve showed that the antibodies disrupt functional recovery in injured nerves (Remsen et al., 1990). These data implicate N-CAM interactions between Schwann cells and axons as significant components of nerve regeneration, whereas L1 plays a key role in Schwann cell myelination.

In addition, N-CAM and L1 may act in concert since it has been shown that L1 can bind to N-CAM and form a heterodimeric complex (Kadmon et al., 1990a,b).

Polysialic acid (PSA) moieties on N-CAM also appear to regulate the cell-cell and cell-substrate interactions, thereby influencing the axonal fasciculation

(Rutishauser et al., 1988; Acheson et al., 1991). If PSA is removed from N-CAM with a specific neuraminidase (endo-N), the spinal cord axons defasciculate on laminin-substrate (Acheson et al., 1991). Transition from fasciculated to defasciculated growth seems necessary to produce the normal pattern of innervation, so PSA on N-CAM may play an important role in determining the pattern of neurite outgrowth (Landmesser et al., 1990; Acheson et al., 1991b; Rutishauser and Landmesser, 1991; Tang et al., 1992, 1994; Landmesser, 1994).

L2/HNK-1 is a common carbohydrate epitope associated with a number of cell adhesion molecules, including L1, N-CAM, peripheral major myelin protein P0, myelin associated protein (MAG), J1 and integrins (Nieke and Schachner, 1985; Kruse et al., 1985; Martini and Schachner, 1986; Pesheva et al., 1987; Bollensen and Schachner, 1987; Martini et al., 1988; Schachner, 1989). This carbohydrate acts as ligand in neural cell adhesion to laminin (Kunemund et al., 1988; Hall et al., 1993) and is also involved in myelin protein P0 homophilic binding mechanism (Griffith et al., 1992). In developing sciatic nerves, L2/HNK-1 is not detectable. It appears 2 weeks after birth and is associated with the outer profiles of thick myelin sheets. It is also seen in adult sciatic nerves. After nerve transection, the L2/HNK-1 epitope remained undetectable until the transected nerve had returned to its normal state of myelination (Nieke and Schachner, 1985). L2/HNK-1 is found only on motor axon-associated Schwann cells and promotes outgrowth of cultured motor but not sensory neurons (Brushart, 1988; Brushart et al., 1992). It may

provide regenerating motor axons with a selective advantage over others resulting in appropriate reinnervation of motor pathways (Brushart, 1993; Martini et al., 1994b).

Calcium-depandent CAMs The cell adhesion molecule N-cadherin is the predominant mediator of calcium-dependent adhesion in the nervous system (Takeichi, 1988). Its signalling involves cell type-specific calcium changes in responding cells and it can cause calcium increases in neuronal growth cones (Bixby et al., 1994).

Investigations using antibodies to block N-cadherin function or transfection of the N-cadherin gene into heterologous cell lines have provided evidence that N-cadherin, alone or in combination with other molecules, can participate in the induction of neurite extension (Bixby et al., 1987; Bixby et al., 1988; Matsunaga et al., 1988). In vitro, N-cadherin has been shown to be an extremely potent factor for the promotion of neurite outgrowth (Bixby and Zhang 1990).

In sciatic nerve, N-cadherin is widely distributed on the surface of myelinated fibres and on myelinating Schwann cells. At the ultrastructural level, this molecule was detected at the inside, at the surface and in the basal lamina of Schwann cells and also associated with endoneurial collagen. These distributions suggest a role of N-cadherin in the structuring and stabilization of the myelin sheaths. After nerve injury, N-cadherin continued to be expressed by proliferating Schwann cells in the distal stump providing a substratum for regenerating axons

(Cifuente-Diaz et al., 1994).

It has also been shown that N-cadherin mediates the interactions between Schwann cells and between Schwann cells and neurites (Letourneau et al., 1991). In cultured chick dorsal root ganglia and sciatic nerves, Schwann cells usually remained extended, and the growth cones often advanced onto the Schwann cell surface if culture medium contained normal concentration of calcium. However, in a low calcium medium where the calcium-dependent cadherins are inactive, the withdrawal of the Schwann cell's processes after contact with a growth cone often occurred. In addition, when motile leading margins of two Schwann cells touched in medium with normal calcium concentration, they often formed stable areas of contact. However, in low calcium medium or the presence of anti-N-cadherin, interacting Schwann cells usually pulled away from each other (Letourneau et al., 1991).

1.3.3.3. Schwann cells' basal lamina and extracellular matrix

Schwann cells are recognised ultrastructurally by the presence of a distinct basal lamina which remains after Wallerian degeneration. Neurites are seen to grow between Schwann cell surface and basal lamina (Ide et al., 1983; Kuffler, 1986; Martini and Schachner, 1988; Ide and Kato, 1990). If the basal lamina of Schwann cells of a graft is denatured by heating in addition to freezing and thawing, regenerating axons will not enter the graft until Schwann cells migrate from the proximal stump into the graft (Sketelj et al., 1989). Once in the graft, the

Schwann cells are used preferentially for contact by growing axons (Sketelj et al., 1989).

It is thought that the basal lamina plays a dual function in nerve regeneration: 1) it provides a physical guidance which leads the regenerating nerves to their targets, and 2) it contains various molecules, such as laminin, fibronectin and tenascin/J1, which support nerve regeneration. Schwann cells secret these molecules to both basal lamina and the ECM of basal lamina (Bunge, 1993). The ensheathment and myelination of Schwann cells are entirely dependent on deposition of this basal lamina (Bunge, 1993). The expression of these molecules by Schwann cells is modulated by axonal signals, as well as the basal lamina components (Bunge and Bunge, 1983; Bunge et al, 1990).

Following adult nerve injury, the proliferating Schwann cells in the distal nerve stumps express these molecules (Salonen et al., 1987; Neuberger and Cornbrook, 1988; Tona et al., 1993; Fruttiger et al., 1995) and support nerve growth (Seckel, 1990; Toyota et al., 1990; Wang et al.,1992; Bailey et al., 1993; Kauppila et al., 1993; Martini, 1994).

Laminin A key component of ECM is a glycoprotein, laminin, which is also a major component of the basal lamina of myelinating and non-myelinating Schwann cells (Martin and Timpl, 1987; Lettle et al., 1995). It is one of the most effective promoters of neurite outgrowth. In vitro experiments have shown that laminin has several important functional roles, including axonal guidance during development

(Sanes, 1989; Jaakkola et al., 1993; Webster, 1993), promotion of the growth of regenerating axons along basal lamina of Schwann cells (Manthorpe et al., 1983; Rogers et al., 1983; Salonen et al., 1987; Seckel, 1990; Toyota et al., 1990; Wang et al.,1992; Bailey et al., 1993; Martini, 1994), and in the elaboration of a myelin sheath by Schwann cells (Bunge et al., 1989; Bunge et al., 1990).

The role of laminin in functional recovery of a peripheral nerve injury was investigated in vivo using electrophysiological and behavioral methods to determine success of rat sciatic nerve regeneration (Kauppila et al., 1993). Using a graft containing laminin, comparing with a control graft containing collagen, it was shown that the laminin graft supported the functional recovery of an injured peripheral nerve, but collagen graft did not. The laminin graft was as effective as neurorrhaphy in promoting regeneration. This result provided the first in vivo evidence for the functional role of laminin in peripheral nerve regeneration. In addition, it suggests that the laminin graft surgery may be a useful method for clinical restoration of injured peripheral nerves (Kauppila et al., 1993).

The effect of laminin on promoting neurite growth is involved in the pathway of protein kinase C (Bixby and Jhabvala, 1990). When protein kinase C is inhibited, the outgrowth of neurite on lamina is inhibited, too.

Fibronectin Fibronectin is an another glycoprotein which is intimately associated with basal lamina. It may mediate cell attachment to other ECM molecules, such as heparin sulphate and collagen (Riggott and Moody, 1987). In vitro, it supports

neurite outgrowth and survival of several types of neurons, by either alone or combination with laminin (Baron-Van Evercooren et al., 1982; Carbonetto et al., 1983; Roggers et al., 1983; Akeson and Warren, 1986; Millaruelo et al., 1988; Lander, 1989; Pesheva et al., 1994). However, the enhancing effect of neurite outgrowth on fibronectin alone is much less than that on laminin or on a substrate of fibronectin/laminin combination (Baron-Van Evercooren et al., 1982; Millaruelo et al., 1988; Rossino et al., 1990). In vivo, when fibronectin is combined with laminin, the two molecules enhance the peripheral nerve regeneration and Schwann cell migration (Willimas and Varon, 1985; Woolley et al., 1990; Bailey et al., 1993; Tong et al., 1994).

After peripheral nerve injury, the expression of fibronectin is increased in the distal nerve stumps and it appears that endoneurial fibroblasts contribute to this elevated fibronectin (Siironen et al., 1992; Mathews and French-Constant, 1995). Endogenous laminin and fibronectin not only regulated the growth of nerve fibers in the early stages of nerve regeneration but exert a positive influence on perineurial cells and macrophages since antibodies blocking the endogenous laminin or fibronectin resulted in reducing the number of regenerated axons in the grafts, the fibroblasts did not recognize empty basal lamina and macrophages closely confronted the naked axons which elongated outside the basal lamina (Wang et al., 1992; Mathews and French-Constant, 1995). Fibronectin also modulates neuronal survival and neurite outgrowth induced by growth factors (Millaruelo et al., 1988; Schwarz et al., 1989; 1990; Rossino et al., 1990).

Tenascin/J1 Tenascin/J1 is suggested to influence peripheral nerve regeneration (Martini, 1994). In developing peripheral nerves of the chick embryo, tenascin/J1 is expressed at a high level. The maximal synthesis of its mRNA peaks at the time period of axonal growth (Chiquet and Wehrle-Haller, 1994). In vitro experiments have shown that tenascin/J1 can support neurite outgrowth from both motor and sensory neurons (Lochter et al., 1991; Taylor et al., 1993; Wehrle-Haller and Chiquet, 1993). The ability to grow neurites on tenascin/J1 is developmentally regulated (Chiquet and Wehrle-Haller, 1994).

Following nerve injury, Schwann cells in the distal stumps strongly express tenascin/J1. It has been shown that the expression of tenascin/J1 is associated with Schwann cell basal lamina along which the regenerating axons grow (Martini et al., 1990). It is thought that this elevated expression is involved in nerve regeneration since the tenascin/J1 upregulation is delayed in C57basal lamina/Wld mice, paralleling the delayed Wallerian degeneration (Fruttiger et al., 1995). Other experiments showed that the regeneration was also delayed in C57basal lamina/Wld mice which was associated with delayed Wallerian degeneration (Brown et al., 1994). Therefore, tenascin/J1 may play a role in nerve regeneration.

Integrins The neurite growth promoting effects of the components of ECM, including laminin, fibronectin and tenasin/J1, are mediated by the interaction with their receptors, the integrins, present on the membranes of growing axons and Schwann cells (Reichardt and Tomaselli, 1991; Lefcort et al., 1992; Jaakkola et al.,

1993; Chiquet and Wehrle-Haller, 1994; Feltri et al., 1994). These receptors are all similar in structure having 2 transmembrane proteins, the α and ß chains. Different receptors contain different permutation of α and ß chains. Each integrin receptor binds to one or more ECM glycoproteins (Darnell et al., 1990) which enable extracellular signals to reach the cytoplasm of the axons and Schwann cells in the PNS.

1.3.4. Reversal of denervation changes in Schwann cells by nerve regeneration

When regenerating axons grow into the distal nerve stump, there is a second proliferation of the Schwann cells in the distal nerve stumps (Reynolds and Woolf, 1993) and a reversal of denervation related gene expression. The Schwann cells downregulate the expression of growth factors and the p75 receptor (Heumann et al., 1987b; Tanuichi, et al., 1988; Jessen and Mirsky, 1992; Glazner et al., 1994; Jessen, et al., 1994), as well as expression of N-CAM and L1 (Martini and Schachner, 1988). Concurrent with this downregulation, they upregulate the myelin associated proteins to ensheath the regenerating axons (Mitchell, et al., 1990; Mirsky and Jessen, 1990). Contact with axons is required for Schwann cell reexpression of myelin proteins (Bunge et al., 1990). Contact with axons is also important for assembling of basal lamina, although it is not nessessary for Schwann cells to secrete the basal lamina proteins (Bunge et al., 1990).

1.3.5. Schwann Cell markers

S100 S100 is a group of low molecular weight (10-12 KD) calcium-binding proteins which is highly conserved among vertebrates (Baudier et al., 1982; Patel et al., 1983; Goto et al., 1988; Fano et al., 1995). Although their function in Schwann cells is not yet clear, S100 is the most used Schwann cell marker.

This protein is a dimer of two subunits α and ß with homologous amino acid sequences (58% identity). Three forms exist in brain: αα, αß, and ßß. The αß and ßß are dominant, but αα is only 3-4% of total S100 in the brain. In the peripheral nerves, intense immunoreactive product of the ß subunit is observed in Schwann cells and satellite cells of the autonomic ganglia, but not in any neurons. Granular immunoreaction product for the α subunit is observed clearly in neurons, but not in Schwann cells and satellite cells (Isobe et al., 1984).

Since the antibody which is most be used reacts to both α and ß subunits, the antibody may also detect the α subunit which is localized in neurons, even though it is present only in small amounts. Neuberger et al (1989) observed that S100 immunoreactivity is localized in outer ring of Schwann cell cytoplasm as well as the inner cytoplasmic ring of myelinating Schwann cells in the cross-section of intact nerve. However, they also observed that S100 is seen in the occasional axon. Recently, availability of a purified antibody against S100ß subunit has provided a powerful tool to distinguish Schwann cells.

Glial fibrillary acidic protein (GFAP) GFAP is a intermediate filament protein which is found in astrocytes in the CNS and nonmyelin-forming Schwann cells in PNS. During development, all the Schwann cells express GFAP, but in adult nerve expression of GFAP is confined in nonmyelin-forming Schwann cells (Mirsky and Jessen, 1992). Therefore, GFAP is often used as a cell marker for glia cells including astrocytes and nonmyelin-forming Schwann cells.

1.3.6. Rationale for the study of expression of RAGs by non-neuronal cells in long-term denervated nerve sheaths

Comparison of nerve regeneration and expression of RAGs in normal and CBL57/Ola mice, as well as antibody perturbation in vivo and in vitro studies have provided important insights into the molecular basis for neural development and regeneration of peripheral nerves. Yet the perplexing issue of poor functional recovery after nerve injuries is still poorly understood. Although the incomplete recovery has been attributed to muscle atrophy (Gutmann, 1948; Irinchev et al., 1990), recent experiments have demonstrated that deterioration of the nerve stump is likely to be the major contributing factor to poor regeneration (Fu and Gordon, 1995b).

Schwann cells, other non-neuronal cells and the many molecules that are synthesized by these cells in the distal nerve stumps have been associated with nerve regeneration (Hansson et al., 1986; Martini and Schachner, 1986; Mirsky et

al., 1986; Heumann et al., 1987a; Raivich and Kreutzberg, 1987; Martin and Timpl, 1987; Kanje et al., 1989; Acheson et al., 1991a; Chiquet and Wehrle-Haller, 1994; Lettle et al., 1995). The objective of this study is to determine the expression of RAGs in long-term denervated stumps of rat hindlimb nerves in which our laboratory has previously demonstrated poor regeneration (Raji, 1994; Fu and Gordon, 1995b).

Although light and electron microscopic studies have indicated that Schwann cells and their basement membranes become severely atrophic (Weinberg and Spencer, 1978; Roytta and Salonen, 1988; Giannini and Dyck, 1990; Vuorinen et al., 1994), little is known about their long-term capacity to express RAGs.

