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Induction of Postnatal Schwann Cell Death by the Low-Affinity Neurotrophin Receptor *In Vitro* and after Axotomy

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Schwann cells express the low-affinity neurotrophin receptor (p75), but no role for either the neurotrophins or their cognate receptors in Schwann cell development has been established. We have found that Schwann cells isolated from postnatal day 1 (P1) or P2 mice that were p75-deficient exhibited potentiated survival compared to wild-type cells after growth factor and serum withdrawal. There was, however, no disparity in the survival of p75-deficient and wild-type Schwann cells isolated at embryonic day 15, suggesting that the death-inducing effects of p75 are developmentally regulated. A comparable degree of cell death was also observed in the sciatic nerves of both wild-type and p75-deficient mice at P1. However, 24 hr after axotomy, there was a 13-fold increase in the percentage of apoptotic nuclei in the distal nerve stumps of the transected sciatic nerves of neonatal wild-type but not p75-deficient mice. The expression of both the p75 and nerve growth factor (NGF) genes was upregulated after axotomy in neonatal wild-type nerves. Collectively, these results suggest that NGF-mediated activation of p75 is likely to be an important mediator of Schwann cell apoptosis in the context of peripheral nerve injury.

Key words: Schwann cells; cell death; apoptosis; p75; low-affinity neurotrophin receptor; p75 knock-out

During postnatal development, Schwann cell numbers are regulated to establish a precise association with the axons that they ensheath. The balance between Schwann cell proliferation and apoptosis influences this process (Grinspan et al., 1996; Syroid et al., 1996; Trachtenberg and Thompson, 1996). Schwann cell apoptosis is modifiable by several mechanisms, first by autocrine survival factors such as leukemia inhibitory factor (Dowsing et al., 1999), insulin-like growth factor-1 (Syroid et al., 1999), insulin-like growth factor-2, platelet-derived growth factor, and neurotrophin-3 (Meier et al., 1999). Second, Schwann cell apoptosis is inhibitable by axonally produced neuregulin-1 (NRG-1). This finding, together with the observation that apoptosis in axotomized neonatal nerve is markedly increased, has suggested that peripheral nerve modeling is consequent to the death of a subpopulation of Schwann cells that have lost access to axonally derived growth factors (Marchionni et al., 1993; Grinspan et al., 1996; Syroid et al., 1996). A third and unexplored potential mechanism is that Schwann cell apoptosis is actively induced.

One molecule implicated in the induction of Schwann cell apoptosis is the low-affinity neurotrophin receptor p75 (Johnson et al., 1986), and it is of note that the expression of p75 is upregulated by Schwann cells in mature peripheral nerve after axotomy (Taniuchi et al., 1986). The cytoplasmic domain of p75 exhibits sequence similarity with the cytoplasmic domains of Fas/Apo-1 and tumor necrosis factor receptor-1 (Goodwin et al., 1991; Oehm et al., 1992; Carter and Lewin, 1997), which act as cell death effectors (Watanabe-Fukunaga et al., 1992; Ashkenazi and Dixit, 1998). Furthermore, p75 has already been implicated in the death of several neural cell types, including sensory and retinal ganglion neurons and oligodendrocytes (Rabizadeh et al., 1993; Barrett and Bartlett, 1994; Casaccia-Bonnefil et al., 1996; Frade et al., 1996; Bamji et al., 1998; Frade and Barde, 1998), and transgenic mice expressing the intracellular domain of the p75 neurotrophin receptor exhibit increased neuronal apoptosis (Majdan et al., 1997).

It is also established that p75 can act in concert with a second class of receptors, the tyrosine kinase (Trk) family, to potentiate Trk-mediated signal transduction and neural cell survival after ligand binding (Berg et al., 1991; Hempstead et al., 1991). Each of the Trk family members preferentially interacts with different neurotrophin ligands [TrkA interacts with nerve growth factor (NGF), TrkB with both brain-derived neurotrophic factor (BDNF) and neurotrophin-4 (NT-4), and TrkC with neurotrophin-3 (NT-3)] (Hanks et al., 1988; Kaplan et al., 1991; Klein et al., 1991, 1992; Lamballe et al., 1991, 1993; Middlemas et al., 1991). All four neurotrophins bind to p75 (Rodriguez-Tebar et al., 1990, 1992).

In oligodendrocytes, the death-promoting activity of p75 occurs in the absence of TrkA expression (Yoon et al., 1998). It is thus potentially relevant that postnatal sciatic nerves express truncated, kinase-deficient TrkB, together with truncated and full-length TrkC but not TrkA (Offenhauser et al., 1995). Furthermore, TrkA is not expressed by either intact or axotomized adult peripheral nerve (Funakoshi et al., 1993). Schwann cells do, however, express the NGF gene (Matsuoka et al., 1991), suggesting that autocrine, ligand-dependent signaling via p75, in the absence of TrkA, might induce Schwann cell apoptosis.

