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Transforming Growth Factor β (TGFβ) Mediates Schwann Cell Death *In Vitro* and *In Vivo*: Examination of c-Jun Activation, Interactions with Survival Signals, and the Relationship of TGFβ-Mediated Death to Schwann Cell Differentiation

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In some situations, cell death in the nervous system is controlled by an interplay between survival factors and negative survival signals that actively induce apoptosis. The present work indicates that the survival of Schwann cells is regulated by such a dual mechanism involving the negative survival signal transforming growth factor β (TGFβ), a family of growth factors that is present in the Schwann cells themselves. We analyze the interactions between this putative autocrine death signal and previously defined paracrine and autocrine survival signals and show that expression of a dominant negative c-Jun inhibits TGFβ-induced apoptosis. This and other findings pinpoint activation of c-Jun as a key downstream event in TGFβ-induced Schwann cell death. The ability of TGFβ to kill Schwann cells, like normal Schwann cell death *in vivo*, is under a strong developmental regulation, and we show that the decreasing ability of TGFβ to kill older cells is attributable to a decreasing ability of TGFβ to phosphorylate c-Jun in more differentiated cells.

Key words: autocrine signals; apoptosis; nerve development; peripheral nerve; nerve injury; nerve regeneration

It is likely that two sets of signals play a major role in promoting the survival of developing Schwann cells. These are β-neuregulins (NRGβs) (Dong et al., 1995; Grinspan et al., 1996; Syroid et al., 1996; Trachtenberg and Thompson, 1996) and autocrine Schwann cell signals, which include a synergistic combination of insulin-like growth factor 2 (IGF-2), neurotrophin-3 (NT3), and platelet-derived growth factor-BB (PDGF-BB) (Meier et al., 1999) in addition to leukemia-inhibitory factor (LIF) (Dowsing et al., 1999; Jessen and Mirsky, 1999). NRGβ is provided mainly by axons and is probably of paramount importance in embryonic and early postnatal nerves, whereas the autocrine circuits are active in postnatal cells and likely to be especially significant after injury and consequent loss of axonal NRGβ. It is possible to envisage that Schwann cell survival is regulated exclusively by these and other positive survival factors, a view that would imply that Schwann cell death, seen for example in normal and, especially, injured neonatal nerves, is caused by a limited availability of such signals, in line with the classical neurotrophic theory. More recently, an alternative view of why cells die has emerged (Cassacia-Bonnefil et al., 1999; Raoul et al., 2000). These experiments indicate that cell death can be caused not only by the absence of survival signals but also by the advent of active cell killing mediated by factors that trigger apoptosis. In the nervous system, nerve growth factor (NGF) is one of the factors that may act in this way, both in retinal development and in Schwann cells (Xia et al., 1995; Frade et al., 1996; Cassacia-Bonnefil et al., 1999; Frade and Barde, 1999; Soilu-Hanninen et al., 1999; Raoul et al., 2000).

Transforming growth factor βs (TGFβs) are expressed by Schwann cells and have various proliferative and phenotypic effects on these cells (for review, see Mirsky and Jessen, 1996; Scherer and Salzer, 1996). In the present work we have explored the idea that TGFβ might act as a death signal for Schwann cells. We find that TGFβ induces Schwann cell apoptosis under a number of different conditions *in vitro*. This effect is blocked by the combined presence of NRGβ and autocrine signals, and, in line with this, TGFβ kills Schwann cells in the distal stump of cut neonatal nerves but not in normal nerves. We provide evidence that TGFβ induces apoptosis by activating c-Jun in Schwann cells and that overexpression of a dominant negative c-Jun inhibits TGFβ-induced apoptosis in Schwann cells. A resistance to TGFβ killing emerges in tandem with Schwann cell differentiation, and this is related to a failure of TGFβ to activate c-Jun in differentiated cells. We show that nerve transection leads to elevation of TGFβ1 mRNA and protein in the distal stump of neonatal animals, in line with observations in the adult which suggest that this factor is involved in events that follow nerve damage. Taken together, this information builds a case for TGFβ as a negative Schwann cell survival signal in perinatal nerves.

MATERIALS AND METHODS

Materials. OX7 hybridoma cell line secreting Ig recognizing Thy1.1 was from the European Collection of Animal Cell Cultures (DERA, Wilt-

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shire, UK). Rabbit polyclonal antibody to S-100 was from Dakopatts (Copenhagen, Denmark), mouse monoclonal antibody to myelin basic protein was from Roche Diagnostics (Lewes, UK), and goat anti-mouse Ig and anti-rabbit Ig conjugated to fluorescein were from Cappel Labs (Durham, NC). Rabbit polyclonal antibody to c-Jun was a gift from G. Evan (University of California San Francisco). Antibody to LexA was from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-FLAG antibody was from Sigma (Poole, UK). Recombinant human TGFβ1, pan-specific TGFβ, and TGFβ1 antibodies were from R & D Systems (Minneapolis, MN). Purified TGFβ2 (porcine) was from British Biotechnology, IGF-1 was from PeproTech EC Ltd (London, UK), neuregulin β1 was from Amgen (Thousand Oaks, CA), and puromycin was from Sigma. Monoclonal antibody SM 1.2 was from the Developmental Studies Hybridoma Bank (Iowa City, IA). Polyclonal antibody CM1 specific for active cleaved caspase-3 was from BD PharMingen (Oxford, UK). Sources of other reagents used in immunocytochemistry, RT-PCR, Western blotting, and cell cultures have been detailed in previous papers (Jessen et al., 1994; Morgan et al., 1994; Dong et al., 1995, 1999; Stewart, 1995; Blanchard et al., 1996).