Many immunocytochemical studies have examined the early changes in expression of various molecules in Schwann cells including the ECM components, membrane antigens and receptors, and cytoplasmic proteins after nerve injury (Taniuchi et al., 1986; Neuberger and Cornbrooks, 1989; Siironen et al., 1992a,b). The time period of these studies was limited to early denervation and reinnervation. Similarly, studies of denervation induced changes in gene expression of p75, EGFr and S100 was limited to a period of 3-20 days (Heumann et al., 1987b; Toma et al., 1992).

To understand whether non-neuronal cells can maintain their expression of RAGs for prolonged periods in the absence of regenerating axons, we have used antibodies against p75, EGFr and S100 protein, and anti-sense RNA probe for p75

to examine the expression of RAGs in long-term denervated rat sciatic nerve. Although this study does not address the question of the potency of the remaining Schwann cells to support regeneration, the aim is to establish the condition of the distal stumps and the optimal time for nerve regeneration in denervated nerve stumps in which we have made physiological estimation of the number of regenerating axons which grew through the denervated nerve stumps (Fu and Gordon, 1995b; Raji, 1994).

MATERIALS AND METHODS

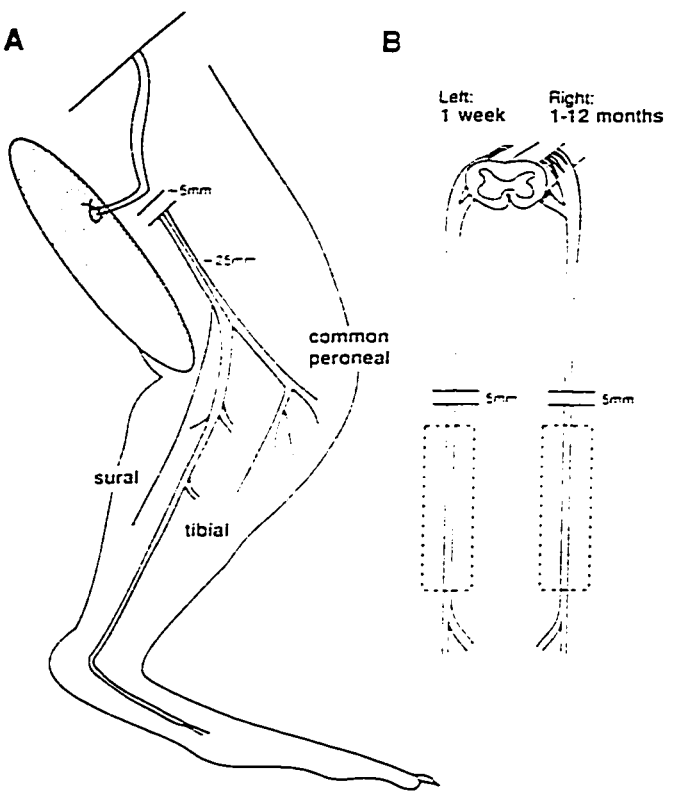
2.1. Animal surgery and tissue preparation

Forty-eight adult female Sprague-Dawley rats (200-250g) were operated and used as experimental animals. Nine rats were used as unoperated controls.

Rats were anaesthetized with sodium pentobarbital (Somnotol, 45mg/kg administered i.p. as 0.07 ml/100g body weight). Using sterile precautions, the sciatic nerve in the right hindlimb was exposed and transected in the middle thigh level 25 mm from the trifurcation of the nerve into the CP, tibial and sural nerves (Fig. 1). Nerve regeneration was prevented by removing a 5 mm piece of nerve between the proximal and distal stumps and suturing the proximal stump to the nearby innervated thigh muscles. At the time periods of 1, 3, 4, 6, 9, 12 months postoperative (n=3-4 at each time point), the rats were anaesthetized and sciatic nerve in the left hindlimb was exposed and cut at the same level as the cut right sciatic nerve.

One week after the second surgery, the rats were sacrificed by intracardiac perfusion with 100ml saline followed by 500ml 4% ice-cold paraformaldehyde under deep pentobarbital anaesthesia (0.12ml/100g body weight) for 30-45 minutes. Both proximal and distal sciatic nerve stumps on both sides were dissected, pined in gelatin coated dishes and treated with 10% (w/v), 20% (w/v)

Figure 1. Schematic drawing of (A) the surgical procedure to section the rat sciatic nerve bilaterally and to prevent regeneration by suturing the proximal nerve stumps to the nearby innervated muscle. (B) The segments from the distal nerve stumps were removed from the right (long-term denervated) and left (short-term denervated) nerve stumps for immunocytochemistry and *in situ* hybridization.


A
B
Left:
1 week
Right:
1-12 months
~5mm
~25mm
common
peroneal
sural
tibial
5mm
5mm

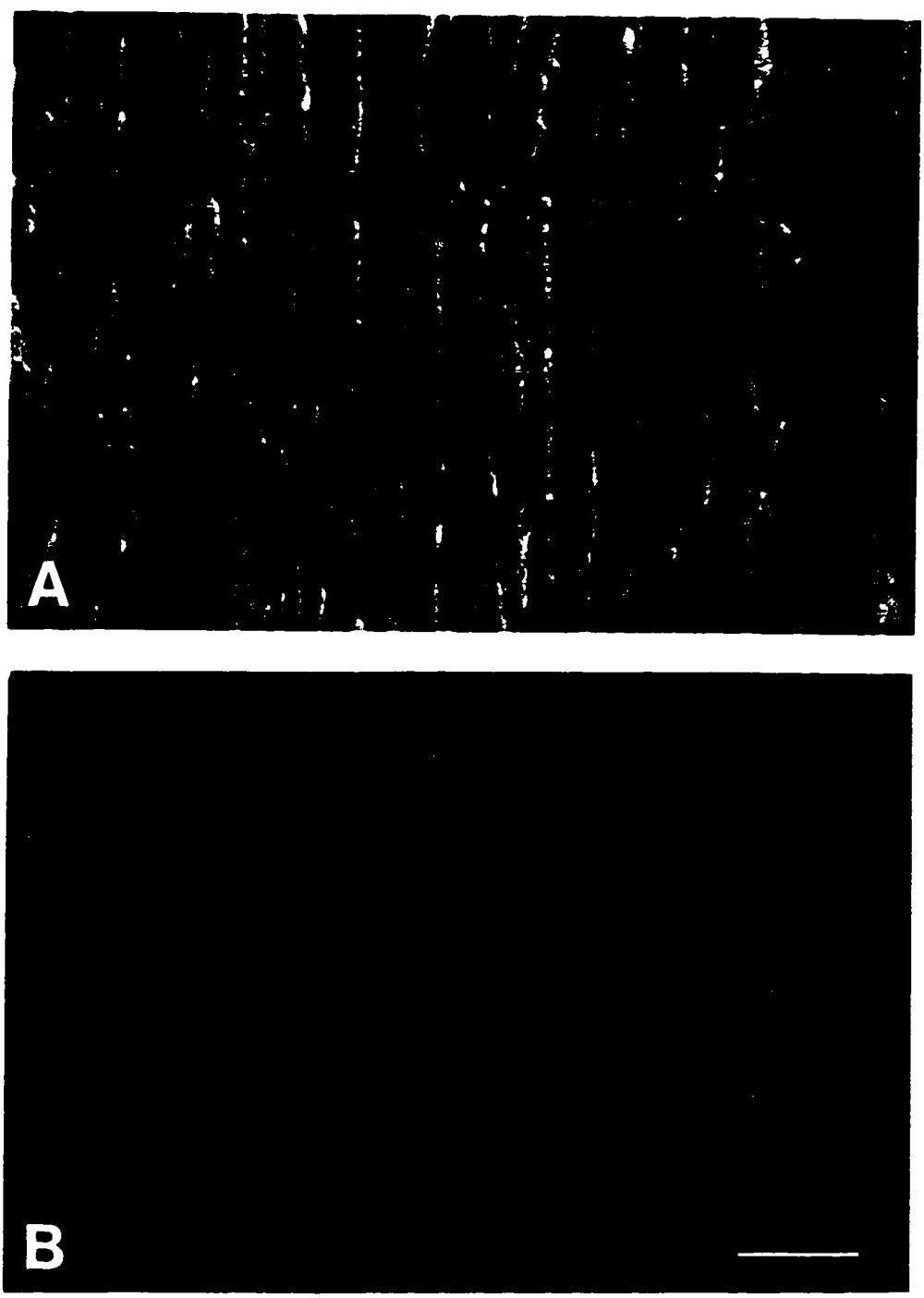
and 30% (w/v) sucrose solution for cryoprotection. After freezing in isopentane that was pre-cooled at -75°C, nerves were cut on a cryostat (14 μm in thickness). Longitudinal sections of one week denervated left sciatic, long-term denervated right sciatic (1-12 months), and intact nerves were mounted on the same gelatin coated slide for either immunocytochemistry or *in situ* hybridization.

A common problem in such experiments using long-term denervation is spontaneous nerve regeneration derived from the proximal stump. Suture of the proximal stump to the nearby innervated muscle is effective in preventing regeneration (Fu and Gordon, 1995a). Nonetheless, to assure that changes in p75 expression were associated with long-term denervation and not regeneration, we firstly removed a 5mm piece of nerve between proximal and distal stumps and secondly used a neurofilament rabbit polyclonal antibody (1:200, Chemicon Intl. Inc., Temecula, CA), followed by anti-rabbit FITC-conjugated secondary antibody (1:50, Jackson Immunoresearch Labs. Inc., PA) to detect any regenerating axons which may have grown into the distal stumps from the proximal stump. As indicated by the supplier this antibody is particularly suitable for the detection of axons. Since no neurofilament was detected in any of the denervated nerve stumps as shown in the example of a 3 month denervated nerve stump in Figure 2, our technique of maintaining sheath denervation was effective.

2.2. Immunocytochemistry

P75, EGFr, and S100 protein immunoreactivity were detected with the

Figure 2. Immunofluorescent staining for neurofilament in a longitudinal section of intact nerve (A) and in the denervated distal sciatic nerve 3 months following transection (B). In contrast to the intense fluorescence in intact nerves (A), there is no neurofilament immunoreactivity in B indicating that spontaneous regeneration did not occur in long-term denervated distal nerve stumps. Bar=50 μm.


A
B

conventional method of Hsu et al. (1981), using diaminobenzidine (DAB) as chromogen so that immunoreactive profiles were rendered brown as previously described (Petrov et al., 1992; Petrov et al., 1994). Tissues were incubated overnight at 4^0C with either a mouse monoclonal anti-p75 antibody (Ig 192, Boehringer Mannheim, Laval, Quebec, 1: 500), a rabbit polyclonal anti-S100 antibody (Dakopatts, Carpinteria, CA, 1:1500), or a mouse monoclonal anti-EGFr antibody (Sigma, St. Lous, MO, 1:100). Tissues were sequentially incubated for 1 hour with anti-mouse or anti-rabbit biotinylated antibody (1:200, Sigma, St. Lous, MO) followed by ABC reagent (1:100, Vector Labs, Burlingame, CA), and 0.05% DAB, 0.01% H_2O_2 in 0.1 M PBS for 5 min. Control sections were processed after omission of primary antibodies; no corresponding brown immunoreactivity was observed. After the immunocytochemical reaction was completed, the tissues were stained with haematoxylin in order to detect the distribution of p75 immunoreactivity within tissue compartments.

For double immunofluorescence, sections were incubated overnight at room temperature in a cocktail of mouse anti-p75 (1:20) and anti-S100 raised in rabbit (1:200, Dakopatts, Denmark) antibodies. P75 immunoreactivity was revealed by incubating the tissues sequentially in anti-mouse biotinylated antibody (1:200) and Texas Red conjugated to avidin (1:200, Amersham U.K.). S100 was detected by incubating the tissues in anti-rabbit IgG conjugated to AMCA fluorophore (1:50, Jackson Immunoresearch Labs. In.,PA).

To control for a possible cross-reactivity of the antibodies, tissues were

incubated as described above with alternating omission of primary antibodies so that in one control series the p75 antibody was omitted, whereas in the other series the anti-S100 antibody was omitted. Nonspecific immunoreactivity was not detected in these control experiments.

In the case while we detected simultaneously S100, neurofilament and non-neuronal cell nuclei, the antigens were visualized by using FITC, Texas Red and DAPI, respectively.

The sections were coverslipped with Cytoseal (Stephens Sci., Denville, NJ) and observed with a Leica epifluorescence microscope equipped with appropriate filter combination for the detection of Texas Red and AMCA.

2.3. *In situ* hybridization

P75 mRNA expression was determined by incubating nerve sections with antisense RNA probe which was generated as previously described (Miller et al.,1991). Briefly, the probe was prepared by incubating p75 cDNA which was subcloned into PGEM3 with SP6 RNA polymerase (Premega, Madison, Wisc.), ^{35}S-CTP (Dupon, Mississauga, Ont.), mixed nucleotides containing GTP, UTP and ATP, and RNase inhibitor (Premega, Madison, Wisc.) at 37°C for 1 hour. The probe was washed three times with isopropanol (Fisher, Canada) and ammonium acetate (Fisher, Canada).

In situ hybridization was performed as reported (Miller et al.,1989). Nerve

sections were fixed in the 37% (w/v) formaldehyde for 15 min, and then digested in proteinase K solution (10μg/ml) for 7.5 min. After dehydration in graded ethanol, sections were incubated with prehybridization solution containing 50% (v/v) formamide (Fisher, Canada), 25mM pipes (Boehringer Mannheim, Laval, Quebec), 5x Denhardt's solution (Maniatis et al., 1982), 0.2% SDS (Boehringer Mannheim, Laval, Quebec), 100mM dithiothreitol (Boehringer Mannherm, Laval, Quebec), 125μg/ml salmon sperm DNA (Sigma, St. Lous, MO) (denatured), 125μg/ml herring sperm DNA (Sigma, St. Lous, MO)(denatured) and 250μm/ml yeast tRNA (Sigma, St. Lous, MO) at 45°C for 2 hours. For hybridization, the same solution was used containing 10^6 cpm ^{35}S-radiolabeled antisense RNA probe per section. Hybridization with sense probe was performed to ensure the specificity of hybridization. After incubation overnight with the probe at 45°C, sections were washed and treated with RNase A (Sigma, St. Lous, MO) solution (175μg/ml) at 37°C for 30 min to remove the unhybridized RNA probe.

2.4. Autoradiography

After hybridization, sections were exposed to Kodak XAR5 film for 1-3 days to establish the strength of the radioactive signal and to estimate an appropriate exposure time for emulsion radioautography. Then, the sections were subsequently dipped in Kodak NTB-2 emulsion (Kodak, Intersciences Inc) diluted 1:1 with distilled water at 42°C. After exposure for 5-8 days, the sections were

developed in D19 for 2.5 min. at 15°C and fixed in Kodak Rapid fixer for 5 min.. The deposited silver particles appear as dark grains under the light microscope. Counterstaining with haematoxylin and eosin was used to identify the tissue structure.

2.5. Quantification of immunocytochemistry

To determine the intensity of the immunoreactivity of long-term denervated stumps, another p75 immunostaining with DAB was carried on alternate sections on separate slides without counterstaining. The intensities of the immunoreactivity in intact (I), one week denervated (1W) and long-term denervated (LT) nerves on the same slide were compared and determined using a computer-based image analysis system (Java video analysis software, Jandel Scientific, Corte Madera, CA) attached to a Leica microscope. Intensity of the immunoreactivity in LT nerves was compared to the intensity at 1W since p75 has been shown to be highly expressed within a week after denervation (Heumann et al., 1987b). Stronger immunoreactivity associated with expression of p75 in the denervated nerve results in decrease of the light passing through the objective , i.e. the light intensity is reduced. The results were displayed by relative intensity which compared the difference between I and LT with the difference between I and 1W. In each slide, the intact nerve section was used as internal standard, and the intensity of the light penetrating through this section was considered to be 100. The relative intensity of the immunoreactivity was calculated as 100- LT/100-1W.