In this paper we demonstrate that the death of postnatal wild-type mouse Schwann cells is potentiated *in vitro* by p75, probably mediated via binding of endogenously produced NGF. This phenomenon is developmentally regulated, given that there is no difference in the viability of embryonic day 15 (E15) Schwann cells isolated from wild-type and p75-deficient mice. Furthermore, *in vivo*, the apoptosis of Schwann cells in postnatal day 1 (P1) sciatic nerves is not p75-dependent. However, the upregulated death of postnatal Schwann cells after axotomy is p75-dependent, correlating with increased expression of p75 and NGF in this circumstance.

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MATERIALS AND METHODS

Cell culture and survival assays. Cultures of murine Schwann cells were prepared from P1–P2 sciatic nerve and purified to >95% homogeneity essentially as previously described (Brockes et al., 1979), using the 30H12 antibody that recognizes Thy1.2. The cellular populations were expanded by culture in DMEM, 10% fetal calf serum (FCS), and NRG-1 (Amgen, Thousand Oaks, CA; 10–50 ng/ml). Cells used for survival assays were passaged a maximum of four times. The cells were dissociated by trypsinization, then washed once in DMEM containing 10% FCS to inactivate the trypsin, and then washed an additional two or three times in serum-free DMEM. Cells were plated at ~800 cells per well in microwell plates (Nunc, Roskilde, Denmark) in DMEM. Assays were performed serially over a 3 d period using multiple microwell plates, such that numbers of viable cells at each time point (0, 1, 2, and 3 d) were scored from either five or six wells in a single plate using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) survival assay (Barres et al., 1992; Syroid et al., 1996). In this assay, MTT (Sigma, St. Louis, MO) was added to cells at a concentration of 0.5 mg/ml, and the cells were then further incubated at 37°C for 1 hr. In microtiter wells, between 20 and 25% of the plated cells adhere to the base rather than the angled sides of the well. Counts of viable cells were determined by assessing the number of cells exhibiting a blue granular reaction in the base of each well after addition of MTT, using bright-field microscopy. Assays comparing the survival of postnatal wild-type and p75-deficient Schwann cells were conducted using three separate litters obtained from matings of mice heterozygous for the p75 deficiency (Lee et al., 1992). Schwann cells isolated from each mouse were kept separate, and survival assays were performed without knowledge of the genotype. Genotyping was performed on DNA isolated from tails, using PCR.

Schwann cells were dissociated from the sciatic nerves of embryonic day 15 homozygous p75-deficient mice and age-matched wild-type mice, maintained on a Balb/c and 129 outbred genetic background, by incubation with trypsin (0.25%; Life Technologies, Gaithersburg, MD) and collagenase (0.02%; Sigma) for 30 min. Trypsinization was inhibited by the addition of FCS, the cells were then pelleted, and single cell suspensions were generated by mechanical dissociation with passage of the cells through 18, 21, and 23 gauge needles. The cells were then plated onto laminin-treated (20 μg/ml; Collaborative Research, Bedford, MA) wells in flat-bottomed 96 well plates (Linbro) in DME and 10% FCS at a density of $\sim 2.5 \times 10^3$ cells per well. After 24 hr of culture, the wells were washed three times with DMEM, the number of viable cells was then assessed by phase microscopy, and the cells were then cultured for a further 72 hr, at which time the number of viable cells was reassessed.

RT-PCR and ribonuclease protection analysis. Total cellular RNA from Sprague Dawley rat sciatic nerves was prepared and analyzed by ribonuclease protection assay as described previously (Chomczynski and Sacchi, 1987; Krieg and Melton, 1987). RNase digestions were performed for 3 hr at room temperature using RNase A and RNase T_1 (~60 μg/ml and 30 U/ml, respectively; Boehringer Mannheim, Mannheim, Germany). Antisense RNA probes were synthesized using the Maxiscript *in vitro* transcription kit (Ambion, Houston, TX) from templates containing the following inserts: a 770 bp rat β-NGF cDNA fragment encompassing the entire coding sequence (nucleotides 251–1021; Whittemore et al., 1988), a 452 bp rat p75 cDNA fragment encompassing the intracellular domain (nucleotides 939–1391; Radeke et al., 1987), and a 316 bp rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA fragment derived from exons 5–8 (nucleotides 369–685; pTRI-GAPDH-rat; Ambion). The β-NGF RNA probe reproducibly gives rise to a major protected RNA fragment of ~484 bp; three smaller species of 270, 219, and 200 bp are also generated and likely reflect the creation of RNase-sensitive sites caused by the secondary structure of RNA and the stringent digestion conditions used (data not shown). p75 and GAPDH RNA probes of low specific activity were generated by including 0.05 mM UTP in transcription reactions and using only two-fifths (20 μCi) the usual amount of [α-^{32}P]UTP (Amersham, Arlington Heights, IL). Either 1 μg of RNA or 10 μg of tRNA was cohybridized with 120,000 CPM of either p75 or NGF RNA probe and 100,000 CPM of GAPDH RNA probe, as indicated. The relative quantity and integrity of RNA used in each experiment was confirmed on agarose gels (data not shown). Protected probe/RNA hybrids were resolved on 6% polyacrylamide/8 M urea denaturing gels. Gene expression was quantitated using a Molecular Dynamics (Eugene, OR) PhosphorImager (model 425S) and ImageQuant version 5.0 software.