Cell culture. Cultures of Schwann cells were prepared essentially as described previously (Jessen et al., 1994; Gavrilovic et al., 1995; Meier et al., 1999). Sciatic nerves and brachial plexuses were removed from newborn, postnatal day (P) 4 and P8 and adult Sprague Dawley rats, desheathed, and treated either with a mixture of collagenase (4 mg/ml), hyaluronidase (1.2 mg/ml), and trypsin inhibitor (0.5 mg/ml) in calcium and magnesium-free DMEM at 37°C for 70–80 min or alternatively with a mixture of collagenase (2 mg/ml) and trypsin 1.25 mg/ml for 35 min (newborn, P4, P8) or twice for 1.5–2 hr in total (adult). The tissue was then gently dissociated through a plastic pipette tip, and cells were centrifuged and then purified by negative immunopanning on dishes coated with Thy1.1 antibodies as described previously (Dong et al., 1997).

For the survival assays and tests of TGFβ1-induced apoptosis, freshly immunopanned rat Schwann cells were plated on polyornithine or poly-L-lysine (PLL)/laminin-coated coverslips (Meier et al., 1999) as indicated in Results. To test survival in the absence of autocrine signals, cells were plated at low density (300 cells per 20 μl per coverslip). To test survival in the presence of autocrine survival support, cells were plated at high density (3000 cells per 10 μl per coverslip).

In most survival assays, the culture medium was a simple medium containing only a 1:1 mixture of DMEM and Ham's F12 plus BSA (0.3 mg/ml final). In these experiments TGFβ or NRGβ was added 3 hr after plating. In other experiments we used a supplemented defined medium identical to that used in previous work (Jessen et al., 1994), except that dexamethasone and IGF-1 were left out. TGFβ was added 16–18 hr after plating. These experiments are specially indicated in the text. Nearly all the survival assays lasted for 24 hr, timed from the addition of growth factors. Experiments using longer survival times are indicated in the text. At 3 hr and at specified times, cells were fixed in 2% paraformaldehyde in PBS for 20 min, immunolabeled with S100 antibodies, and mounted in Citifluor mounting medium containing 4 μg/ml Hoechst dye. The number of living cells in this assay is expressed as survival percentage. Survival percentage is the number of living cells present at the end of the experiment as a percentage of the number of cells that had plated successfully at the beginning of the experiment, i.e., the number of cells that had attached and begun to flatten on the substrate 3 hr after plating. Routinely, dead cells were identified by observing Hoechst nuclear staining and obvious morphological changes associated with death. Thus cells classified as dead showed either clearly elevated intensity of Hoechst nuclear labeling or nuclei that had fragmented, showing two or more Hoechst-labeled bodies per cell, and in addition had retracted processes and cytoplasm that by phase contrast appeared granulated and most often also vacuolated; the nucleus of these cells appeared condensed and/or fragmented by phase contrast. To validate the classification of these cells as dead, we examined cultures of dying cells that had been labeled with the terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL) method. In addition, apoptosis in cells was confirmed using an antibody (CM1) specific for the activated cleaved form of caspase 3. For the assay of cell death and measurement of TGFβ mRNA or protein after axotomy, newborn rats were anesthetized, the left sciatic nerve was transected, and the proximal stump was dissected and sutured to the muscle. For experiments measuring cell death, the growth factor, TGFβ1, control, or anti-TGFβ antibodies or PBS were applied three times at 8 hourly intervals during 24 hr. TGFβ1 or antibodies diluted in PBS were injected into the relatively large intermuscular space that surrounds the sciatic nerve in the mid-thigh region. For the first injection, a volume of 10 μl was used, followed by the two further injections in 10 μl. After the times indicated, the control or transected sciatic nerves were removed, and cryostat sections were prepared for immunohistochemistry.

Immunocytochemistry. Immunolabeling for S100 and myelin basic protein (MBP) was performed as follows. Cells were fixed in 2% paraformaldehyde for 10 min (MBP) and 20 min (S100), washed in PBS, and then treated with methanol (−20°C) for 10 min. After rinsing in PBS, cells were incubated in S100 (1:100) antibody or MBP (1:100) antibody for 30 min, washed, and incubated in anti-rabbit Ig fluorescein (S100) or anti-mouse Ig fluorescein (MBP) for 30 min, washed, and mounted in Citifluor anti-fade mounting medium. All antibodies were diluted in PBS containing 0.1 M lysine, 0.2% sodium azide, and 10% calf serum. Immunolabeling with c-Jun antibody was performed exactly as described previously (Stewart, 1995). For immunolabeling with antibodies to LexA, FLAG, or ser-63 phospho c-Jun, cells were fixed in 4% paraformaldehyde in PBS for 15 min, then permeabilized in 0.5% Triton X-100/PBS for 5 min. After a block of 50% goat serum/1% BSA in PBS, primary and secondary antibodies were then applied in block solution for LexA, FLAG, or 1% BSA/PBS for ser-63 phospho-Jun. For labeling of cells with CM1 antibody for active caspase 3, cells were fixed in 4% paraformaldehyde for 20 min, followed by a block of 20% goat serum/0.4% Triton X-100/PBS for 30 min; primary and secondary antibodies were applied in this block solution. For immunolabeling of sections with TGFβ1 antibody, sections were fixed in 4% paraformaldehyde for 20 min followed by a 10% goat serum/0.1% Tween 20/PBS block for 1 hr. Primary and secondary antibodies were applied in this block solution.