2.6. Quantification of *in situ* hybridization

Silver grains overlying the nuclei were counted also by using the image analysis system. Twenty nuclei were selected randomly in each section. Three-four animals were used for each time point. We only counted the grains over the nuclei since we observed a predominant accumulation of the signal in this cellular compartment. Furthermore, when observed under the light microscope, the contours of Schwann cells' somata are not readily detectable which may lead to misinterpretation of the results.

2.7. Preparation of neural tissue for electron microscope

Six rats were used for electron microscopic study. The rats' right sciatic nerves were denervated for 1 month and 12 months and the left sciatic nerves were denervated for 1 week. One intact nerve was used as control. The fresh denervated distal nerve stumps were taken from both sides and fixed in 3% (w/v) gluteraldehyde at 4°C for 24 hours. Then, the nerves were segmented into 5mm pieces and transfered to numbered test tubes. Nerve segments were treated with 2% (w/v) of osmiun tetroxide for 80 minutes, and then were dehydrated in graded ethanol. Before embedding in araldite, nerve segments were put in propylene oxide for 15 min twice and then in 50% (v/v) propylene oxide with 50% araldite for overnight.

Thin sections (60 nm) were cut and mounted on copper grids using collodium foils. Sections, then, were contrasted with lead citrate and uranyl acetate and examined using Hitachi H 7000 transmission electron microscope.

2.8. Statistics

Mean values of grain counts were compared for the intact, 1 week denervated and long-term denervated distal stumps using a one-way analysis of variance (ANOVA) test. Where the ANOVA revealed significant differences among groups, the Student-Neuman-Keuls test was applied to define which group(s) contributed to these differences. Significance was accepted with $P<0.05$.

To summarize the results from the *in situ* hybridization experiments (Fig. 13) we have plotted the number of grains derived from each experiment in MicroCal Origin software (MicroCal Software, Inc.). The analysis revealed that the decline was fitted by an exponential curve (goodness of fit significant at $p<0.05$) with a time constant of 1.5 months (Fig. 13 A, C).

The relative intensity of the immunoreactivity was also analysed using MicroCal. The results were summarized in Figure 10 where the decline of the immunoreactivity was linear between the first and sixth month ($R=-0.9121$). A second linear relationship reflected the constant background betwen the sixth and twelfth month ($R=-0.8018$).

RESULTS

3.1. Expression of P75 and EGFr in proximal nerve stumps

The proximal stump of the transected sciatic nerve was sutured to the adjacent muscle to prevent reinnervation of the denervated distal nerve stump. One week later, the proximal stump was visibly swollen near the suture. Two of more weeks later, it was difficult to separate the proximal stump from the muscles since the stumps had adhered to the muscles. Most of the stumps had formed neuromas consistent with the well known inability of regenerating nerves to make functional connections with normally innervated muscles (Fu and Gordon, 1995a,b).

P75 and EGFr immunoreactivity in the proximal stumps was examined on slides which were counterstained with haematoxylin. Proximal stumps, 2-4 weeks after sciatic nerve section, were mounted on the same slide with the contralateral left nerves (denervated for 1 week) and an intact sciatic nerve from another unoperated rat in order to directly compare the expression of p75 and EGFr.

3.1.1. p75 In control intact nerves, there was no detectable p75 immunoreactivity (Fig. 3A). One week after sciatic nerve transection the intense p75 immunoreactivity was seen at the site of transection in the proximal nerve stump

(Fig. 3C). The same intense and localized p75 immunoreactivity was seen 1 month after nerve transection (Fig. 3D). Non-specific staining was not observed on sections where the primary antibody was omitted (Fig. 3B) (In order to discern the non-specific binding stain, the sections were not counter stained with haematoxylin). Intense p75 immunoreactivity was associated with an increase in the number of non-neuronal cell nuclei. The expression of p75 was localized to the tips of the proximal stump and decreased sharply 2-6mm proximal to the transection (Fig. 4). Similarly, the number of nuclei declined proximal to the injury site (Fig. 4). This result confirms previous findings that p75 expression is induced in the proximal stump of a transected nerve and that the expression is confined to the level of transection (Toma et al., 1992). The elevated expression of p75 was maintained for up to 1 month after suturing the nerve to nearby innervated muscle to prevent regeneration.

3.1.2. EGFr The pattern of EGFr immunoreactivity in the intact and transected nerves was very similar to that of p75. This was illustrated in figure 5 in which longitudinal sections of the proximal stump at the transection site are shown at a lower magnification than in figure 3. In the intact nerve, there was little EGFr immunoreactivity (Fig. 5A). One week after nerve transection, high EGFr immunoreactivity was found at the site of transection (Fig. 5C) and this high expression of EGFr was maintained at 1 month after nerve transection (Fig. 5D). The intense immunoreactivity of EGFr was associated with increased number of

Figure 3. Longitudinal sections of proximal nerve stumps incubated with p75 monoclonal antibody. The brown colour represents p75 immunoreactivity which is not present in the intact nerve (A), but is similarly intense in nerve 1 week (C) and 1 month (D) following nerve transection. Note the high number nuclei of non-neuronal cells in C and D indicating the proliferation of non-neuronal cells. Tissues were counterstained with haematoxylin (violet nuclei) except in B in which sections only exposed to secondary antibody as control. Bar=50μM

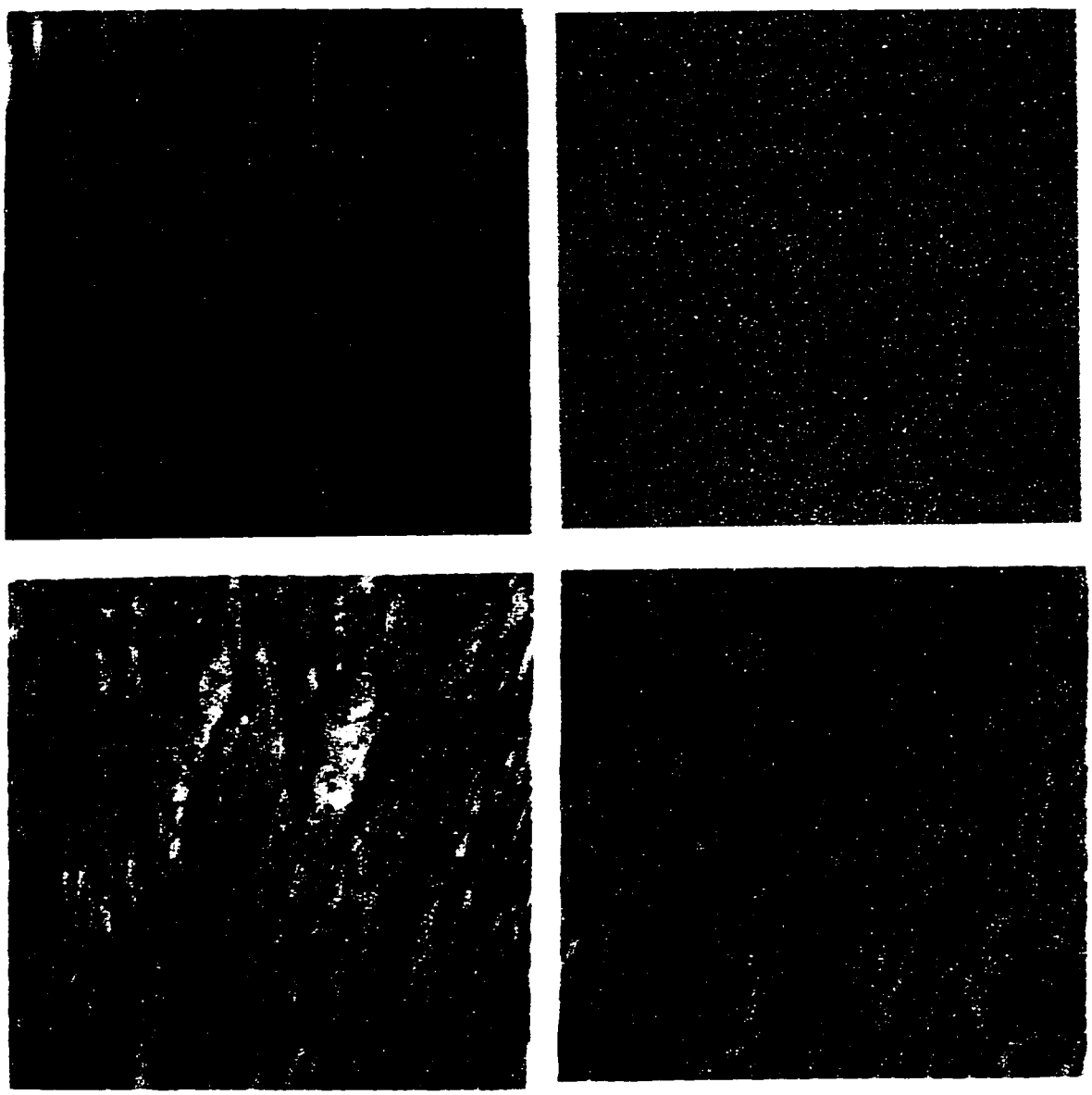
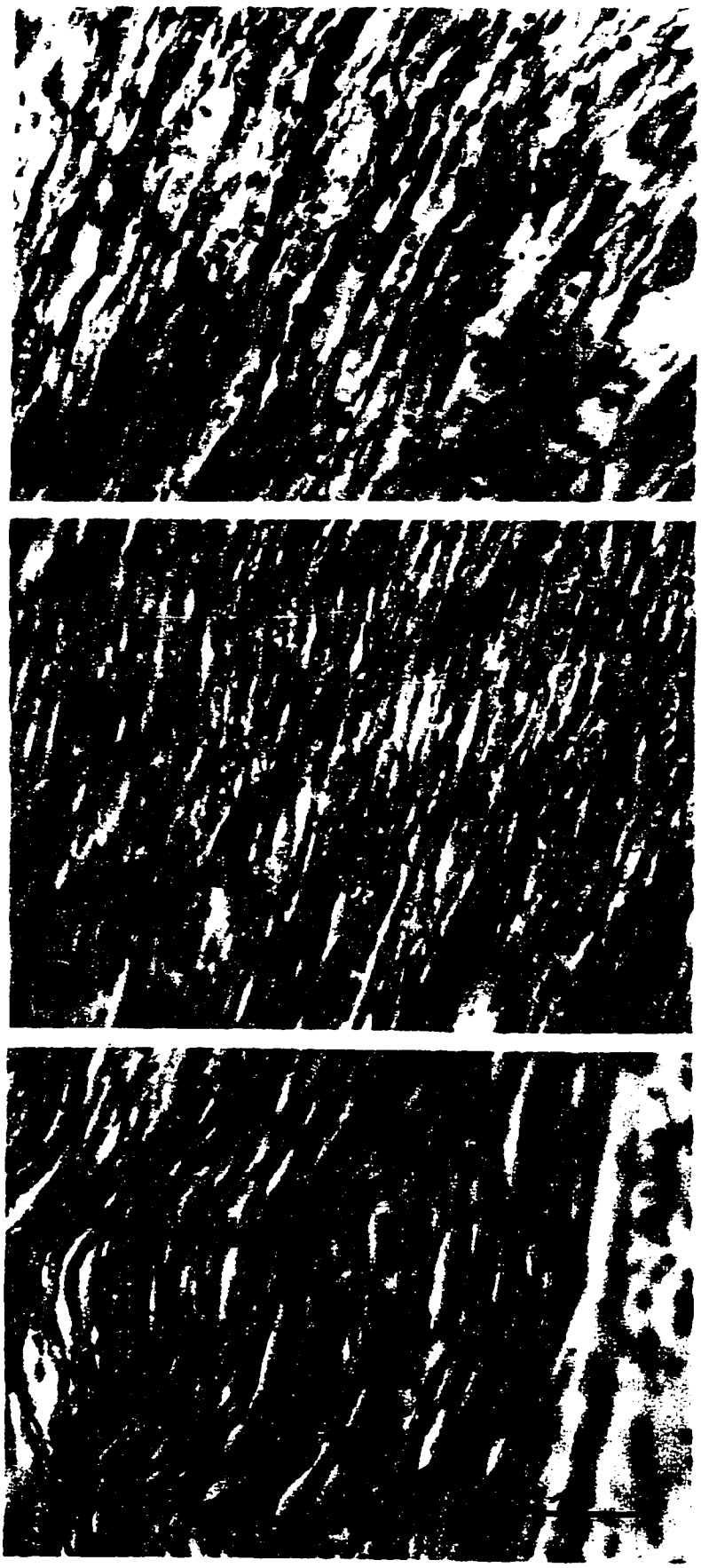


Figure 4. Longitudinal section of proximal nerve stump 1 week after nerve transection showing a gradient of p75 immunoreactivity. The segment closest to the level of transection showed the most intensive immunoreactivity (A). Moderately, intense immunoreactivity was seen 2-3 mm proximal to the segment A (B). However, there was almost no immunoreactivity in the segment 5-6 mm proximal to the segment A. Sections of the nerve in A, B and C were from same section of the transected nerve, proximal to the injury. Bar= 50 μm.



nuclei (Fig. 5C, D). There was no non-specific staining on sections where the primary antibody and haematoxylin were omitted (Fig. 5B). The immunoreactivity of EGFr in the proximal stumps also declined gradually with distance proximal to the site of transection, as described for p75 immunoreactivity.(Fig. 6).

This result indicated that the expression of EGFr in the proximal stumps, which was induced by the nerve transection, was also a local response of non-neuronal cells to injury. This elevated expression of EGFr, similar to the expression of p75, is maintained 1 month after nerve transection.

3.2. Expression of P75 and EGFr in short-term denervated distal nerve stumps

3.2.1. Wallerian degeneration Following nerve transection and suture of the proximal nerve stump to near innervated muscle, the distal nerve stumps undergo Wallerian degeneration. One week after denervation, degeneration of the axons and myelin sheaths was evident from the visibly swollen axons and the myelin debris in the denervated distal nerve stump (Fig. 7A). The number of nuclei was obviously increased demonstrating proliferation of the non-neuronal cells including p75 immunoreactive Schwann cells. The axon and myelin debris were gradually removed with time. At 2 weeks after distal nerve stump denervation, less debris was seen (Fig. 7B) as compared with 1 week denervated nerve. By 1 month, the debris had disappeared leaving spaces between the p75 positive cells (Fig. 7C). These findings indicate that the process of Wallerian degeneration was completed

Figure 5. Longitudinal sections of proximal nerve stumps incubated with EGFr monoclonal antibody at a lower magnification than Figure 4. The brown colour represents EGFr immunoreactivity which was not present in intact nerve (A), but was equally intense in 1 week (C) and 1 month (D) after nerve transection. Note the positive staining located in perineurium in C and D indicating additional expression of EGFr in fibroblasts. Bar=100 μm.