RNA was isolated using the Qiagen RNeasy minikit from Thy-1-sorted Schwann cells initially obtained from P1 wild-type mice that had been subjected to FCS and NRG-1 withdrawal for 4 hr. RT-PCR was performed as described in the Life Technologies SuperscriptII kit using 3 μg of total RNA and Moloney murine leukemia virus reverse transcriptase for cDNA synthesis. Amplification of the cDNA was performed on a Perkin-Elmer (Norwalk, CT) Celtus-DNA thermal cycler 480 machine in the following buffers: Tris/HCl (20 mM, pH 8.4), 50 mM KCl, 200 μM of each dNTP, 5 U of Life Technologies *Taq* DNA polymerase, and 1.5 mM $MgCl_2$. Oligonucleotide primers were used at 10 μM. To amplify NGF, the forward primer was 5′-GGCCCATGGTACAATCCCTTTCA-3′, and the reverse primer was 5′-TCAGCCTCTTCTTGTAGCCTTCCT-3′. The expected RT-PCR product was 409 bp. To amplify p75, the forward primer was 5′-AACAGGGGCACCCTAAGACTCAGG-3′, and the reverse primer was 5′-TTTCAGCTCAGATAGGCC-3′. The expected RT-PCR product was 172 bp. To amplify trkA, the forward primer was 5′-CGTAGTCCCAGCCAGTGTGC-3′, and the reverse primer was 5′-TCAGGGTTGAACTCAAAAGG- 3′. The expected RT-PCR product was 320 bp. For each amplification, 35 cycles of denaturation (94°C, 60 sec), annealing (55°C, 60 sec), and extension (72°C, 120 sec) were performed. One fifth of the total PCR mix was resolved on a 1.5% agarose slab gel, and the PCR products were visualized by ethidium bromide staining. A ΦX174 *Hae*III DNA ladder (Life Technologies) was used to establish the size of the amplified bands.

Immunohistochemistry. To assess for the expression of S-100, Schwann cells were first fixed in 4% paraformaldehyde for 30 min and pretreated with 2% Tween 20 before incubation with rabbit anti-cow S-100 (Dako, Carpinteria, CA) at a dilution of 1:200. The cells were subsequently incubated with biotin-conjugated goat anti-rabbit Ig, and staining was completed using a Vectastain kit (Vector Laboratories, Burlingame, CA).

Sciatic nerve transection. Adult and P1 Sprague Dawley rats were gas-anesthetized using isoflurane, and unilateral sciatic nerve transections were performed just proximal to the sciatic notch as described previously (Zorick et al., 1996). Animals were killed 24 hr after transection, at which time both the unlesioned contralateral sciatic nerve and the entire length of the distal stump of the transected nerve were isolated and processed for RNA preparation.

Postnatal day 1 wild-type [129/SV × Balb/c] and p75-deficient mice were also axotomized. These animals were rendered unconscious under ice-induced hypothermia. The left sciatic nerve was then exposed, and transected distal to the sciatic notch using iridectomy scissors, and the wounds were closed using 9–0 surgical sutures. The pups were allowed to warm, and when fully conscious were returned to their mothers. At either 24 or 72 hr after axotomy, pups were rendered unconscious again, and they were transcardially perfused with PBS followed by 4% paraformaldehyde. The proximal and distal segments of the axotomized left sciatic nerve were removed, along with the intact right sciatic nerve.

Sciatic nerves from P1 wild-type and p75-deficient animals that had not been subjected to axotomy were also harvested. These nerves, together with those obtained from the axotomized animals, were fixed in 4% paraformaldehyde–PBS for 30 min and were then stored in 0.5% paraformaldehyde–PBS before processing. Nerves were first embedded in agar (2.5% in PBS; Sigma) and then incubated overnight in 20% sucrose–PBS at 4°C. The sciatic nerves were then embedded in TissueTek OCT compound (Miles, Elkhart, IN) and frozen on dry ice.

Terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling assays. Preparation of 8 μm longitudinal sciatic nerve sections and terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) assays were performed as described previously (Syroid et al., 1996). Schwann cells were identified by their typical elliptical nuclear morphology in longitudinal sciatic nerve sections. Apoptotic Schwann cells were identified by correlating a TUNEL-positive signal with alterations in nuclear morphology (chromatin condensation, nuclear fragmentation). The number of apoptotic Schwann cells was assessed in both the distal and proximal stumps of axotomized nerves, in addition to the intact contralateral nerves of axotomized mice, as well as the intact nerves of P1 mice that had not been subjected to axotomy. Three wild-type and p75-deficient mice were analyzed from both the unoperated and 24 hr post-axotomy groups, and five wild-type and six p75-deficient mice were assessed at the 72 hr time point. A minimum total of 25,000 Schwann cell nuclei was counted for each condition.

Statistical analysis. The statistical significance of differences between the experimental groups was analyzed by a two-tailed Student's *t* test.