Adenoviral infection of Schwann cells. Adenoviral supernatants for recombinant adenoviral constructs expressing either LacZ or the dominant negative c-Jun molecule FLAGΔ169-Jun in the adenoviral vector pAdCMVpoly A were prepared and titered as described previously (Garnier et al., 1994; Berkner, 1998; J. Whitfield, unpublished observations). Immunopanned Schwann cells from newborn rats were plated at a density of 3000 cells per 10 μl drop on laminin-coated glass coverslips in supplemented defined medium. Approximately 16 hr after plating, adenoviral supernatant corresponding to a multiplicity of infection of ~1500 was added to the cells. Twenty-four hours later the adenoviral supernatant was removed, and the medium of the cells was changed into fresh supplemented defined medium. No toxic effects of addition of the adenoviral supernatant on the Schwann cells were observed. After an additional 24 hr to allow expression of the lacZ or FLAGΔ169-Jun, the time 0 controls were fixed, and the Schwann cells were changed to fresh supplemented defined medium alone or with increasing amounts of TGFβ1. Twenty-four hours later, the cells were fixed and stained with Hoechst dye, and survival was assessed as described previously.

Infection of Schwann cells with retroviral constructs. For retroviral infection experiments, Schwann cells from newborn nerves were purified by culture in DMEM and 10% calf serum containing 10^{-5} M cytosine arabinoside for 3 d as described previously (Morgan et al., 1991). The cDNAs for the LexA and LexA-vJun (Struhl, 1988) were cloned into the retroviral plasmid vector pBABEpuro, and the GP+E ecotropic packaging cell line (Morgenstern and Land, 1990) was then stably transfected with the plasmid DNA. Retroviral supernatant from the GP+E cells was then used to infect rat Schwann cells, and puromycin-selected pools of infected Schwann cells were cultured and used for all experiments. Antibodies against the LexA portion of the fusion protein were used to confirm LexA-vJun protein expression (data not shown).

Transient transfection of Schwann cells. Schwann cells for transfection were grown to semi-confluency on PLL-coated 90 mm tissue culture dishes in DMEM/10% FCS/4 μM forskolin. Just before transfection, the medium of the cells was changed into supplemented defined medium containing 0.5% FCS. Schwann cells were transfected using 3 μg of the AP1-responsive collagenase I gene promoter, Coll(-514)-CAT (Bossy-Wetzel et al., 1997), together with 3 μg of the SV40-driven LacZ plasmid pCH110 (Amersham Pharmacia, St. Albans, UK) together with 18 μl of Fugene 6 transfection reagent (Roche Diagnostics) in 600 μl of DMEM per the manufacturer's instructions and added to the Schwann cells. Twenty-four hours after addition of the transfection mix to the cells, TGFβ 1 was added to a final concentration of 5 ng/ml. After 30 hr, lysates were prepared from the cells and assayed for CAT activity; assay of LacZ activity was used to correct for transfection efficiency. The relative CAT activities shown represent data from duplicate transfections.

Western blotting. Protein extracts were prepared from control and transected newborn rat nerves. Thirty micrograms of protein were elec-