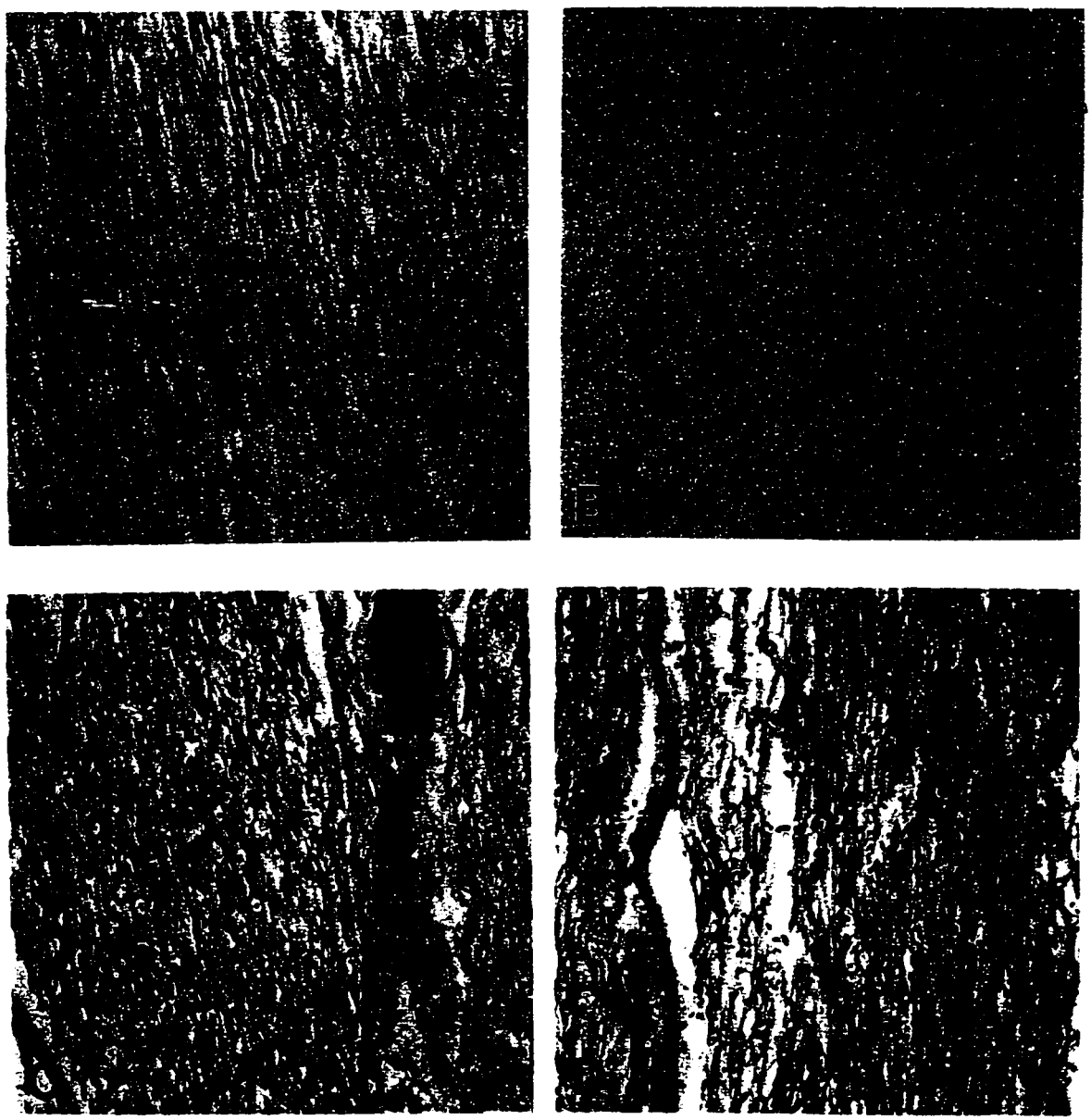
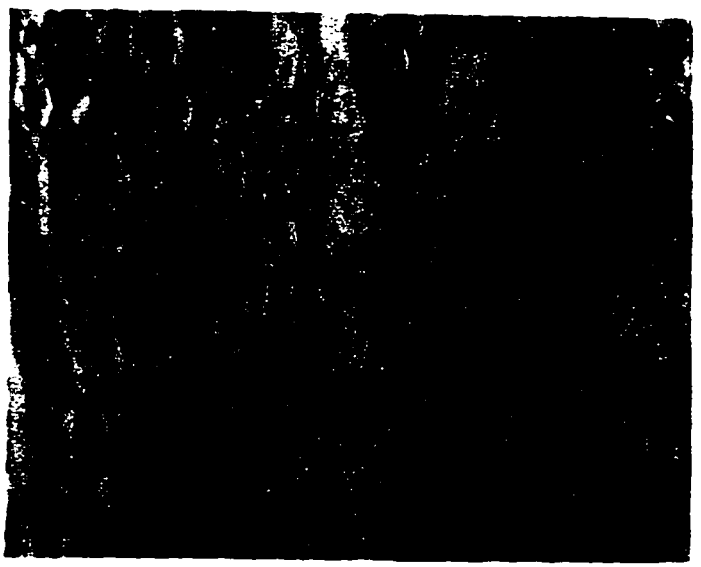
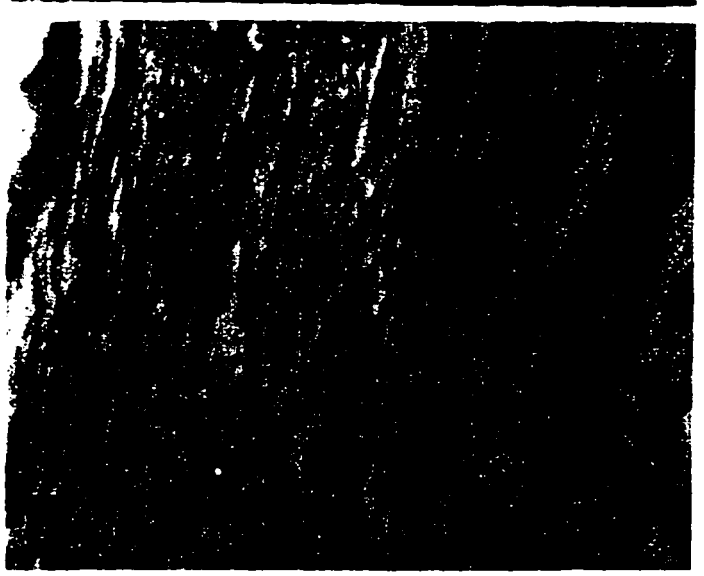


Figure 6. Longitudinal section of proximal nerve stump 1 month after nerve transection, showing the gradient of EGFr immunoreativity. The segment closest to the level of transection showed the most intensive immunoreactivity (A). Moderate intensive immunoreactivity was seen 2-3 mm proximal to the segment A (B). However, the segment 5-6 mm proximal to the segment A was as almost as normal. Sections of the nerve in A, B and C were from same section of the transected nerve, proximal to the injury. Bar=50 μm





during the first month.

3.2.2. p75 P75 immunoreactivity was examined in short-term denervated distal stumps from 1 week to 1 month. Compared with intact nerve (Fig. 3A), a dramatic increase in p75 immunoreactivity was seen in the distal nerve stumps following nerve transection (Fig. 7). After 1 week, the intense immunoreactivity was seen in long strands parallel to axons which were undergoing Wallerian degeneration (Fig. 7A). By 1 month, when axonal and myelin debris had disappeared, the strands of p75 immunoreactivity were separated by the space previously occupied by axons (Fig. 7C). The intense immunoreactivity of p75 could be seen during the entire first month (Fig. 7). Thus nerve transection induced high expression of p75 in the distal nerve stumps which was paralleled by a proliferation of non-neuronal cells. This pattern was maintained throughout the first month of prolonged nerve sheath denervation.

3.2.3. EGFr The pattern of EGFr immunoreactivity was similar to that of p75 immunoreactivity. The EGFr immunoreactivity was markedly increased 1 week after denervation (Fig. 8A), as compared with intact nerve (Fig. 5A) and was maintained up to 1 month (Fig. 8C). This result indicated that expression of EGFr was upregulated in the distal nerve stumps during the first month after nerve injury suggesting that both receptors follow a similar pattern of upregulation.

The elevated expression of p75 and EGFr remained at high levels during the

Figure 7. Longitudinal sections of the distal nerve stumps following nerve transection incubated with p75 monoclonal antibody. The brown colour represents p75 immunoreactivity. Intense immunoreactivity was seen in 1 week after nerve transection (A), 2 weeks after nerve transection (B), and 1 month after nerve transection (C), as compared with no reactivity in intact nerve (Fig. 4A). Degeneration of axons and myelin was evident from the visibly swollen axons and the myelin debris in 1 week after transection (A), but less evident considerably in the nerve 2 weeks after transection (B). Note the decreased tissue density in C as a result of the removal of degenerated debris following Wallerian degeneration. Bar=50 μm.

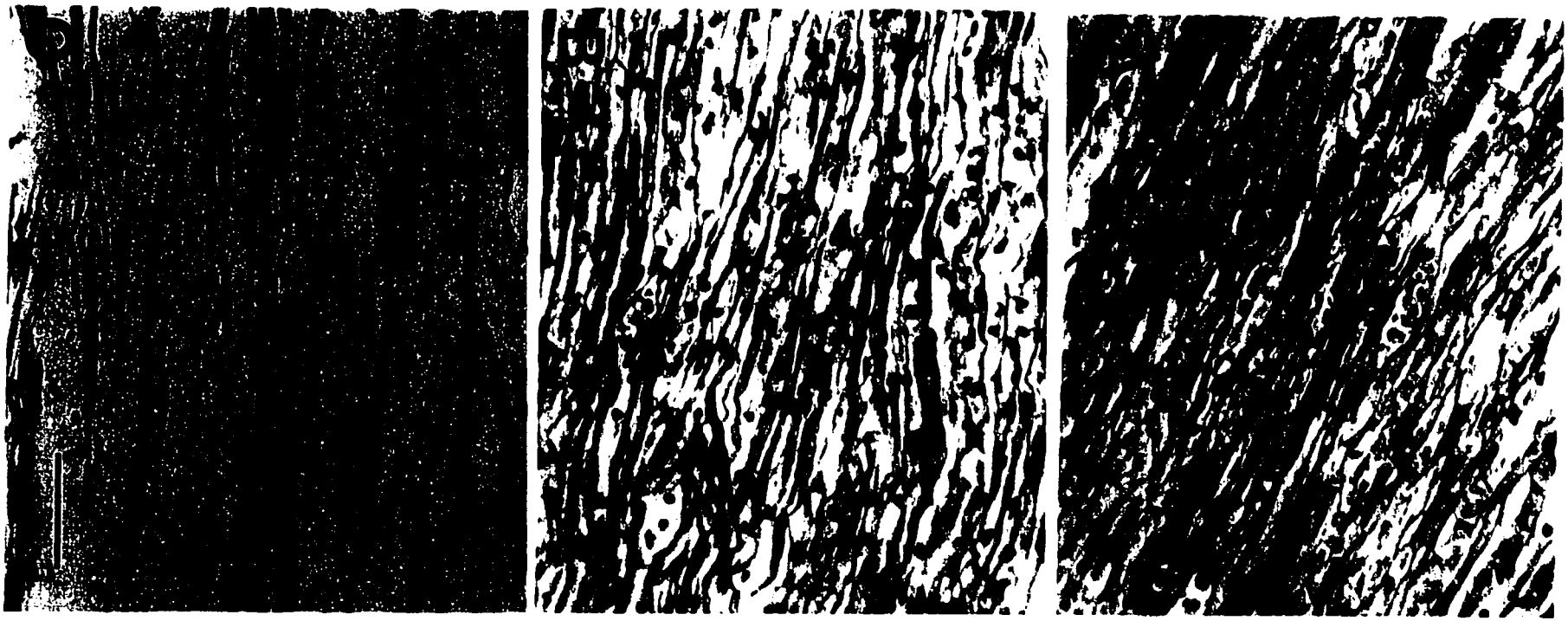
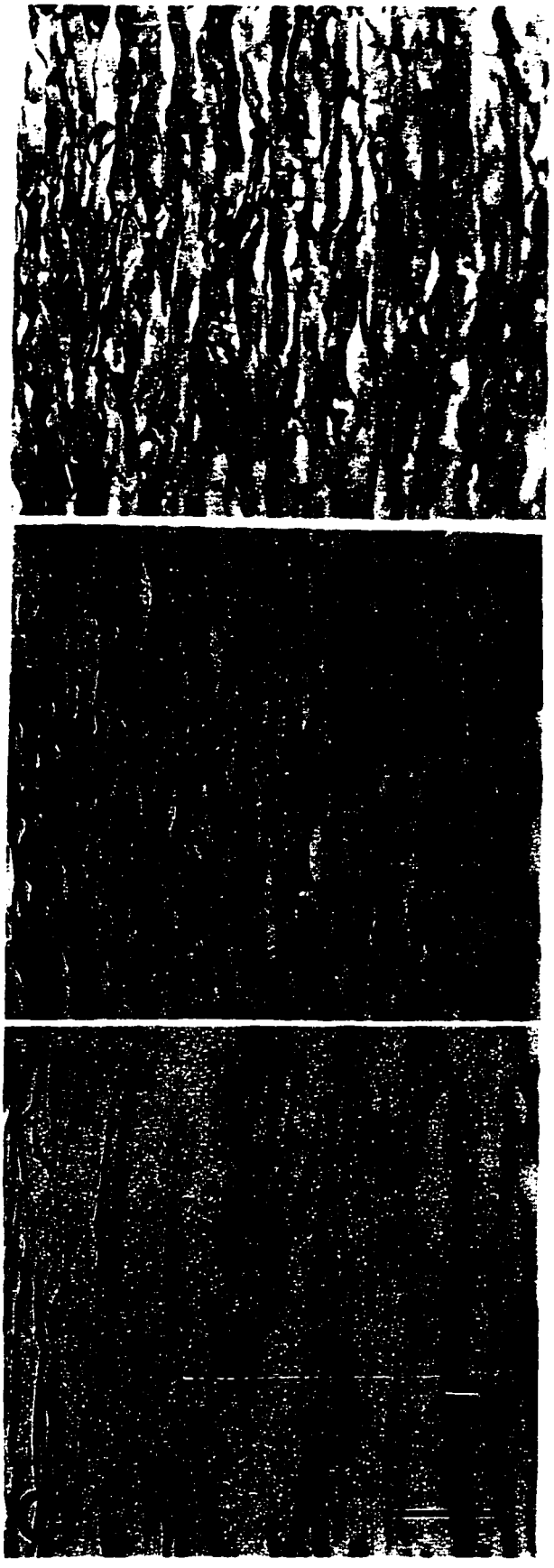


Figure 8. Longitudinal sections of the distal nerve stumps incubated with EGFr monoclonal antibody. The brown colour represents the EGFr immunoreactivity. The pattern of staining was similar to that of NGFr. The intense immunoreactivity and numerous nuclei of non-neuronal cells were seen 1 week (A), 2 weeks (B) and 1 month (C) after nerve transection. At 1 month after transection, the myelin debris were almost all removed (C). Bar=50 μm.