RESULTS

Postnatal Schwann cells isolated from mice deficient for the p75 gene exhibit a survival advantage relative to wild-type cells

We initially assessed the role of p75 in regulating the survival of postnatal (P1–P2) Schwann cells by comparing the viability of Schwann cells isolated and purified from p75-deficient mice (Lee et al., 1992) and that of wild-type littermates. The cells were initially allowed to proliferate in serum and NRG-1, and to set up survival assays, the cells were then dissociated and resuspended in unsupplemented DMEM. The plating density was ~800 cells per well in microwell plates, and the viability of the cells was assessed daily, for 3 d, using an MTT assay. The p75-deficient Schwann cells displayed a modest but significant survival advantage, with ~60% of these cells surviving the 3 d culture period, in comparison to the wild-type cultures in which, on average, less than 40% of the cells survived (Fig. 1*A*). There were also more crenated cells in the cultures of wild-type (Fig. 1*B*) than p75-deficient (Fig. 1*C*) Schwann cells, and these cells displayed nuclear condensation, consistent with death by apoptosis, as previously described (Syroid et al., 1996).

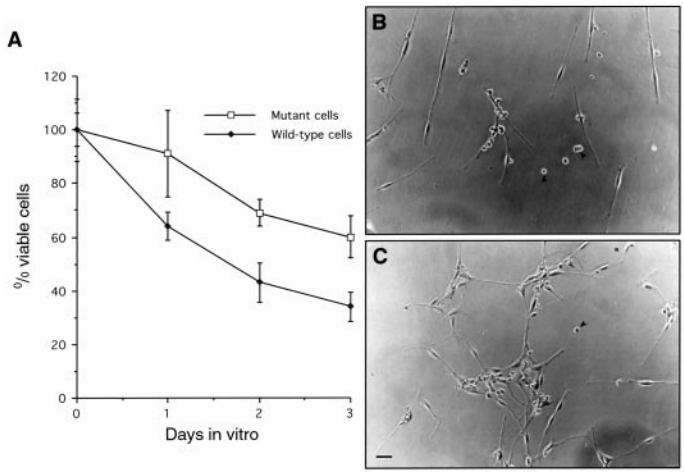

Figure 1. Postnatal Schwann cells isolated from p75-deficient mice exhibit a survival advantage *in vitro*, in comparison to wild-type cells, after growth factor withdrawal. Shown in *A* is a graphical representation of survival of postnatal cells using an MTT assay, from a representative experiment of three independent assessments, with five separate wells assessed daily over a 3 d period. Shown in *B* and *C* are photomicrographs of cultures of postnatal wild-type (*B*) and p75-deficient (*C*) cells grown in serum-free conditions for 48 hr demonstrating a large number of crenated wild-type cells (*arrowheads*), whereas the majority of p75-deficient cells maintain a bipolar morphology. Scale bar, 10 μm.

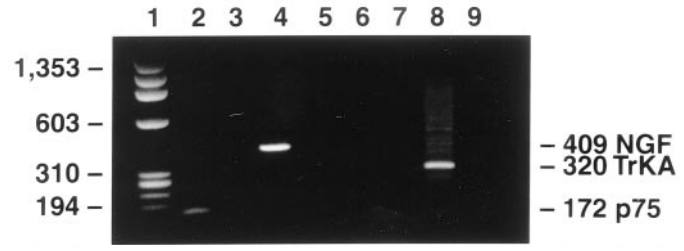

Figure 2. The p75 and NGF genes are expressed by postnatal wild-type Schwann cells after growth factor withdrawal, but the trkA gene is expressed at barely detectable levels. cDNA samples generated from purified wild-type Schwann cells were subjected to PCR. The expected 172 bp p75 (*lane 2*) and 409 bp NGF (*lane 4*) fragments were amplified from the sample. The expected 320 bp (*lane 6*) trkA fragment was also amplified, although at a very low level. This contrasted with the robust amplification of the expected 320 bp trkA fragment in a cDNA sample derived from PC12 cells (*lane 8*). Negative controls were obtained by subjecting reagents to PCR reaction conditions without template cDNA (p75 primers, *lane 3;* NGF primers, *lane 5;* trkA primers, *lane 7*), and there was no amplification of the relevant bands. *Lane 1* represents the ΦX174 *Hae*III DNA ladder.

Postnatal Schwann cells express the p75 and NGF genes

We next confirmed, using RT-PCR, that wild-type mouse Schwann cells cultured under the same conditions as the survival assay (in unsupplemented DMEM) expressed the p75 gene (Fig. 2). This suggested that the survival disadvantage of wild-type cells was likely to be a direct consequence of disparity in the expression of p75 between the wild-type and p75-deficient populations. We also established that the wild-type cells expressed the NGF gene, one of the cognate ligands of p75, whereas the gene encoding the high-affinity NGF receptor (TrkA) was barely detectable (Fig. 2).