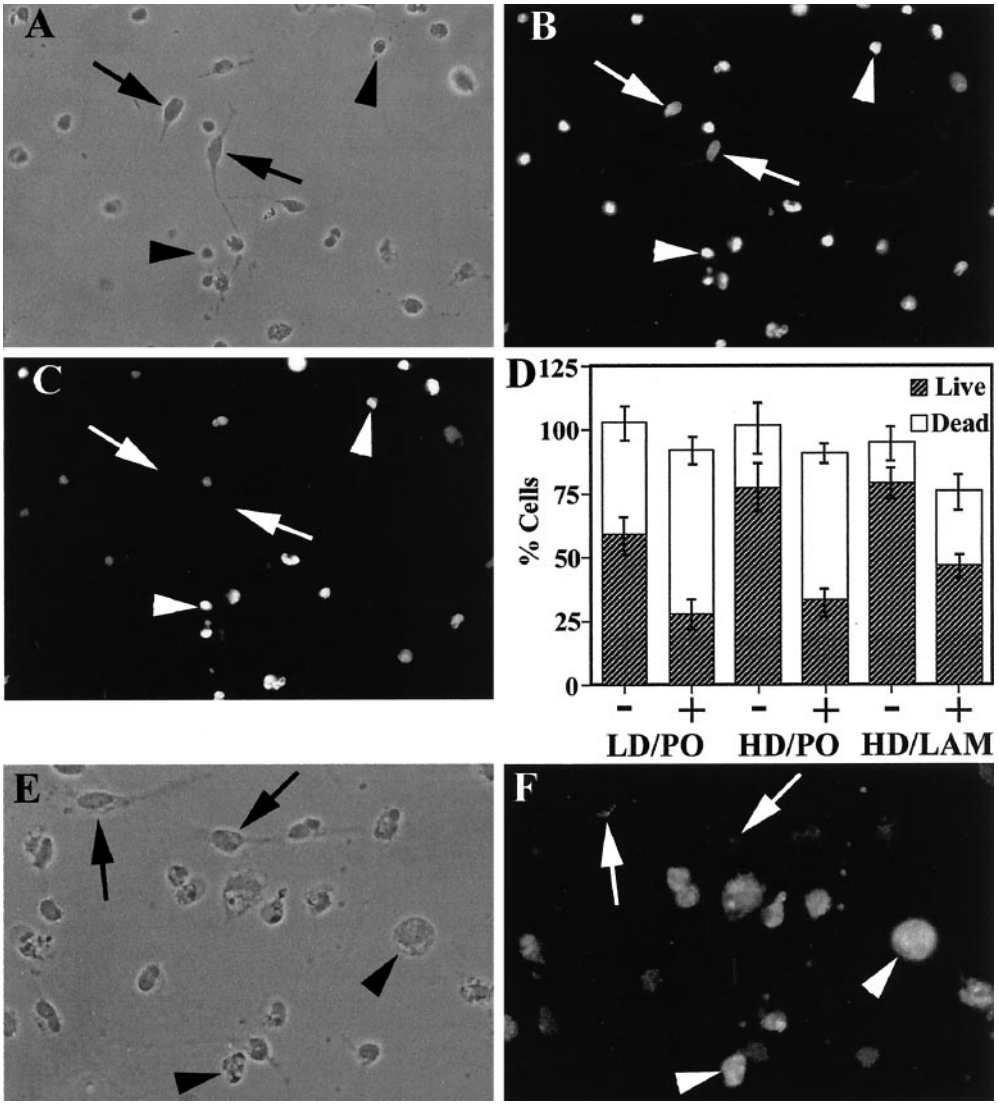

Figure 1. TGFβ induces apoptosis in Schwann cells *in vitro*. *A–C*, Death of Schwann cells at high density on polyornithine induced by TGFβ (2 ng/ml) as measured by criteria of cell morphology (*A*), nuclear condensation viewed by Hoechst stain (*B*), and TUNEL labeling (*C*). *Arrows* indicate live cells with extended processes with nuclei that are round and TUNEL negative. *Arrowheads* show dying or dead cells that are rounded up, have condensed nuclei, and are TUNEL positive. *D*, Counts of live and dead cells in control (−) and TGFβ1 (2 ng/ml)-treated (+) cultures of Schwann cells at low density (*LD*/*PO*) and high density (*HD*/*PO*) on polyornithine, and high density on laminin substrate (*HD*/*LAM*). *E*, *F*, Activation of caspase 3 in dying Schwann cells treated with TGFβ1 (2 ng/ml). *Arrows* indicate live cells as viewed by phase contrast (*E*) that are not labeled for active caspase 3 (*F*). Examples of dying/dead cells, which are labeled for active caspase-3, are indicated with *arrowheads*.

trophoresed on 12% SDS-polyacrylamide gels. Protein was transferred onto nitrocellulose membrane, blocked with 5% fat-free milk in PBS/0.1% Tween 20, and incubated with primary and secondary antibodies diluted in PBS/Tween 20. After washing, specific protein complexes were revealed using ECL Plus chemiluminescent reagent (Amersham Pharmacia).

Preparation of RNA, cDNA synthesis, and semiquantitative PCR analysis. Total RNA was isolated from freshly dissected tissue (sciatic nerve and brachial plexus) or cultured Schwann cells using Ultraspec RNA reagent (Biotecx Laboratories, Houston, TX) according to the manufacturer's instructions. For the preparation of RNA from transected nerve, newborn rats were anesthetized, the left sciatic nerve was transected, and the proximal stump was dissected and sutured to the muscle. cDNA was prepared from 500 ng of total RNA with random hexamer primers using Superscript II reverse transcriptase (Life Technologies) in a 50 μl reaction containing (in mM): 50 Tris-Cl, pH 7.3, 75 KCl, 3 $MgCl_2$, 10 DTT, and 0.5 dNTPs. One microliter of cDNA, equivalent to 10 ng of total RNA, was used for quantification of cDNA species using semiquantitative PCR analysis. The following primer pairs were used: c-Jun sense 5′-CTGATCATCCAGTCCAGC-3′, antisense 5′-CGTAGAC CGGAGGCTCAC-3′; ALK1 sense 5′-TTCTCCTCACGAGATGAGCAGTC-3′, antisense 5′-TCCCAGGTCTGCAATGCAAC-3′; ALK2 sense 5′-GCAGGGGAAGATGACGTGTAAGAC-3′, antisense 5′-CGACACACTCCAACAGGGTTATCTG-3′; ALK5 sense 5′-AGCTGTCATTGCTGGTCCAGTC-3′, antisense 5′-TCTGCCTCTCGGAACCATGAAC-3′; TGFβ1 sense 5′-ACCTGCAAGACCATCGACATGG, antisense 3′-CGTCAAAAGACAGCCACTCAGG; TGFβ2 sense 5′-GAATCTGGTGAAGGCAGAGTTCAG, antisense 3′-GCAACAACATTAGCAGGAGATGTG; TGFβ3 sense 5′-GAGTTGCTGGAAGAGATGCACG, antisense 3′-CAGAGTGGCTGTCCTTCGATGT; cyclin D1 sense 5′-GAAGTTGTGCATCTACACTGACAAC-3′, antisense 5′-CCGGGTCACACTTGATGACTCTGG-3′. 18S rRNA primers were as described by Owens and Boyd (1991).