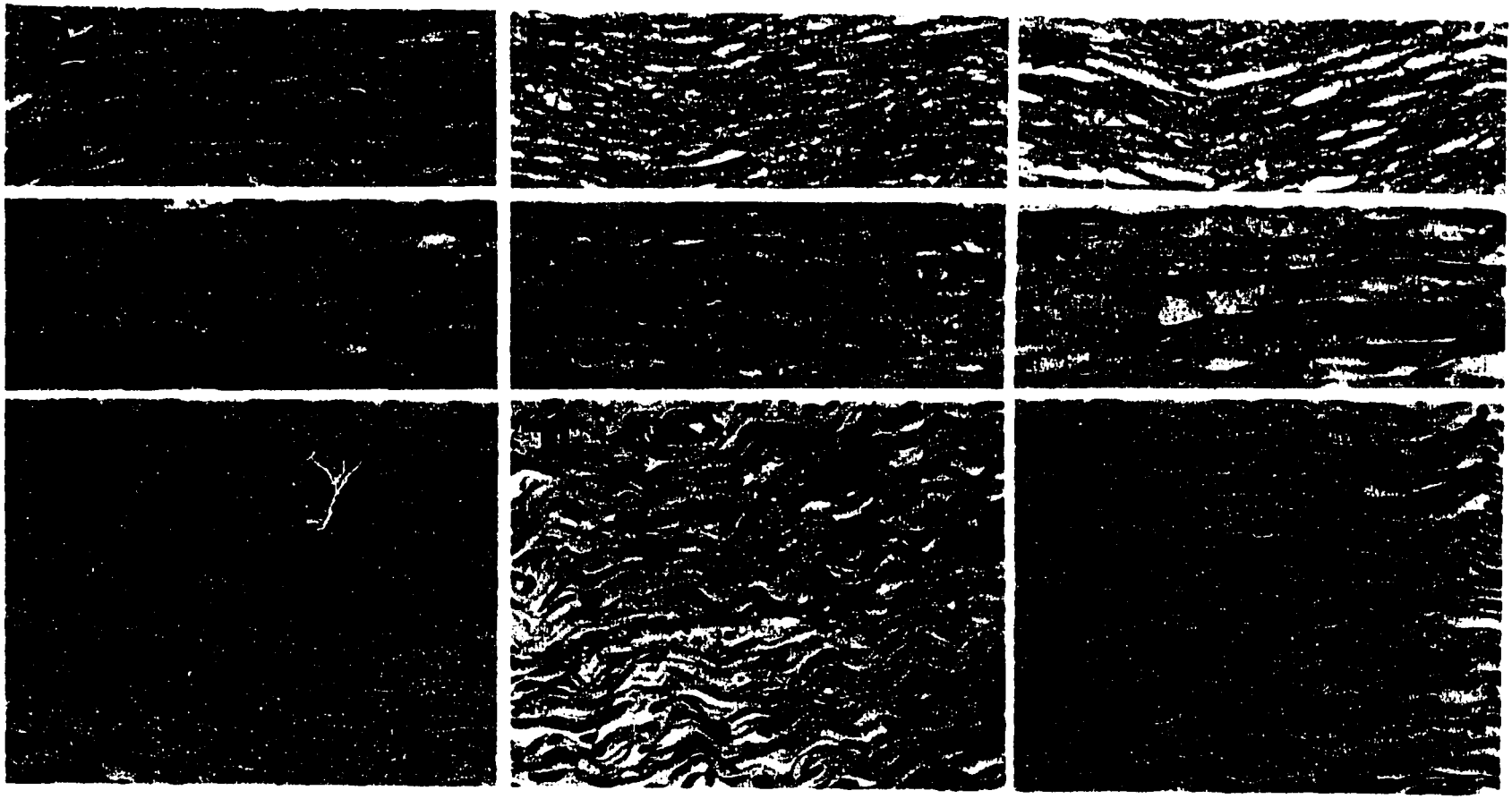
first month after nerve transection in the distal and proximal stumps. In the latter, the elevation is confined to the transection site.

3.3. Expression of P75 in long-term denervated distal nerve stumps

In order to determine whether the elevated expression of RAGs is maintained in long-term denervated nerve stumps, we examined the expression of p75 in the long-term denervated nerve stumps 1-12 months after sciatic nerve transection. Because the pattern of EGFr expression was similar to the expression of p75 in both proximal and short-term denervated distal nerve stumps, and the expression of these proteins at the site of transection of proximal stumps was similar to their expression in distal stumps, we studied the expression of p75 as the marker for RAG expression by non-neuronal cells in the long-term denervated distal nerve stumps. In addition, as shown below (see data 3.5.) p75 expression is confined to S100 positive Schwann cells and is therefore a good marker of RAG expression by Schwann cells.

3.3.1. immunochemistry In order to control for variations in the intensity of the immunocytochemical reaction in comparisions of sciatic distal nerve stumps 1-12 months after denervation, long-term denervated nerve stumps were mounted on the same slide with the 1 week denervated nerve stump and an intact sciatic nerve from another unoperated rat. As shown in Figure 9, immunoreactivity in the right

Figure 9. Longitudinal sections of long-term denervated distal nerve stumps (3-6 months) with p75 immunoreactivity. The immunoreactivity in intact (A, D, G), short-term denervated (1 week: B, E, H), and 3 (C), 4(F) and 6(I) months denervated distal sciatic nerve stumps were compared. Tissues in B, C; E, F; and H, I respectively were taken from the same animal and examined on the same slide. At 3 months (C), the intensity of immunoreactivity was still comparable with the one in 1 week (B). However, the intensity of the immunoreactivity was decreased at 4 months (F) as compared to 1 week (E), and was almost undetectable 6 months after denervation (I). Note the high number of non-neuronal cells on the long-term denervated nerve stumps (C, F, I). Bar=50 μm.



experimental nerve was compared at each time point with the left short-term (1 week) denervated nerve from the same animal and an intact nerve from a control unoperated animal.

P75 immunoreactivity was almost undetectable in intact nerves (Fig. 9A,D,G) in contrast with the intense immunoreactivity seen 1 week after denervation (Fig. 9B,E,H). The faint immunoreactivity in the intact nerve was detected between the intact axons in alignment with the non-neuronal nuclei (Fig. 9A). Three months after sciatic nerve denervation, when the endoneurial tubes were visibly collapsed, the brown immunoreactive strands lie in parallel rows interspersed with many nuclei (Fig. 9C). The intensity of the immunoreactivity appeared similar to that observed in the left sciatic distal nerve stump 1 week after denervation (Fig. 9B). At 4 months, immunoreactivity was high relative to 1 week (Fig. 9D-F) but lower than at 3 months (Fig. 9C). By 6 months, however, the p75 immunoreactivity was almost undetectable (Fig. 9I) resembling the intact unoperated nerve (Fig. 9G). Note that the 6 month denervated nerve stump differs from the intact in the high number of nuclei in the denervated stump which was maintained as high as at 7 days.

To evaluate the changes in p75 immunoreactivity as a function of time, we examined the density of the immunoreactive product on alternate tissue sections from the same nerves without haematoxylin counterstain (as described in the methods). The relative intensity of immunoreactivity of long-term denervated nerve stump was compared to the immunoreactivity at 1 week of denervation. P75 immunoreactivity was not significantly different at 1 and 3 months ($P>0.05$) (Fig.

Figure 10. Relative intensity of the p75 immunoreactivity where the intensity of the DAB reaction in long-term denervated (≥1 month) sciatic nerve stumps was compared to short term denervated (1 week) distal nerve stumps. Tissues were mounted on the same slide for each time point to calculate relative intensity on the abscissa. The high intensity of the immunoreactivity at 1 month declined to baseline levels by 6 months. The slope of the regression line fitted to the fall in relative intensity of immunocytochemistry was -0.28 and regression coefficient was -0.9121.

10). Thereafter, immunoreactivity decreased linearly to background level by 6 months.

3.3.2. in situ hybridization To determine whether the decline in p75 protein in long-term denervated nerve stumps was due to a decline in synthesis and/or breakdown, p75 gene expression was examined by measuring mRNA levels using *in situ* hybridization from the same nerves which were used for immunocytochemistry. To quantitate differences in mRNA levels, long-term denervated distal nerve stumps were processed together with 1 week denervated sciatic nerves and intact sciatic nerves from unoperated rats as described above for the immunohistochemistry. Figure 11 showed an X-ray film which was apposed to the nerve sections which were hybridized to an anti-sense RNA probe specific for p75 mRNA. P75 signals in the distal nerve sections which were denervated for 1 week (Fig. 11. left) were obviously higher than in the distal nerves which were denervated for 6 months (Fig. 11. right).

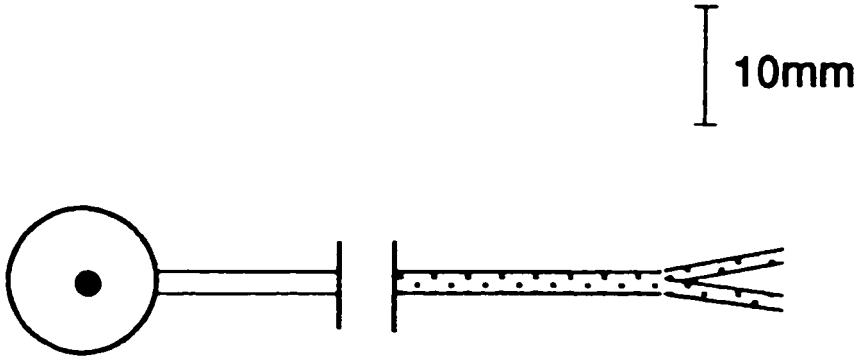
The nerve sections which were counterstained with haematoxylin and p75 was visualized as silver grains after autoradiography. As shown in figure 12, grain density reflecting gene expression of p75 was dramatically increased above background levels (Fig. 12A) at 1 week after denervation (Fig.12B) and maintained for at least 1 month after denervation (Fig. 12C).

In the intact nerve, the silver grains were scattered evenly over the nuclei and cytoplasm. The density of silver grains over the nuclei was similar to that

Figure 11. Distal nerve stumps 1 week and 6 months after sciatic nerve transection. The nerve sections were hybridized to an anti-sense RNA probe specific for p75 mRNA and apposed to X-ray film . The one week denervated nerve showed intense signals (left), as compared to weak signals in nerve which denervated for 6 months (right).

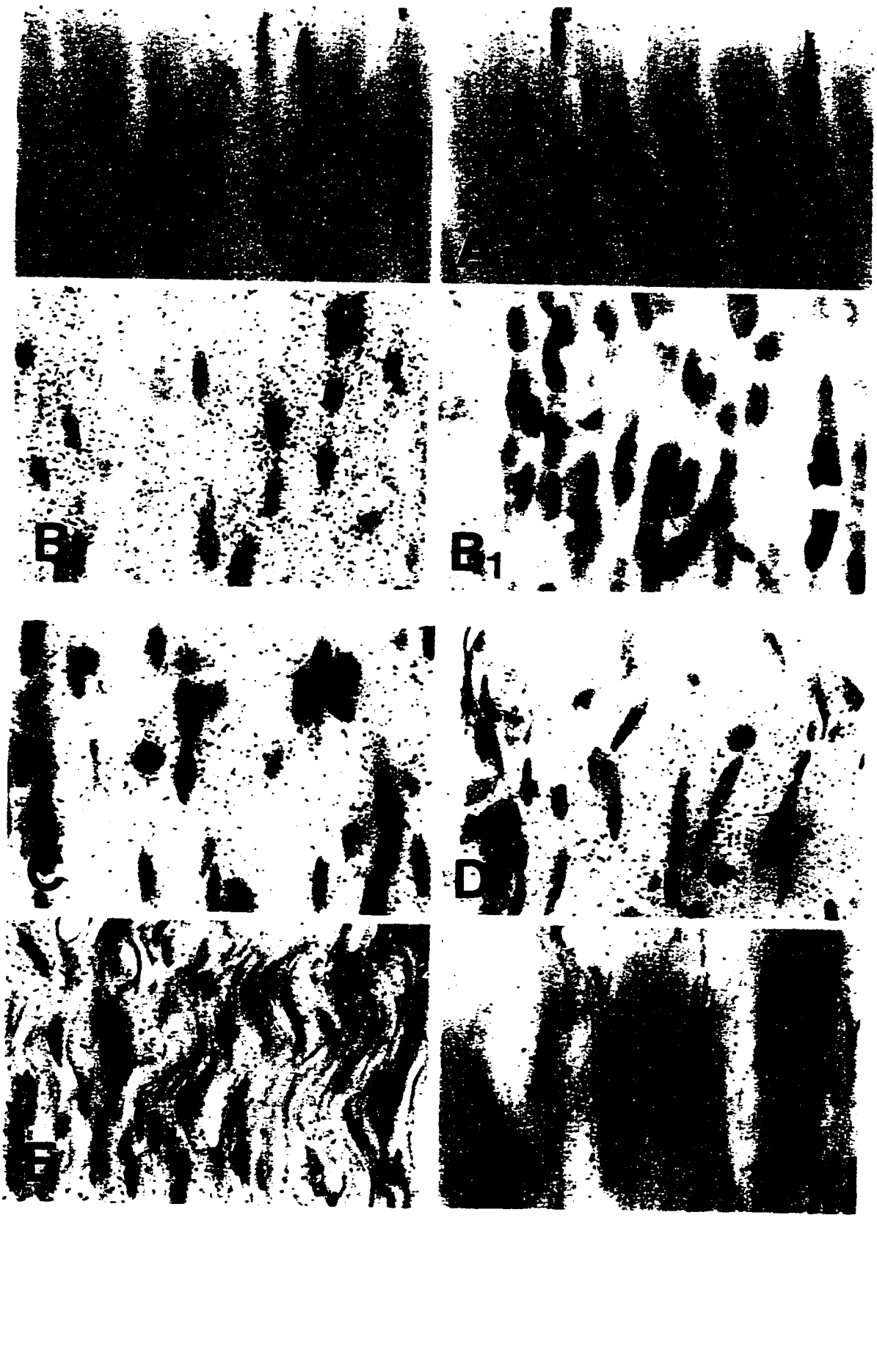
p75: in situ hybridization

L: 7d R: 180d



sciatic

Figure 12. Autoradiographs of P75 mRNA after *in situ* hybridization in intact (A) and distal nerve stumps of 1 week (B), 1 (C), 3 (D), 4 (E) and 6 (F) months following the nerve transection. Sections from intact (A1) and 1 week (B1) denervated distal nerve stump which were hybridized with a sense RNA probe showed weak background labelling. The amount of p75 mRNA is greatly increased after 1 week (B) and remains elevated until the first month (C) where the grains are accumulated over the nuclei (Counterstained with haematoxylin). At 3 (D) and 4 (E) months, the density of the grains over the nuclei is reduced. The density and distribution of the grains over the nuclei in the intact nerve and 6 months after nerve transection (F) are comparable and close to the background labelling. Bar=20 μm.

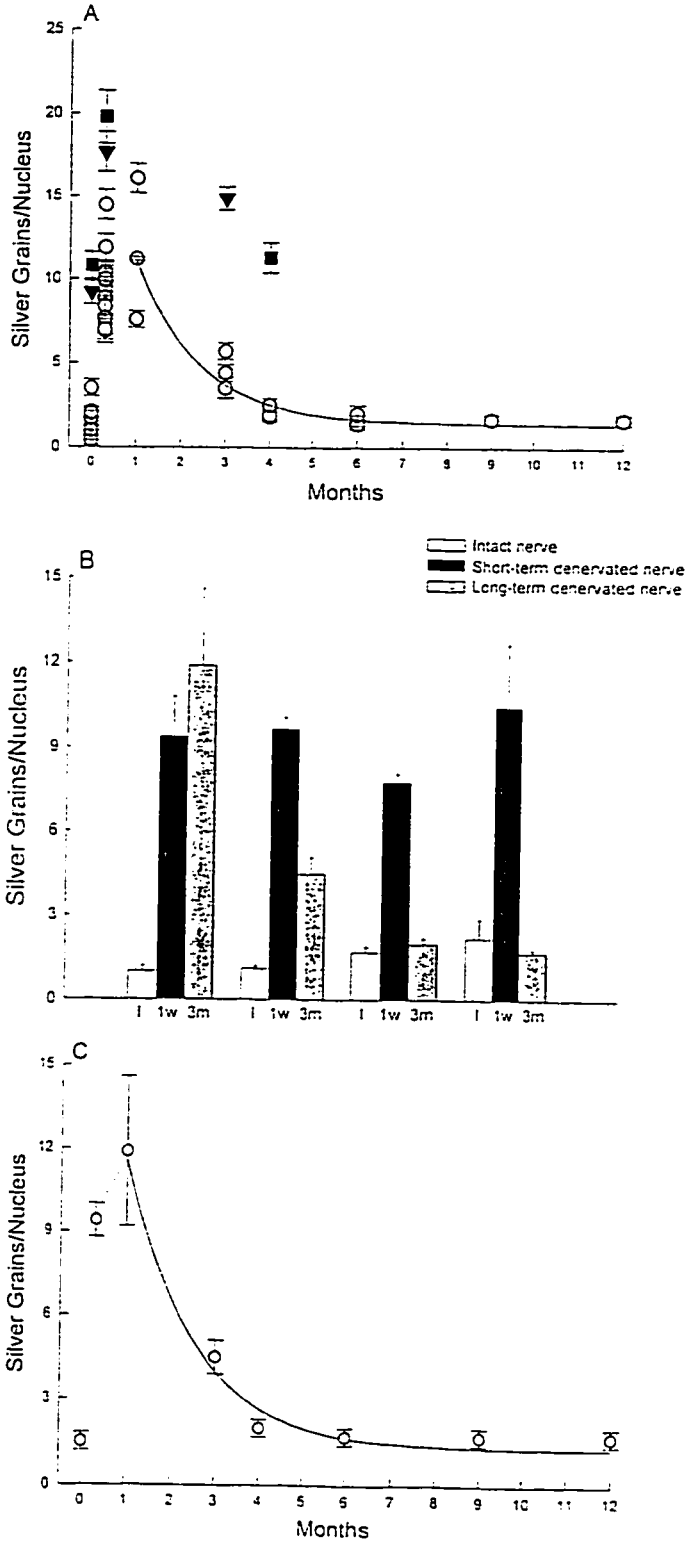

B
B1
D
E

observed in the background, as shown by the comparison with serial sections which were processed with sense RNA probe (Fig 12A, A1). In the denervated nerve stumps, the density of silver grains over the nuclei was visibly higher than in the cytoplasm. At 3 months after sciatic nerve denervation, the density of silver grains was lower and appeared to decrease to background levels by 4 months (Fig. 12E).