Both embryonic wild-type and p75-deficient Schwann cells exhibit potentiated *in vitro* viability in comparison to that of postnatal wild-type cells

It was possible that postnatal p75-deficient Schwann cells represented a selected subpopulation of the Schwann cells generated during embryogenesis. To investigate this possibility, Schwann cells were isolated from the sciatic nerves of both wild-type and p75-deficient mice at embryonic day 15, corresponding to the developmental stage at which definitive Schwann cells are thought to be first generated (Jessen et al., 1994). The cells were cultured in DMEM and 10% FCS for 24 hr, at which time 92% of plated p75-deficient cells and 87% of wild-type cells expressed the Schwann cell-specific marker, S100. After 24 hr, the cells were transferred to unsupplemented DMEM, and the viability of the cells at this time was compared to the viability after a further 3 d of culture. The p75-deficient and wild-type cells exhibited similar viability [80.1 ± 10% of wild-type cells and 91.4 ± 7.8% of p75-deficient cells remained viable after 3 d of culture in DMEM ($n = 6$ and 4, respectively; $p > 0.10$)]. This suggested that the survival advantage of postnatal p75-deficient Schwann cells was not the consequence of deletion in embryogenesis of a death susceptible subpopulation of cells that otherwise survived in wild-type mice until postnatal development.

During postnatal development the percentage of Schwann cells undergoing apoptosis is similar in the sciatic nerves of wild-type and p75-deficient mice

We and others had previously identified that Schwann cell death occurs in a stochastic fashion in the peripheral nerves of the postnatal rat (Grinspan et al., 1996; Syroid et al., 1996). We therefore investigated whether peripheral nerves isolated from postnatal p75-deficient mice exhibited reduced cell death. To study this, we isolated sciatic nerves from both P1 wild-type and p75-deficient mice. Using TUNEL assays, we found that the percentage of apoptotic cells was similar to that which we had previously observed in the neonatal rat sciatic nerve (Syroid et al., 1996). However, there was no significant difference in the percentage of apoptotic nuclei in normal (0.13%) and p75-deficient nerves (0.14%) (Tables 1, 2). This demonstrated that although deficiency of the p75 gene renders Schwann cells less susceptible to cell death *in vitro* in basal conditions, apoptosis can still occur in the Schwann cells of p75-deficient mice *in situ*.

After axotomy the percentage of Schwann cells undergoing apoptosis is significantly upregulated in the distal stumps of postnatal wild-type sciatic nerves in comparison to p75-deficient mice

It remained possible that activation of p75 accounted for the upregulated Schwann cell apoptosis that occurs in the sciatic nerves of neonatal rodents after axotomy, previously documented by Grinspan et al. (1996). To investigate this, sciatic nerves from both P1 wild-type and p75-deficient mice were transected distal to the sciatic notch. Both the distal and proximal nerve stumps, together with the intact contralateral nerve, were then harvested either 24 or 72 hr after axotomy. As indicated in Tables 1 and 2, transection resulted in a very significant (13-fold) increase in the percentage of apoptotic Schwann cells in the distal nerve stumps of wild-type animals at 24 hr after axotomy and a twofold increase 72 hr after axotomy. There was, however, no significant increase in the percentage of apoptotic nuclei in the p75-deficient mice at either time point (Fig. 3). On the other hand, there was no increase in the percentage of apoptotic nuclei in the proximal nerve stumps of either the wild-type or the p75-deficient nerves. Collectively, these findings suggest that p75 is implicated in mediating the death of Schwann cells that have lost contact with axons and, hence, neuronal-derived growth factors.

The expression of the p75 and NGF genes is upregulated after axotomy

Given that p75-mediated postnatal Schwann cell death *in vitro* was likely to be consequent to NGF binding, we next confirmed that the NGF gene was expressed within postnatal and adult rodent sciatic nerves. Using RNase protection, we confirmed that P1 rat nerves expressed NGF mRNA and that the level of expression of the NGF gene was upregulated approximately threefold 24 hr after axotomy (Fig. 4). This result, together with our *in vitro* findings, suggests that Schwann cells are themselves a source of NGF within the developing nerve, enabling cell death to be initiated by either paracrine

Table 1. Percentage of Schwann cell apoptosis in intact P1 wild-type and p75-deficient sciatic nerves

	Wild-type		p75-deficient	
Nerve	Frequency apoptosis (%)	Mean ± SD	Frequency apoptosis (%)	Mean ± SD
Nerve 1	0.15		0.15	
Nerve 2	0.16	0.13 ± 0.05%	0.18	0.14 ± 0.05%
Nerve 3	0.07		0.08	

TUNEL assays were conducted on longitudinal sections of P1 sciatic nerve from p75-deficient mice and wild-type littermates. No difference in Schwann cell apoptosis was observed in the intact postnatal sciatic nerves of wild-type and p75-deficient mice.