RESULTS

TGFβ induces cell death of Schwann cells *in vitro*

Because our preliminary observations *in vitro* suggested that TGFβ adversely affected Schwann cell numbers [our unpublished results; see also Cheng and Mudge (1996); Stewart et al. (1995a,b); Skoff et al. (1998)], we set out to analyze the effects of TGFβ on Schwann cell survival using immunopurified primary Schwann cells. First, we examined the effect of TGFβ on cells plated on polyornithine under conditions similar to those that we have used previously to demonstrate the existence of autocrine survival loops in Schwann cells (Meier et al., 1999). Cell death in these cultures was assayed in three ways: first by cell morphology, second by nuclear condensation as viewed by Hoechst stain, and third by TUNEL analysis. In comparing these three methods for assessing cell death (Fig. 1*A–C*), we found an essentially complete overlap between cells judged to be dead by morphology and cells with condensed, strongly Hoechst-stained nuclei. Furthermore,

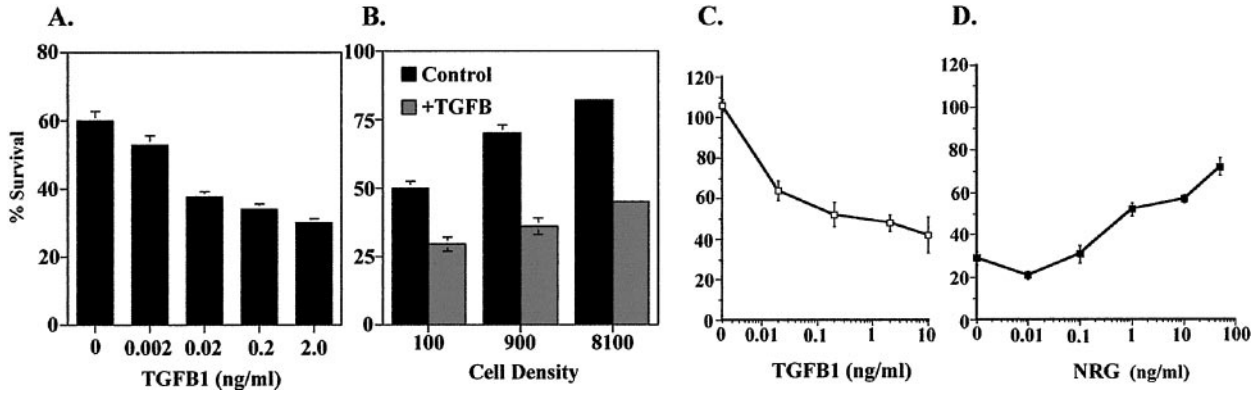

Figure 2. *In vitro* survival assays. TGFβ-induced cell death is not blocked by high cell density or by NRGβ alone. *A*, TGFβ1 causes death of Schwann cells in a dose-dependent manner. Schwann cells plated at low density (300 cells per coverslip) on polyornithine-coated coverslips were treated with increasing concentrations of TGFβ1, and survival was assessed after 24 hr. *B*, Cell death caused by TGFβ1 is density independent. Survival of Schwann cells plated on polyornithine at different densities as indicated and treated with TGFβ1 (2 ng/ml) for 24 hr is shown. *C*, Dose-dependent TGFβ1-induced death occurs even in high-density cultures (3000 cells per coverslip) on a laminin substrate. *D*, In low-density cultures (300 cells per coverslip), NRGβ is relatively ineffective at inhibiting TGFβ1-induced death. Schwann cells were treated with TGFβ1 (2 ng/ml) in the presence of increasing concentrations of NRGβ; survival was assessed after 24 hr. Compare this curve with that shown in Figure 3*A*.

combined Hoechst/TUNEL analysis showed that all cells considered to be alive on the basis of Hoechst staining were TUNEL negative, whereas >95% of the cells judged to be dead by Hoechst staining were TUNEL positive. In control experiments for the TUNEL analysis in which the terminal transferase enzyme was omitted from the reaction, no labeling of nuclei was observed (data not shown). Thus all three methods for assessing Schwann cell death gave similar results. Hoechst staining/morphological analysis was used in most of the following experiments, whereas in a number of instances, TUNEL staining or active caspase-3 immunolabeling was used as well. These are indicated in the text.