To determine the changes in gene expression as a function of time after denervation, silver grains were counted over the nuclei in tissue sections processed on the same slide. As shown in Figure 13A, nuclear density of silver grains increased dramatically within the first month after denervation and declined thereafter to the same background level as in intact nerves by 4 months. The mean $\pm$ SEM number of grains per nucleus counted in different rats was generally consistent with the few exceptions of higher grain densities which were shown as filled symbols in Figure 13A and which had corresponding high grain densities in the intact nerves. The trend for the initial upregulation of p75 gene to decline with time was evident from data for every animal which were plotted individually in Figure 13A. When the data for animals in which unusually high background levels of mRNA were excluded, the decline follows a simple exponential with a time constant of 1.5 months.

The initial upregulation of p75 and subsequent decline in expression in long-term denervated distal nerve stumps was obvious when p75 mRNA in long-term denervated stumps (1-12 months) was compared directly with short-term (1 week)

Figure 13. Quantification of the *in situ* hybridization signal for p75 mRNA in the intact nerve (0 on the ordinate in A and C) and denervated distal nerve stumps. A: Each symbol represents the mean number of grains (±SEM) counted over 20 nuclei in 14 intact nerves, 14 distal stumps 1 week after denervation, 3 at the 1st month, 4 at the 3rd month, 4 at the 4th month and 3 at the 6th month. The circles represent values which do not differ substantially between animals, whereas the black triangles and squares represent numbers of grains from tissues where a relatively higher background was observed. The decline in number of silver grains for long-term denervated stumps (1 month) was fitted by a simple exponential curve with a time constant of 1.5 months. The distal nerve stumps with unusually high background (black symbols) were excluded. B: Comparison of the mean number of grains per nucleus (±SEM) among groups of animals whose sciatic nerve distal to the transection were examined. Although there is approximately a 9 fold increase in the numbers of grains per nucleus at 1 month post transection as compared to the intact sciatic nerve, there is no significant difference between the mean numbers at 1 week and 1 month. At 3 months after the denervation the number of grains is approximately 5 times greater than in the intact sciatic nerve, whereas after longer periods of denervation (4 and 6 months) the numbers are similar to the intact nerve. C: A summary of the p75 expression reflecting the rapid upregulation of the p75 gene which is sustained until the 1 st month denervation and than decreases to baseline levels at the 4 th and the following months. The mean values are well fitted by the same exponential curve fitted to all data in A.


A
Silver Grains/Nucleus
Months
B
Intact nerve
Short-term denervated nerve
Long-term denervated nerve
Silver Grains/Nucleus
I 1w 3m
C
Silver Grains/Nucleus
Months

in the same animal and an intact nerve in an age-matched rat (Fig.13B). At one month after denervation, the number of grains per nucleus was not significantly different from 1 week but more than 8-fold higher than in the intact sciatic nerve (paired t-test: $p<0.05$). In contrast, the grain density in 3 month denervated nerve stumps was less than 50% that in 1 week denervated stumps ($p<0.01$). This number declined to levels which were not significantly different from the intact nerve by 4 months ($p>0.05$). Densities in intact and short-term denervated stumps in each group were not different (ANOVA, $P> 0.05$). The data was collated in Figure 13C to show the time course of upregulation and downregulation of p75 expression in denervated nerve stumps.

These results show that the upregulation of p75 is not maintained in long-term denervated nerve stumps beyond 3 months.

3.4. Expression of S100

S100 is frequently used as an immunocytochemical marker for Schwann cells (Stefansson et al., 1982; Karlsson et al 1993). As illustrated in Figure 14, S100 immunoreactivity is seen outside of axons in cross sections (Fig. 14A) and longitudinal sections (Fig. 14B) of intact nerve. In longitudinal sections,the S100 immunoreactivity was seen as continuous, rod-like structures around the axons (Fig. 14B, Fig. 15). The spatial relationship between S100 positive Schwann cells, neurofilament containing axons and DAPI positive non-neuronal cell nuclei in

Figure 14. Intact sciatic nerve with S100 immunoreactivity. The brown colour represents the immunoreactivity to S100. (A) Cross sections showed that the immunoreactivity of S100 was localized on exterior of axons. Occasionally, the immunoreactivity can be seen in the axons (arrow). (B) Longitudinal section showed S100 immunoreactivity in continuous, rod-like structures around the axons. Bar=50 μm.

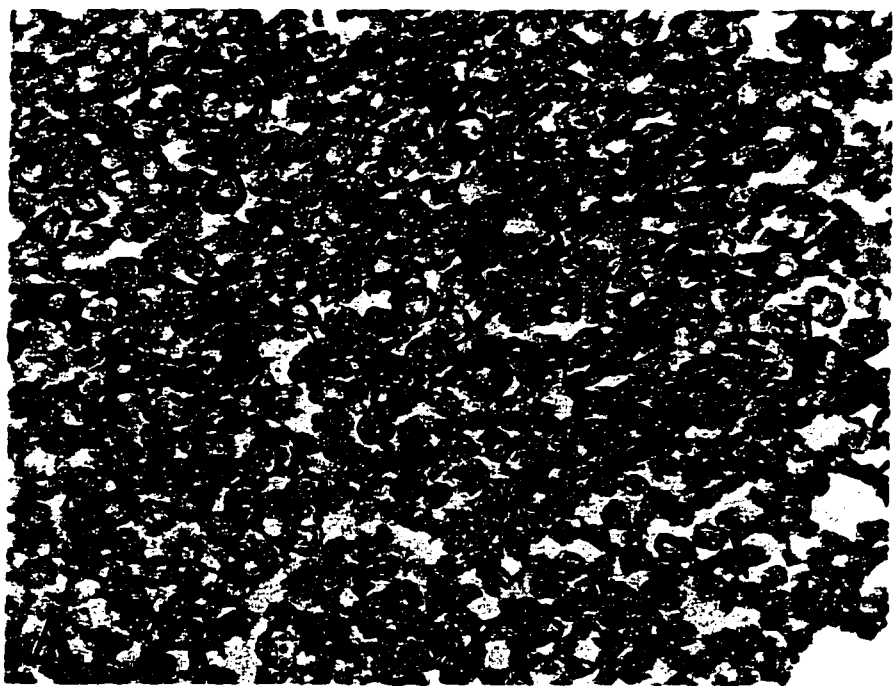
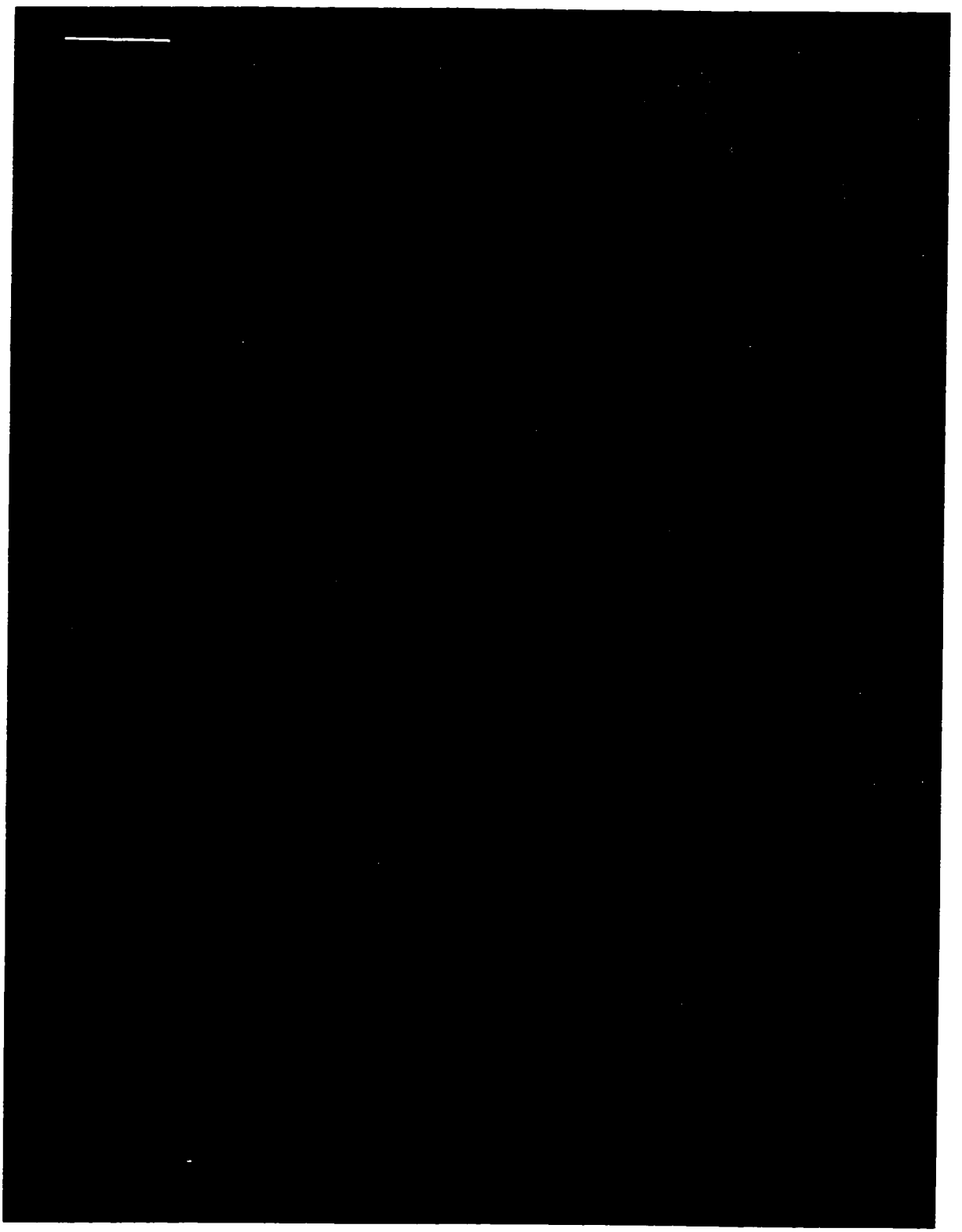


Figure 15. Triple immunostaining of intact nerve with S100, neurofilament, and DAPI for Schwann cells, axons and nuclei respectivelly linked to 3 different fluorescent probes: red colour represents neurofilament; green colour represents S100; blue colour represents non-neuronal cell nuclei. Bar=20 μm.



normal nerves was illustrated using triple immunofluorescent staining with 3 different fluorescent probes: S100 (green colour in Fig. 15), neurofilaments (red colour in Fig. 15), and DAPI (blue colour in Fig. 15). Only occasionally was that S100 was seen within axons (Fig. 14A, arrows).

Within the first month after nerve transection, S100 immunoreactivity in the distal nerve remained intense (Fig. 16A-C). One week after axotomy when the axons became fragmented in the distal stumps and disorganized and the number of nuclei were markedly increased, the Schwann cells lay within the axon debris (Fig.16A). By 2 weeks, more of the debris had been removed, leaving only S100 immunoreactivity and nuclei (Fig. 16B). By the 1 month, there was little sign of axonal cytoplasm and rows of Schwann cells were evident as the parallel rows of S100 immunoreactive staining (Fig.16).

3.5. Double immunofluorescent staining

To determine whether the expression of p75 is confined to Schwann cells, we used double immunofluorescent staining for both S100 protein and p75. The immunoreactivity of S100 was seen in the photomicrographs as dense patches in the intact nerve, possibly reflecting the particular distribution of Schwann cell cytoplasm in the clefts of Schmidt-Lantermann within the myelin sheath (Fig.17). In confirmation of the preceding experiments where p75 immunoreactivity was detected by using DAB as a chromogen, p75 immunoreactivity was barely

Figure 16. Longitudinal sections of denervated distal nerve stumps incubated with S100 polyclonal antibody. Intense immunoreactivity was seen throughout the first month. (A) 1 week after nerve transection. (B) 2 weeks after nerve transection. (C) 1 month after nerve transection. As illustrated in figure 7, degeneration of the axons and myelin sheaths was evident from the visibly swollen axons and the myelin debris in the denervated distal nerve stump (Fig. 16A,B). The number of nuclei was obviously increased demonstrating proliferation of the non-neuronal cells including the S100 immunopositive Schwann cells. In C, less debris remains indicating the process of Wallerian degeneration was completed. Bar=50 μm.

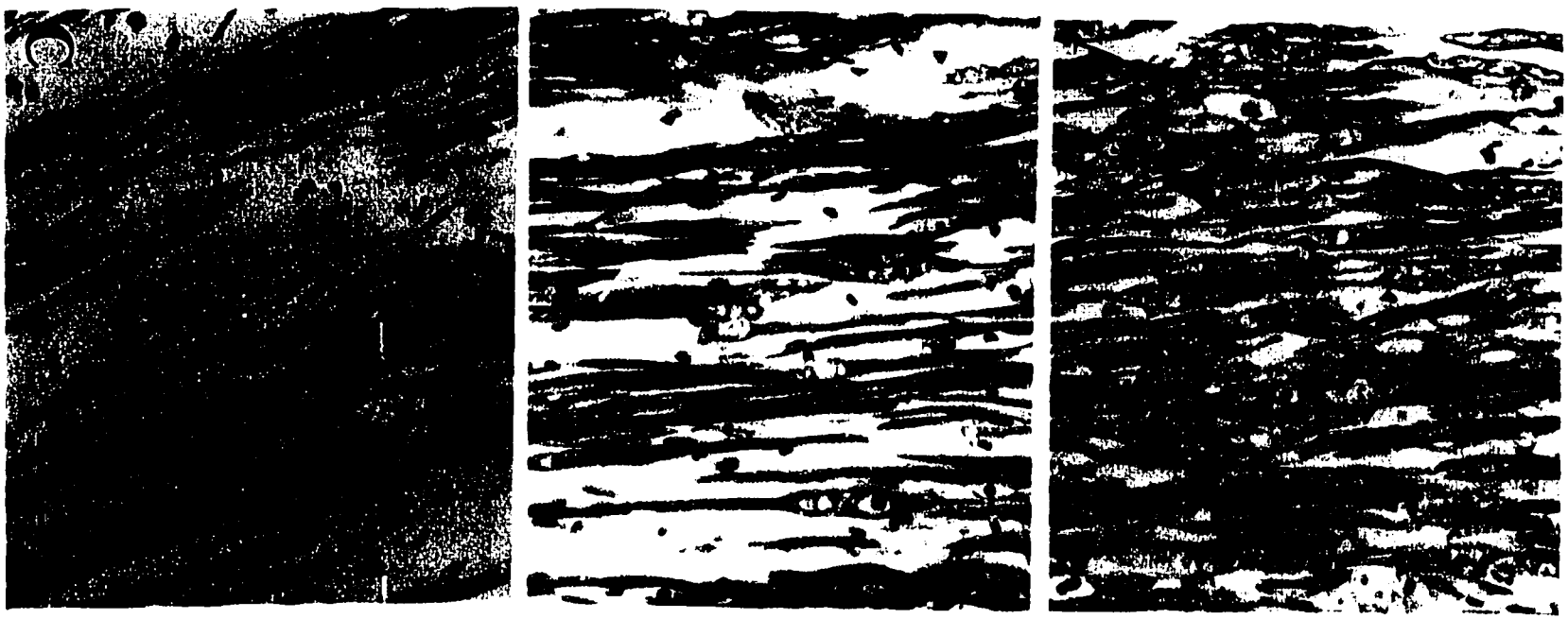


Figure 17. Darkfield photomicrographs of intact sciatic nerve (A, B) and denervated distal nerve stumps (C,D; E,F; G,H) following double immunocytochemical staining for S100 (left column, UV filters) and p75 (right column, RITC filters). The intensely fluorescent patches of S100 in the Schwann cells correspond to the Schmidt-Lantermann clefts (A), and lack of p75 immunoreactivity (B) was observed in the intact nerve. 1 week following denervation S100 (C) and p75 (D) were colocalized in the Schwann cells (arrows). 4 months following denervation both immunoreactivities were colocalized (arrows in E and F) while the intensity of the fluorescence was reduced. 6 months following denervation only occasionally S100 (G) and p75 (H) immunoreactivities were observed (arrows). Exposure time was identical for the photomicrographs in the right and left column respectively. Bar=50 μm.