Table 2. Percentage of Schwann cell apoptosis 24 and 72 hr after transection of P1 wild-type and p75-deficient sciatic nerves

	24 hr after transection		72 hr after transection	
Nerve	Wild-type (n = 3) Mean ± SD (%)	p75-deficient (n = 3) Mean ± SD (%)	Wild-type (n = 5) Mean ± SD (%)	p75-deficient (n = 6) Mean ± SD (%)
Distal nerve stump	1.72 ± 0.93*	0.17 ± 0.03*	0.30 ± 0.16**	0.16 ± 0.06**
Proximal nerve stump	0.14 ± 0.05	0.15 ± 0.03	0.17 ± 0.08	0.04 ± 0.02
Contralateral nerve	0.21 ± 0.08	0.16 ± 0.06	0.16 ± 0.07	0.04 ± 0.05

P1 nerves from p75-deficient mice and wild-type littermates were transected and analyzed by TUNEL. Transection results in a 13-fold increase in apoptosis 24 hr after sciatic nerve transection in the distal nerve stump of wild-type animals. In comparison, there was a twofold increase in Schwann cell apoptosis occurring 72 hr after transection of wild-types, whereas no such increase is observed in the distal stump of p75-deficient nerves at either time point. No differences in Schwann cell apoptosis were observed in the proximal nerve stumps or in contralateral control nerves. Sciatic nerve sections were stained with Hoechst nuclear stain to identify Schwann cell nuclei: a minimum of 25,000 Schwann cells were counted for each condition. (*,**$P \leq 0.05$; two-tailed t test.)

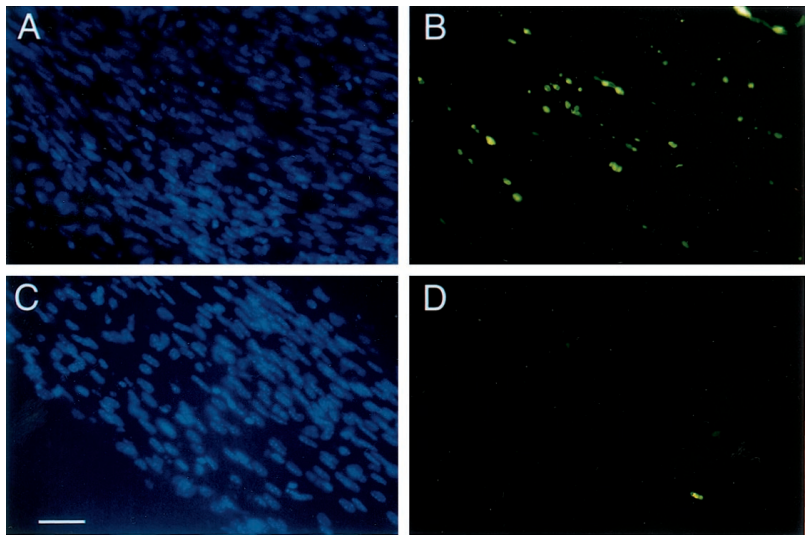

Figure 3. Schwann cell apoptosis is upregulated in the axotomized nerves of P1 wild-type mice in comparison to that exhibited in the axotomized nerves of p75-deficient mice. The sciatic nerves of P1 mice were axotomized, and the distal stumps were harvested 24 hr later. The sectioned nerves were assessed using DAPI staining to identify nuclei (*A, C*) and by TUNEL (*B, D*). A significantly increased number and percentage of condensed and TUNEL-positive nuclei were present in the wild-type nerves (*A, B*) in comparison to the number observed in nerves isolated from p75-deficient mice (*C, D*). Scale bar, 10 μm.

or autocrine mechanisms. The result also suggests that an increase in the availability of NGF is unlikely to be the sole cause of the upregulated cell death that occurs in the transected postnatal nerve. Consistent with this view, we found that the level of expression of the p75 gene was upregulated ~14-fold 24 hr after axotomy (Fig. 4). This suggested that an increase in p75 expression was likely to be a significant contributory factor to the induction of potentiated Schwann cell death observed within postnatal nerves undergoing Wallerian degeneration. In contrast, although expression of the NGF and p75 genes were also upregulated in adult nerve after axotomy, the expression was only increased to levels similar to those found in the intact postnatal nerve.

DISCUSSION

Our initial results established that postnatal Schwann cells isolated from mice with a deficiency for the p75 gene exhibit a survival advantage over wild-type cells in basal conditions. This differential survival potential could have either been attributable to signaling via p75 in wild-type cells or, alternatively, could have been the consequence of the premature loss, during embryogenesis in p75-deficient mice, of a subpopulation of Schwann cells with increased susceptibility to death. Analysis of E15 Schwann cells suggested that there was no differential survival potential between newly generated wild-type and p75-deficient cells, and both embryonic populations exhibited potentiated survival in comparison to postnatal wild-type cells. This result argued against heterogeneous survival potential among newly generated Schwann cells in embryonic p75-deficient mice and also indicated that p75-mediated effects within the Schwann cell lineage are developmentally regulated. Thus, the parsimonious explanation for the differential survival of the postnatal wild-type and p75-deficient cells is that p75 is directly implicated in the mediation of Schwann cell death.