In an initial set of experiments, immunopurified Schwann cells from the sciatic nerve of newborn rats were exposed to TGFβ (2 ng/ml) for 1 d under three experimental conditions: high (3000 cells per coverslip) density and low (300 cells per coverslip) density on a polyornithine substrate and high density on a laminin substrate. High and low cell density cultures were compared because Schwann cell survival *in vitro* is subject to autocrine, density-dependent regulation, and polyornithine and laminin were compared because laminin can support Schwann cell survival (Meier et al., 1999). At the end of the experiment, the TGFβ-treated cells and control untreated cultures were fixed, Hoechst stained, and TUNEL labeled. In addition, sister coverslips were fixed and immunolabeled with antibodies to active caspase-3, because caspase-3 activation is associated with apoptotic cell death (Nicholson and Thornberry, 1997; Cryns and Yuan, 1998). Under all three conditions TGFβ caused significant Schwann cell death (Fig. 1*D*). On polyornithine, >90% of the cells could be accounted for throughout the assay because even the dead cells remained attached to the coverslip at the end of the experiment, as seen previously (Meier et al., 1999). Because of this it could be seen unambiguously that TGFβ killed Schwann cells, rather than causing living cells to detach from the coverslips. Even on laminin substrate, although there was slightly more loss of cells from the coverslips with TGFβ treatment (24%), analysis of the supernatant of the tissue culture revealed cells with condensed nuclei or just cellular debris, thereby excluding the possibility that TGFβ1 was causing a significant number of live cells to detach under these conditions. To further characterize the cell death caused by TGFβ, we immunolabeled Schwann cells with an antibody specific for the active cleaved form of caspase-3. We observed that ~80% of cells judged to be dead by morphological criteria in TGFβ-treated cultures were labeled for active caspase 3 (Fig. 1*E*,*F*), indicating that TGFβ is causing apoptotic death of Schwann cells. Having shown that TGFβ will cause cell death in Schwann cells, we next performed a series of experiments to further characterize this effect and the relationship between TGFβ and positive survival signals for Schwann cells.

In a 24 hr survival assay of Schwann cells at low density on polyornithine, TGFβ1 induced dose-dependent cell death in newborn rat Schwann cells over and above that which occurs because of the absence of positive survival signals under these conditions (Meier et al., 1999) (Fig. 2*A*). At 2 ng/ml, as observed previously, TGFβ1 reduced survival to approximately half that seen in control cultures and correspondingly increased the number of TUNEL-positive cells (from 35 ± 7% in control to 62 ± 3% in TGFβ-treated cultures; $p < 0.001$). To examine whether the remaining cells represented a TGFβ-resistant subpopulation, cells were counted after an additional 24 hr in one experiment using TGFβ1 at 5 ng/ml. At this time point (48 hr) <2% of the cells remained alive in the presence of TGFβ, indicating that all cells can be killed in this assay (data not shown).

To further test whether the death induced by TGFβ in these sparse cultures could be blocked by the autocrine signals that support Schwann cell survival at high cell densities (Meier et al., 1999), we tested the effect of TGFβ1 (2 ng/ml) in high-density cultures using polyornithine substrate as before (Fig. 2*B*). TGFβ still induced cell death: at very low density (100 cells per coverslip), survival of cells in TGFβ-treated cultures was 59% of that in the control cultures, whereas at very high density (8100 cells per coverslip) the same statistic was 57%, indicating that the proportion of cells killed in the TGFβ1-treated cultures was just as striking at high density as at low density. This suggests that autocrine factors alone cannot block TGFβ-induced death of Schwann cells (see below) (Fig. 3).

We have shown previously that autocrine signals alone are insufficient for maintaining Schwann cell survival for a long time and that longer term survival of Schwann cells requires both laminin and autocrine signals (Meier et al., 1999). We therefore