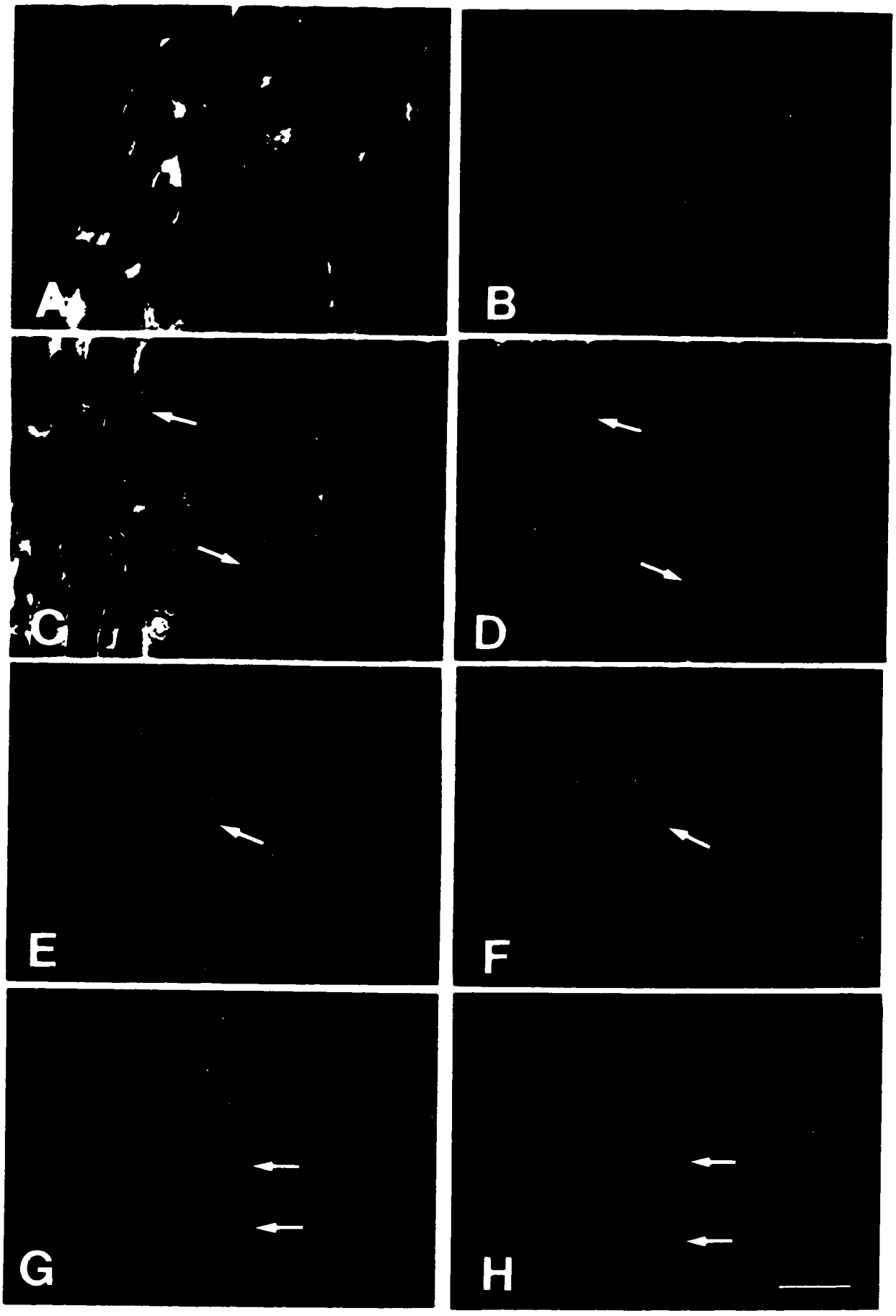

A
B
C
D
E
F
G
H

Figure 18. Electron micrographs of the distal nerve segments 1 week (A,B) and 1 month (C) after sciatic nerve transection. Evidence for early degeneration of axons and myelin was seen at 1 week after nerve transection (Fig. 18A,B). The Schwann cell nucleus (Fig. 18A, arrowhead), many phagocytic vacuoles representing lipid droplets (Fig. 18B, arrowhead), and basal lamina (arrows) are included the the micrograph. One month after nerve transection, there was no evidence of myelin debris (Fig. 18C), but a clearly visible basal lamina outside the degenerated Schwann cell column remains (Fig.18. biger arrows).

detectable in the intact nerve (Fig. 17B). One week after sciatic nerve transection, S100 immunoreactivity was more continuously distributed along the nerve and almost all Schwann cells showed corresponding p75 staining (Fig.17A,C,E). As described above, p75 immunoreactivity was intense. The correspondence between S100 and p75 remained in long-term denervated stumps even though both immunoreactivity to S100 and p75 declined.

Thus expression of S100 and p75 by Schwann cells declined after long-term denervation despite the large number of non-neuronal cells remaining in the long-term denervated stumps.

We examined the non-neuronal cells at the electron microscopic level to determine whether the decline in expression was due to death of Schwann cells after long-term denervation as suggested by Weinberg and Spencer (1978) or the decline was due to inability of Schwann cells to maintain the expression of these proteins as they atrophy.

Myelin and axonal degeneration in distal denervated nerve stumps (Fig. 18A, B) was evident 1 week after nerve transection. Schwann cell nucleus (Fig. 18A) and many translucent phagocytic vacuoles representing lipid droplets (Fig. 18B) were seen. By 1 month after nerve transection, almost all the myelin debris had been removed (Fig. 18C). However, the basal lamina was still intact outside degenerated Schwann cell column during this period (Fig.18. arrows).

After prolonged nerve stump denervation (12 months), some Schwann cell

Figure 19. Electron micrographs of long-term (12 months) denervated distal nerve stumps. The Schwann cell column shown in A is filled with cytoplasm and contains several nuclei (arrow) with condensed chromatin. Schwann cell basal lamina is discontinuous. The Schwann cell columns shown in B are more clearly seperated.

B

Figure 20. Electron micrographs of fibroblast-like cells which form extensive cytoplasmic processes (A, B) and contain many electron dense particles in the cytoplasm (A).


1.0μm

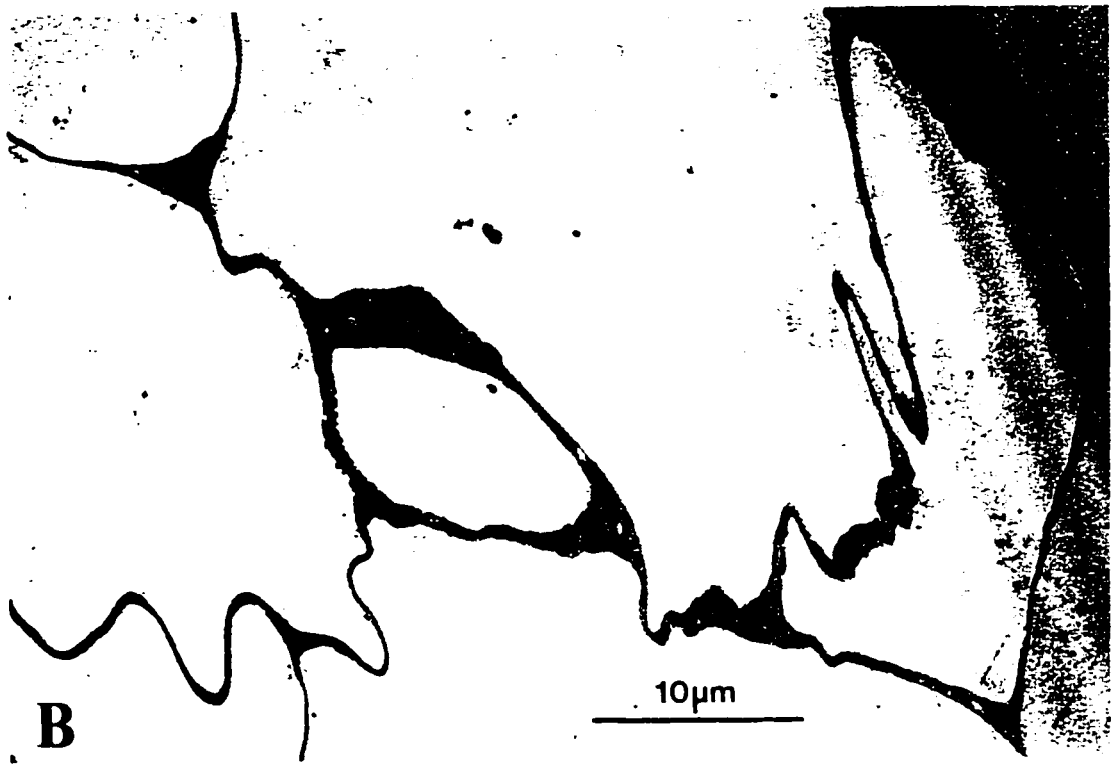
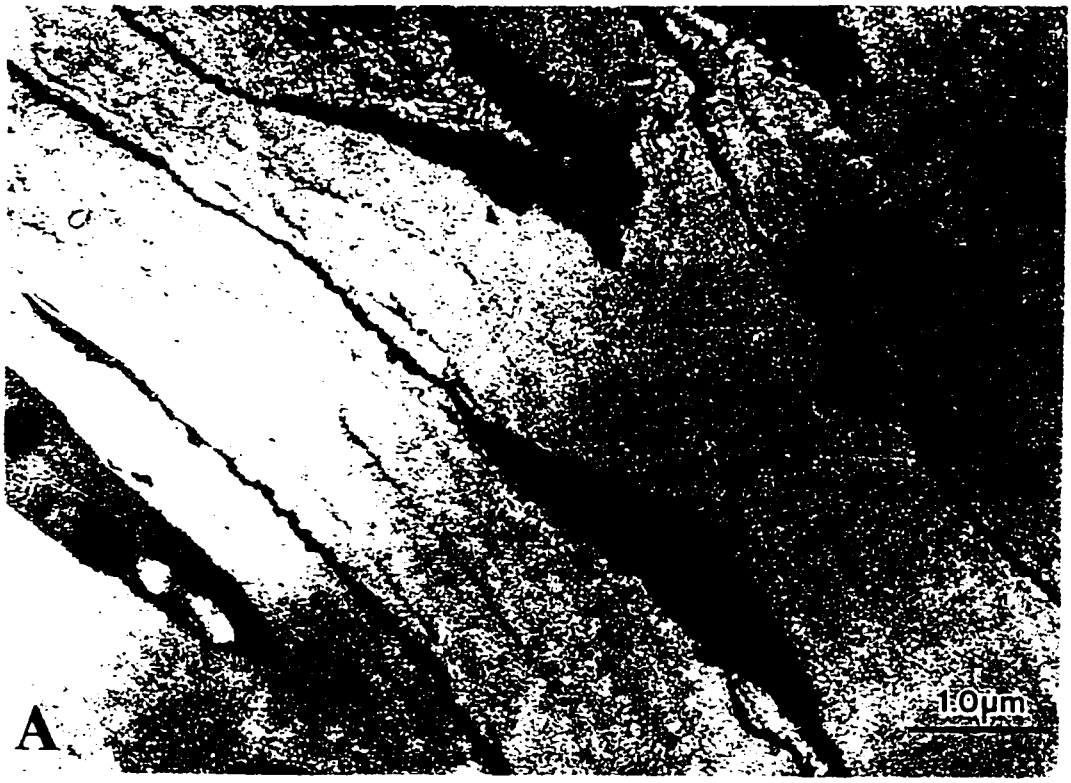

10μm
B

Figure 21. Fibroblast-like cells formed long processes which form many compartments (A) which are filled with collagen fibers (B).


A
1.0μm


1.0μm

columns were entirely filled with the Schwann cell cytoplasm. Often these cytoplasmic masses were multinucleuted (Fig. 19A). The nuclei were irregular and contained condensed chromatin. Other Schwann cell columns contained one nucleus (Fig. 19B). The diameter of these Schwann cell columns was reduced. The distinct and continuous basal lamina was not seen in these long-term denervated nerve stumps (Fig. 19A,B). Fibroblast-like cells were distinguished by their extensive cytoplasmic processes and inclusion of electron dense particles (Fig. 20A,B). The extensive processes form many compartment which were filled with collagen (Fig. 21B).

These results showed that, in the long-term denervated distal nerve stumps, Schwann cells and fibroblasts can be distinguished but the Schwann cell columns are highly atrophic. Thus reduced expression of p75 cannot be attributed to death of Schwann cells but rather to reduced synthesis of RAGs by atrophic Schwann cells.

DISCUSSION

In this study of the molecular expression of non-neuronal cells in long-term denervated nerve stumps, we have demonstrated that the early expression of RAGs by non-neuronal cells in the denervated distal nerve stump is not maintained if denervation is prolonged for longer than 1 month. In addition, the characteristic cytoplasmic marker of Schwann cells, S100, is also downregulated as Schwann cells atrophy. However, the large number of nuclei and the electron microscopic evidence of surviving Schwann cells indicate that deterioration of the growth support of long-term denervated stumps is due to atrophy of non-neuronal cells rather than their death.

In contrast to previous studies of p75 expression after nerve transection, using northern blotting, in situ hybridization, immunocytochemistry and antibody cross-linking (Tanuichi, et al., 1986, 1988; Heumann et al. 1987b; Toma et al. 1992) which was limited to the first 2 months after transection, this study follows p75 expression in denervated nerve stumps over one year after nerve transection.

Short-term denervation

Our immunocytochemical demonstration of increased expression of p75

and EGFr at the transection site of the proximal stump (Fig. 3, 5) and in the distal stump (Fig. 7, 8), in the first month after sciatic nerve transection, is in good agreement with previous findings (Tanuichi, et al., 1986, 1988; Heumann et al. 1987b; Toma et al. 1992). Examination of longitudinal sections over a 20 mm length of proximal stump demonstrated that the elevated expression was confined to the final 4-6 mm (Fig. 4,6) which is associated with "die-back" to the first node of Ranvier. The "die back" involves calcium influx into degenerated nerves, macrophage invasion, proliferation of Schwann cells and expression of RAGs (Sunderland, 1978; Hall, 1986, 1988). Consistent with the known migration of Schwann cells from the proximal stump and their proposed role in providing trophic and substrate support and guidance (see introduction), the expression of RAGs by the Schwann cells in the proximal stump at the site of injury is an integral component of the Schwann cell response to injury.