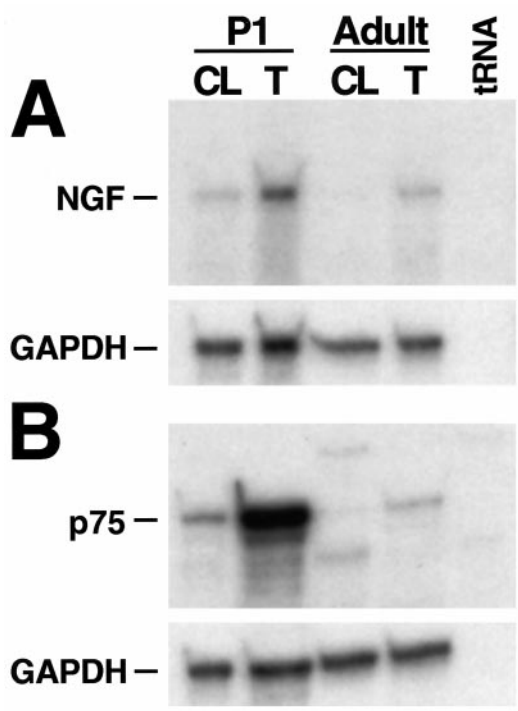

Figure 4. Expression of NGF and p75 mRNA is upregulated in rat sciatic nerve during Wallerian degeneration. Unilateral transections of P1 and adult rat sciatic nerves were performed, and ribonuclease protection analyses were performed using total RNA (1 μg per hybridization reaction) isolated from both the intact contralateral nerve (*CL*) and the transected distal nerve stump (*T*), 24 hr after transection, or using 10 μg of tRNA as a negative control, as indicated. NGF expression (*A*) and p75 expression (*B*) in the degenerating neonatal nerve is upregulated by ~3-fold and 14-fold, respectively, relative to that in the contralateral control nerve. NGF and p75 expression is also upregulated in degenerating adult nerves, but the level of expression only reaches that found in the intact P1 nerve. The major 484 bp protected NGF RNA fragment and the 452 bp protected p75 RNA fragment are indicated. Autoradiographic exposure was for 66 hr. The bottom panels in *A* and *B* show the relative GAPDH expression from corresponding cohybridization reactions using the GAPDH RNA probe. Autoradiographic exposure was for 20 hr.

This effect could have been mediated by constitutive activity of the p75 receptor, as suggested by Rabizadeh et al. (1993); alternatively, the p75-mediated activity could have been ligand-dependent. Ligand-induced activation of p75 would indeed appear to be a potential contributory mechanism, given that preliminary results indicate that anti-NGF antibodies increase the viability of wild-type Schwann cells *in vitro* by up to 50% after 48 hr in DMEM, whereas they have no effect on the viability of p75-deficient cells (T. J. Kilpatrick, unpublished data). This would suggest that endogenously produced NGF can inhibit Schwann cell viability by either autocrine or paracrine mechanisms.

Although our results implicate a role for p75 in the death of wild-type cells *in vitro*, there was no difference in the survival of wild-type and p75-deficient Schwann cells within intact P1 sciatic nerves. Furthermore, there was a basal level of cell death in the p75-deficient mice, indicating that either there is no role for p75 in the induction of Schwann cell apoptosis in postnatal development or that alternative signal transduction pathways mediating cell death can be induced in the context of p75 deficiency.

What could account for the disparity between the *in vitro* and *in vivo* results? First, it is well established that the expression of the p75 gene is upregulated during *in vitro* culture (Lemke and Chao, 1988), suggesting that axonal contact could, of itself, downregulate p75 expression. Second, Schwann cells *in vivo* are exposed to axonally derived factors, such as NRG-1 and insulin-like growth factor-1, which are known to act as Schwann cell survival factors (Syroid et al., 1996, 1999). It is of note, however, that rat Schwann cells grown *in vitro* with the survival factors NRG-1 and IGF-1 remain susceptible to p75-induced killing (Soilu-Hänninen et al., 1999). Ultimately, it is likely that the balance between the supply of trophic factors and NGF, together with the expression profile of p75, are critical determinants of whether a postnatal Schwann cell survives or dies.

After axotomy, the percentage of Schwann cells undergoing apoptosis was considerably increased in the distal stumps of postnatal wild-type nerves at 24 hr after axotomy and also increased, although to a lesser extent, at 72 hr after axotomy, data essentially in agreement with those generated by Grinspan et al. (1996). We also found that the percentage of Schwann cells undergoing apoptosis was significantly greater within the distal nerve stumps of postnatal wild-type nerves in comparison to the distal nerve segments of p75-deficient mice. The absence of p75 appears to prevent rather than delay apoptosis because there was a clear disparity in the percentage of apoptotic Schwann cells within wild-type and p75-deficient distal sciatic nerve segments at 72 hr as well as 24 hr after axotomy. In summary, this set of observations strongly suggests that p75 is implicated in mediating the Schwann cell death observed in the wild-type animals.