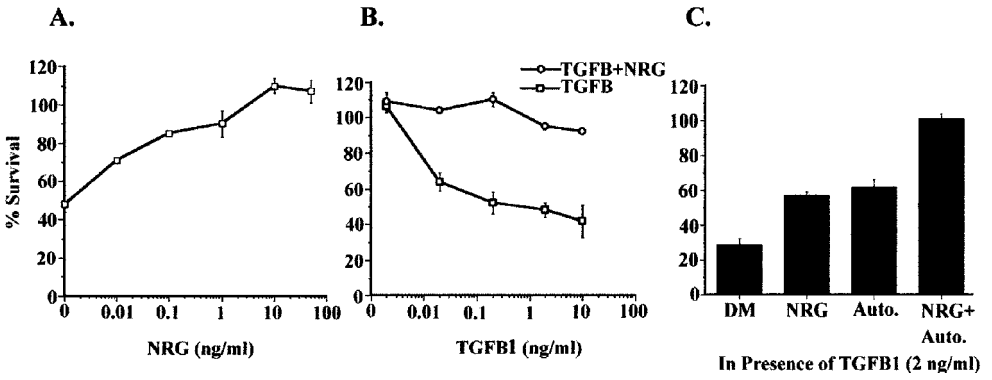

Figure 3. *In vitro* survival assays. TGFβ-induced cell death is blocked by a combination of NRGβ and autocrine signals. *A*, In high-density Schwann cell cultures (3000 cells per coverslip), NRGβ inhibits TGFβ1 (2 ng/ml)-induced apoptosis in a dose-dependent manner. *B*, In sister cultures, application of NRGβ at 10 ng/ml in the presence of increasing amounts of TGFβ1 (*TGFB+NRG*) inhibits apoptosis. Shown also are data for TGFβ1 alone (*TGFB*). *C*, In the presence of TGFβ1 (2 ng/ml) in low-density cultures (300 cells per coverslip), NRGβ (*NRG*, 10 ng/ml) alone, or a combination of IGF-2 (1.6 ng/ml), NT3 (0.8 ng/ml), and PDGF-BB (0.8 ng/ml) (*Auto*) alone, inhibits TGFβ1-induced apoptosis only partially, whereas application of both (*NRG+Auto.*) blocks TGFβ1-induced death.

tested the effect of TGFβ on Schwann cells plated at high density on a laminin substrate (Fig. 2*C*). TGFβ1 was equally effective in inducing death in these cultures after 24 hr as in cultures plated on polyornithine. In cell death assays of Schwann cells on laminin substrate treated with TGFβ1, a higher proportion of dead cells (24% for 2 ng/ml TGFβ1 after 24 hr) detached from coverslips. Analysis of cells still attached to the coverslip also revealed increased apoptosis with TGFβ1 treatment as measured by combined Hoechst/TUNEL staining (data not shown).

In addition to autocrine signals, NRGβs are likely to be a major regulator of the survival of developing Schwann cells *in vivo*, and they act as potent survival factors for cultured Schwann cells (Grinspan et al., 1996; Syroid et al., 1996; Trachtenberg and Thompson, 1996). We therefore tested whether NRGβ alone (i.e., in low-density cultures and the consequent absence of autocrine signals) could block the death-promoting effects of TGFβ. Sparse cultures were prepared on either polyornithine- or laminin-coated coverslips and exposed to 2 ng/ml TGFβ1 and varying concentrations of NRGβ. Under these conditions, NRGβ was relatively ineffective at promoting Schwann cell survival except at very high doses (Fig. 2*D*, compare with 3*A*).

TGFβ1 and TGFβ2 were equipotent in their ability to induce Schwann cell death, consistent with their indistinguishable effects observed in other *in vitro* assays (Ten Dijke et al., 1990). At 2 ng/ml, a dose used frequently in this study, Schwann cell survival averaged 25 ± 1.29% for TGFβ1 ($n = 6$) and 28 ± 3.77% for TGFβ2 ($n = 3$) in an assay identical to that described in Figure 2*A*. Each isoform was reconstituted in an acid–BSA mix. When TGFβ was omitted from this mix and cells were treated with the appropriate volume of carrier solution, the percentage survival was not reduced from the level observed in control cultures, indicating that TGFβ is the active killing ingredient. Although Schwann cells in culture make TGFβ, this is secreted in an inactive from (Stewart et al., 1995b). It is also important to note that expansion of newborn Schwann cells with cAMP elevating agents and growth factors is accompanied by a reduction in TGFβ-mediated cell death (data not shown).

Together these experiments show that TGFβ kills primary Schwann cells from nerves of newborn rats under various conditions in culture. Individually, neither of the two major signals likely to totally regulate the survival of these cells *in vivo*, NRGβ or autocrine signals, prevents TGFβ-induced death.

TGFβ-induced cell death is blocked by a combination of NRGβ and autocrine signals

Having shown that neither NRGβ nor autocrine survival signals could completely prevent TGFβ-induced death when present separately (above), we now tested, in three different ways, whether the combination of these survival factors could block the effect of TGFβ.

First, immunopurified cells from newborn animals were plated at high density (and therefore exposed to autocrine signals) on a laminin substrate. All cells were maintained with a constant concentration (2 ng/ml) of TGFβ1, whereas the concentration of NRGβ varied from 0.01 to 50 ng/ml. Under these conditions, NRGβ prevented Schwann cell death in a dose-dependent manner (Fig. 3*A*, compare with 2*D*). Conversely, in other experiments also performed at high density, the concentration of NRGβ was held constant at 10 ng/ml whereas the TGFβ1 concentration varied: almost complete survival was seen even at TGFβ concentrations of 10 ng/ml (Fig. 3*B*).

Second, Schwann cells were plated at low density on a laminin substrate and maintained with 10 ng/ml NRGβ and autocrine factors in the form of 1:10 dilution of medium conditioned by dense Schwann cell cultures as described previously (Meier et al., 1999). Addition of 2 ng/ml TGFβ to these cultures did not induce cell death (data not shown).