In the distal nerve stump, upregulation of p75 and EGFr coincides with cellular proliferation and Wallerian degeneration (Fig. 7,8; Toma et al., 1992). The increased expression of p75 is associated with a dramatic increase in the levels of mRNA within the first week (Fig. 12B; Heumann et al., 1987b). Consistent with the data of Heumann et al (1987b), mRNA levels remained the same within the first month in our study (Fig. 12C). The similar spatiotemporal expression of p75 and EGFr suggests that the change in gene expression of these proteins are a general characteristic of Schwann cell responses to nerve injury.

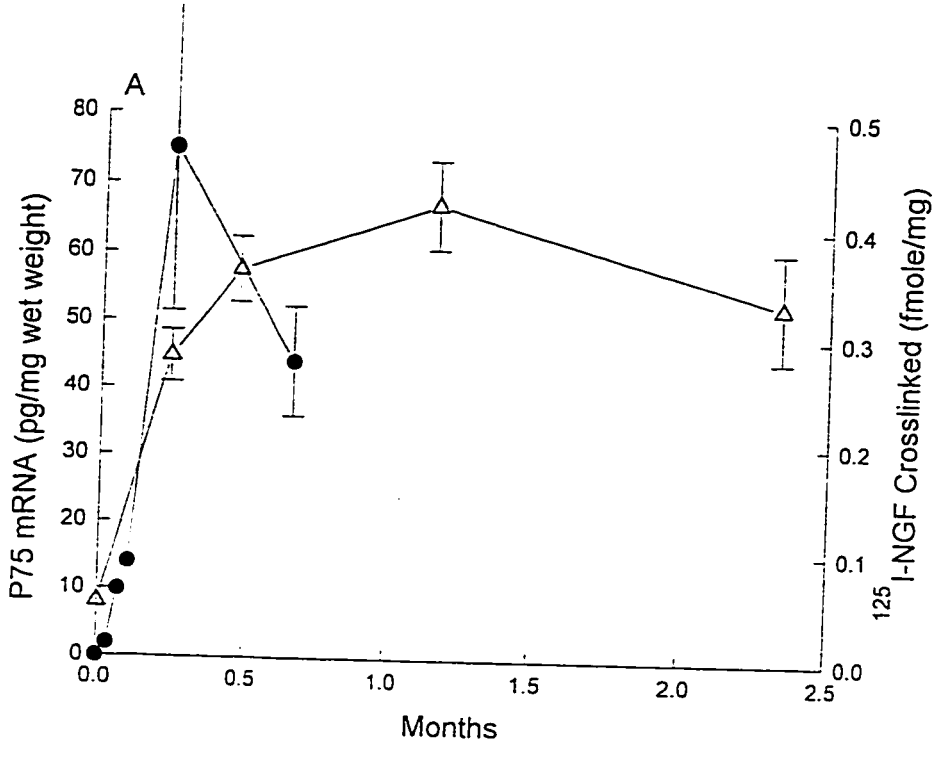
The expression of these molecules may reflect, at least in part, the

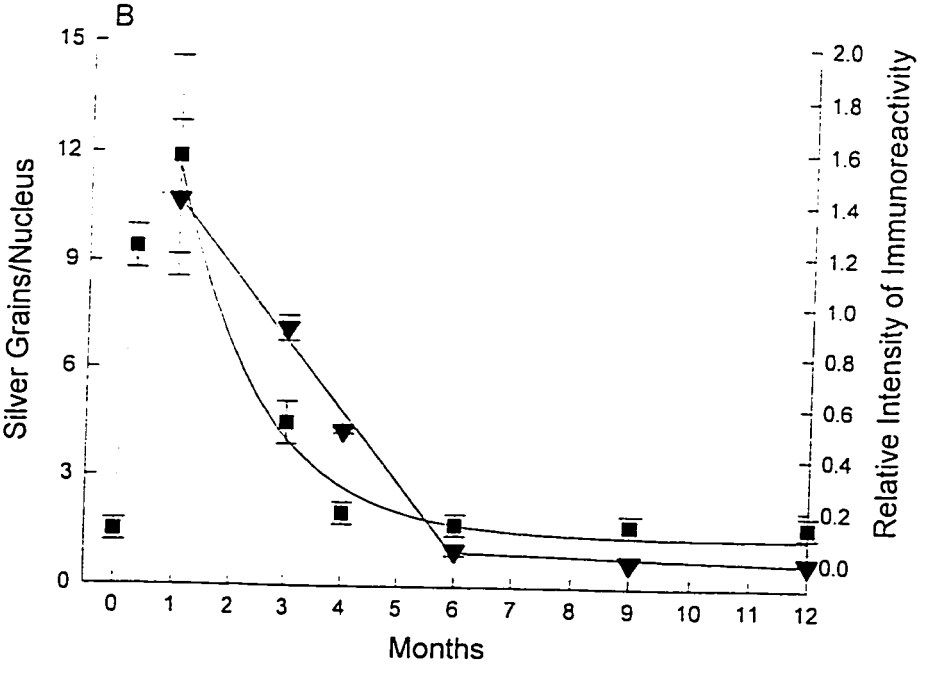
expression of other RAGs. The elevation in p75 and EGFr coincides with the upregulation of expression of several RAGs including growth factors NGF, BDNF, LIF, PDGF and IGFs (Richardson and Ebendal, 1982; Hansson et al., 1986; Heumann et al., 1987a; Raivich and Kreutzberg, 1987; Kanje et al., 1989; Acheson et al., 1991a; Hendry et al.,1992; Curtis et al., 1994), CAMs including N-CAM, L1, and basal lamina and ECM proteins including laminin, fibronectin and tenascin (Martini and Schachner, 1986; Martin and Timpl, 1987; Chiquet and Wehrle-Haller, 1994; Lettle et al., 1995). It is at this time that gene expression for myelin associated proteins such as P0, MAG, L2/HKN1 are downregulated (Martini and Schachner, 1986, 1988).

It is during this first month that regeneration is the most successful. Morphological studies which suggest that predegeneration of nerve grafts for 7 days to 1 month indicate some benefit of predegeneration for regeneration (Holmes and Young, 1942; Kerns et al., 1993; Sorenson et al., 1993). Yet counts of functional reinnervated motor units indicate that regeneration is not significantly improved (Fu and Gordon, 1995b). Nonetheless, successful regeneration is maintained in the first month during which time Schwann cell numbers (Fig. 7, 8; Salonen et al., 1988) and expression of RAGs are high (Tanuichi et al., 1986, 1988; Heumann et al., 1987a,b; Toma et al., 1992).

IL-1 released from macrophages has been shown to trigger NGF synthesis, but the molecular trigger for p75 and EGFr gene expression is not well understood. Whether the mitogens for Schwann cells are also signals for

Figure 22. Upregulation and downregulation of expression for p75 mRNA and protein. (A) Reploted from the data of Heumann et al (1987b) and Tanuichi et al (1988), showing the increase in expression of mRNA and protein immediately after nerve transection. Solid circles represent p75 mRNA, which increases more rapidly than p75 protein. (B) Data from the present study showing the decline of expression p75 mRNA and protein in denervated sciatic distal nerve stumps with time after sciatic nerve section. The square symbol represents the expression of p75 mRNA and the triangle represents the p75 protein. The decline in p75 mRNA follows a simple exponential with a time constant of 1.5 months, whereas the p75 protein declines with a linear time course. A high level of p75 immunoreactivity was seen 2 (A) and 3 (B) month after nerve transection when mRNA levels were significantly lower, suggesting that p75 protein has a longer half life than its mRNA.


A
P75 mRNA (pg/mg wet weight)
125I-NGF Crosslinked (fmole/mg)
Months
0
10
20
30
40
50
60
70
80
0.0
0.1
0.2
0.3
0.4
0.5
0.0
0.5
1.0
1.5
2.0
2.5


B
Silver Grains/Nucleus
Relative Intensity of Immunoreactivity
Months
0
3
6
9
12
15
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
0
1
2
3
4
5
6
7
8
9
10
11
12

upregulation of p75 and EGFr is not known at this time. The mitogens include myelin debris, macrophages and PDGF (Reynolds and Woolf, 1993).

Long-term denervated stumps

The most novel and important finding of this study is the exponential fall in expression of p75 when denervation is prolonged beyond 1 month (Fig. 12, 13B). mRNA levels fall exponentially and precede the linear decline in p75 immunoreactivity (Fig. 22B), suggesting that p75 protein has a longer half life than its mRNA. The small delay between appearance of p75 protein product and increased mRNA levels during the first week is the expected result for protein synthesis (Fig. 22A). Decline in p75 is normally seen after nerve regeneration. However in this study, there was no nerve regeneration as resection of 5 mm of the sciatic nerve and suture of the proximal stump to an innervated muscle was effective in preventing reinnervation of the long-term denervated stump.

The decline of S100 with prolonged nerve sheath denervation described here (Fig. 17) was consistent with the observations of Salonen et al (1988) in so far as S100 positive cells fell from a high level in the first week of distal nerve sheath denervation. However, their data indicate that S100 levels fell to control levels which was certainly not the case in our study where S100 was undetectable at 6 months (Fig. 16). Furthermore, the number of nuclei remained 3-4 times higher than normal in this study (Fig. 8, 12) in contrast to the findings of Salonen

et al (1988). These conflicting results are difficult to reconcile particularly in view of our observation that S100 is downregulated below visual detection after 4 months. Gutmann and Young (1942) also observed many nuclei in long-term denervated nerve stumps and distinguished atrophic Schwann cells from fibroblasts at the light microscopic level. Yet at the electron microscopic level, some authors claimed that Schwann cells eventually die (Thomas and Hones, 1967; Weinberg and Spencer, 1978).

There are several possible explanations for the downregulation of S100. Either Schwann cells die, dedifferentiate to an embryonic or fetal phenotype which does not express S100 (Jessen et al., 1994), or the cells atrophy and fail to express any known Schwann cell markers. GFAP immunoreactivity which is characteristic of nonmyelinating Schwann cells (Neuberger and Cornbrooks, 1989; Jessen and Mirsky, 1992), vimentin immunoreactivity (for intermediate filaments; Neuberger and Cornbrooks, 1989) were negative in long-term denervated sheaths (Petrov et al., unpublished findings) showing that dedifferentiation to a known phenotype is unlikely. GAP43 immunoreactivity as a marker for Schwann cells in short-term denervated nerve stump (Curtis et al., 1992) was also negative in long-term denervated stumps. In the absence of a marker, the fate of the Schwann cells has to be determined by exclusion (from fibroblasts and macrophages) and by examination the ultrastructural level. ED-1 immunoreactivity was absent in the long-term denervated stumps showing that macrophages were unlikely to account for any significant number of the cells.

Although Thy 1, a classic immunocytochemical marker of fibroblasts was barely detectable (Pethov et al., unpublished finding), fibroblasts were distinguished from Schwann cells in long-term denervated stumps at electron microscopic level by their extensive processes and inclusion of electron dense particles (Fig. 20A; Vuorinen et al., 1994). After nerve injury, fibroblasts infiltrate the distal stump during the earlier stages of degeneration and differentiate into perineurial cells in the later stages of degeneration, even as late as after 12 months of degeneration. They then form minifasicle-like structures around the remaining atrophic Schwann cell columns (Fig. 19; Thomas and Hones, 1967; Weinberg and Spencer, 1978; Roytta and Salonen, 1988; Vuorinen et al., 1994).

Remaining Schwann cells in long-term denervated nerve stumps are highly atrophic and are not surrounded by the characteristic basal lamina of normal Schwann cells (Fig. 19) presumably because of the progressive fragmentation with long-term denervation (Giannini and Dyck, 1990). While disappearance of Schwann cells of normal appearance has been used as evidence for death of Schwann cells after long-term denervation (Weinberg and Spencer, 1978), the presence of increased numbers of nuclei at the light microscopic level and the clear distinction of fibroblasts from the atrophic putative Schwann cell at the electron microscopic level in long-term denervated nerve stumps argues for the survival of Schwann cells. Nonetheless the dramatic downregulation of RAGs and the atrophic appearance of the nonfibroblast cells demonstrates that Schwann cell function is severely curtailed by long-term denervation.

Findings in our laboratory that at least 10% of regenerating axons are successful in growing through highly atrophic nerve stumps (Fu and Gordon, 1995b) demonstrate that a small but limited trophic and substrate support remains. Schwann cells can only migrate for distances of 10-20 mm from proximal stump to support regeneration (Naimd et al., 1990) so that it is unlike that Schwann cell migrate. Distance is not sufficient to account for regeneration of 20-30 mm in the denervated CP nerve (Fu and Gordon, 1995b). The Schwann cell migration can explain the higher success of regeneration through 10-15 mm CP grafts after prolonged denervation as compared with much poorer regeneration through longer predegenerated nerve stumps (Raji, 1994). Preliminary observations of regenerated axon profiles in predegenerated stumps 12 months after nerve repair show that regenerated axons are myelinated (Petrov and Gordon, unpublished data). Thus, atrophic Schwann cells retain a capacity, though limited, for trophic and substrate support and myelination. Since Schwann cells show a second proliferative response when axons regenerate into the stump (Reynolds and Woolf, 1993), it is possible that the atrophic Schwann cells may also respond to mitogens.

Thus, although the time course of upregulation of different RAGs may differ, our study of p75 and EGFr indicates that p75 provides a reasonable molecular marker of the reduced capacity of Schwann cells to express RAGs in long-term denervated stumps. Our results also provide a good molecular and cellular basis for our physiological measurements of the number of axons which successfully regenerate through long-term denervated nerve stumps (Fu and Gordon, 1995b).

The results demonstrate that the severe Schwann cell atrophy is preceded by a dramatic fall in RAGs expression by Schwann cells.

Expression of RAGs is unlikely to be driven by an absence of a repression signal because their expression is not maintained. It is more likely that RAG expression is induced by a positive trigger, particularly as Schwann cell induction involves expression of immediate early genes and RAGs. The severe curtailment of Schwann cell proliferation and expression of RAGs in C57BL/Ola mice was initially attributed to reduced macrophage invasion suggesting that macrophages were essential both for proliferation and induction of RAGs (Perry et al., 1987; Lindholm et al., 1987; Guenard et al., 1991; Rotshenker et al., 1992; Brown et al., 1991, 1994). However, the axonal membrane itself is more resistant to phagocytosis in these mice (Glass et al., 1993). Thus, it appears that macrophages, axonal and myelin debris together are required to activate Schwann cells. The substances involved include PDGF, FGF, TGF, IL-1, LIF and CNTF (Reynolds and Woolf, 1993).

Perhaps it is the clearance of myelin, axon debris and macrophages after Wallerian degeneration which believes the induction of RAGs in the Schwann cells and allows Schwann cell dedifferentiation. The inflammatory response at the injury site which includes macrophage invasion and penetration into the injured nerve resembles many other inflammatory responses in being short lived. Therefore there may be only a small window of opportunity for successful regeneration. The challenge for the future is to prolong this window by prolonging the positive

signals of reduction.

It would be interesting to determine whether the upregulation of growth factors including BDNF and LIF is maintained in the atrophic Schwann cells. Since p75 has been implicated in an autocrine regulation of Schwann cell function (Schecterson and Bethwell, 1992), it is important to establish whether a decrease in neurotrophins and/or their receptors is responsible for their deterioration. In addition, the relative time course of expression of NGF and p75 is important since NGF has recently been shown to promote Schwann cell migration (Anton et al., 1994). Finally, p75 is important in remyelination suggesting a short window of opportunity for the capacity of Schwann cell to form an effective myelin sheath on regenerated axons.

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