What accounts for the increased susceptibility of neonatal wild-type Schwann cells to die after axotomy? First, we have documented that the expression of the p75 gene is very significantly upregulated in the distal stump of postnatal nerves after axotomy. Second, the access of cells in the distal stump to axonally produced growth factors is rapidly abrogated after axotomy. Third, the inflammatory cells that infiltrate peripheral nerve after axotomy can secrete NGF (Lindholm et al., 1987). With regard to this latter phenomenon, it is of note that Frade and Barde (1998) have suggested that microglia secrete the NGF that activates p75 on chick retinal ganglion cells, to expedite the developmentally regulated death of these neurons. It is possible, however, that infiltrating inflammatory cells are not the only source of NGF within postnatal sciatic nerve after axotomy, because we have shown that Schwann cells cultured *in vitro* express NGF mRNA and that levels of NGF mRNA were only threefold greater in the distal stump than in the intact nerve.

One potential caveat with respect to the *in vivo* results is that a significant proportion of dorsal root ganglionic (DRG) sensory neurons express p75 (Buck et al., 1987; Verge et al., 1992) and that the number of DRG neurons is reduced in the p75-deficient mice (Stucky and Koltzenburg, 1997). The responsiveness of these neurons to p75 appears to be developmentally regulated; on the one hand, p75 potentiates the survival of embryonic DRG neurons, presumably by increasing the affinity of neurotrophin binding to the Trk receptors but, on the other hand, p75 also potentiates the death of postnatal DRG neurons (Barrett and Bartlett, 1994). Could the deficiency of DRG neurons in the p75-deficient mice explain the disparity in Schwann cell death observed in the axotomized wild-type and p75-deficient nerves? Such a disparity could occur if in the p75-deficient mice there were a pre-existing loss of a subpopulation of DRG neurons that after axotomy in wild-type mice displayed disproportionately impaired capacity to provide trophic support to Schwann cells within the distal stump. However, our *in vitro* results, in which postnatal p75-deficient cells displayed a significant survival advantage in the absence of axonal influence, argue strongly for an intrinsic difference between the wild-type and p75-deficient Schwann cells. Furthermore, it has been suggested that the loss of sensory neurons in p75 mice is not confined to a functionally defined subpopulation (Stucky and Koltzenburg, 1997). An alternative explanation for the *in vivo* result could be that there are intrinsic differences between wild-type Schwann cells that ensheath the subpopulation of DRG neurons that are deficient in the p75-deficient mice and those Schwann cells that ensheath the neuronal population common to both wild-type and p75-deficient nerves. This did not appear to be the case, because there was a random array of TUNEL-positive Schwann cell nuclei in the wild-type nerves rather than a concentration of these nuclei along the course of individual axons. We conclude that it is most likely

that the differences observed in Schwann cell viability in wild-type and p75-deficient mice after axotomy are a direct consequence of the differential expression of p75 by these cells.

It will be important to establish whether p75-mediated Schwann cell death influences repair after peripheral nerve injury in the postnatal animal. Subsequent to nerve crush, axonal regrowth is dependent on the survival and proliferation of Schwann cells distal to the injury site (Abercrombie and Johnson, 1946; Grinspan et al., 1996). However, it is also possible that regulated apoptosis of Schwann cells is necessary to facilitate nerve repair. Thus, it is uncertain as to whether repair will be potentiated or compromised in the context of p75 deficiency. The situation could be further complicated by the possibility that p75 expressed on Schwann cells might act to enhance the presentation of the neurotrophins to axons, thereby promoting neuronal regeneration (Zhou et al., 1996).

It is well established that Schwann cell apoptosis does not occur in the distal stump of axotomized adult rodent nerves even at intervals as long as 60 d after axotomy (Grinspan et al., 1996). This difference in Schwann cell viability within axotomized postnatal and adult nerves could be accounted for by either environmental differences or, alternatively, by intrinsic differences between the postnatal and adult Schwann cell. With regard to the latter possibility, it is of note that although the expression of the p75 and NGF genes is upregulated within the distal nerve stump of axotomized adult nerves, their levels of expression are much less than that observed within transected postnatal nerves. It is also possible that there are developmentally regulated differences in the ability of Schwann cells to signal transduce via the p75 pathway. We have recently established that there is a disparity in the ability of the anti-apoptotic protein, Bcl-2, and the poxvirus caspase inhibitor, CrmA (cytokine response modifier A) to protect Schwann cells against p75-mediated apoptosis. Bcl-2 fails to provide protection, whereas the CrmA-mediated potentiation of survival suggests that either caspase-1 or -8, both of which act as apoptotic proteases, is involved in the p75-mediated signal transduction pathway (Soilu-Hänninen et al., 1999). It has also recently been suggested that in oligodendroglia, caspase-1, -2, and -3 are activated downstream of p75 (Gu et al., 1999). It will thus be of considerable interest to determine whether there is a difference in the expression profile of these proteins, as well as of various "inhibitor of apoptosis" proteins (Deveraux and Reed, 1999), within postnatal and adult Schwann cells. It will also be important to determine whether there is reactivation of the p75 signal transduction pathway within Schwann cells in the context of peripheral neuropathic disorders in which there is upregulation of Schwann cell apoptosis. In this way, it may be possible to predict the therapeutic utility of modulating p75 expression in the context of peripheral neuropathy.

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