Third, we took advantage of our previous finding that IGF-2, NT3, and PDGF-BB are important components of the autocrine Schwann cell signal. This predicts that the minimal combination of IGF-2 (1.6 ng/ml), NT3 (0.8 ng/ml), and PDGF-BB (0.8 ng/ml), a mixture that mimics the autocrine activity in a number of tests (Meier et al., 1999), should block TGFβ-induced death, provided NRGβ is also present. When this was tested, using sparse Schwann cell cultures on a laminin substrate, we found as expected that the combination of IGF-2, NT3, and PDGF-BB was relatively ineffective on its own but that cell death in TGFβ (2 ng/ml) was completely prevented if NRGβ (10 ng/ml) was also present (Fig. 3*C*).

These results show that in the combined presence of NRGβ and autocrine signals, Schwann cells from newborn nerves are resistant to the killing effects of TGFβ.

Endogenous TGFβ might contribute to Schwann cell death *in vivo*

TGFβ is present in Schwann cell precursors and embryonic and neonatal Schwann cells, and the results above show that TGFβ can kill Schwann cells *in vitro*. This raised the possibility that the increase in cell death triggered by transection of neonatal nerves might be attributable in part to the action of endogenous TGFβ. A number of *in vivo* experiments were performed to explore this idea.

First, expression of TGFβs in nerves of newborn animals was examined. The previous observation that TGFβ is present in

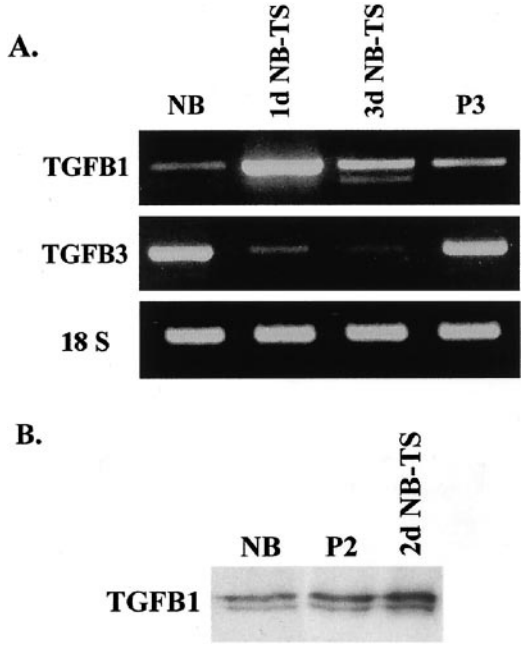

Figure 4. *A*, Nerve transection in newborn rat causes an upregulation of TGFβ1 mRNA and downregulation of TGFβ3 mRNA. Shown is semiquantitative PCR measurement of levels of mRNA for TGFβ1 and TGFβ3 in newborn rat sciatic nerve (*NB*), 1 d (*1d NB-TS*), and 3 d (*3d NB-TS*) after transection, and from postnatal day 3 (*P3*) rat. *B*, Measurement of TGFβ1 protein levels in intact and transected newborn nerve. Shown is Western blot of protein from newborn (*NB*), P2, and newborn rat sciatic nerve 2 d after transection (*2d NB-TS*).

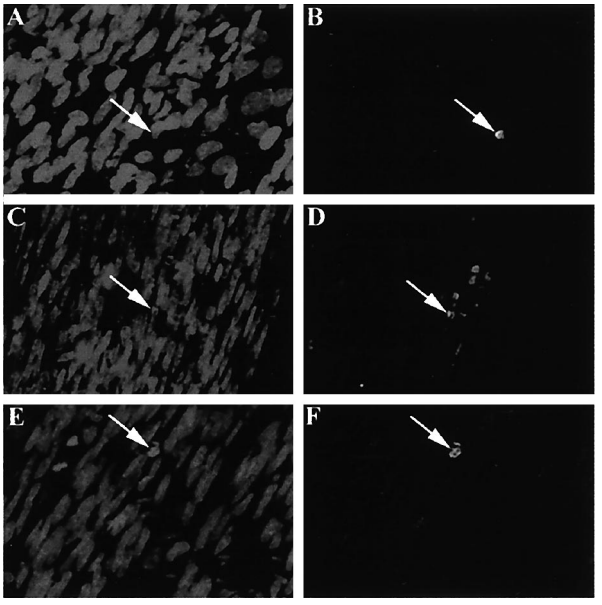

Figure 5. TUNEL analysis of newborn rat sciatic nerve after transection. Shown are representative fields from sections stained with Hoechst dye to reveal nuclei (*A*, *C*, *E*) and TUNEL stained to reveal apoptotic nuclei (*B*, *D*, *F*). *A* and *B* show control untransected nerve, *C* and *D* show sciatic nerve 24 hr after transection, and *E* and *F* show transected nerve 24 hr after transection with injection of pan-TGFβ blocking antibody. *Arrows* point to examples of individual TUNEL-labeled nuclei.

Schwann cells and that TGFβ1 mRNA and protein are elevated in distal nerve stumps after nerve cut is one of the findings that suggests that TGFβ might be involved in events that follow nerve damage (Scherer et al., 1993; Rufer et al., 1994). Because this has been tested only in the adult, we monitored TGFβ1 and TGFβ3 mRNAs in the distal stump of transected nerves in newborn rats using RT-PCR (Fig. 4*A*). Levels of TGFβ1 and TGFβ3 mRNAs by semiquantitative PCR showed that there is no significant change in levels among newborn, P1, and P3 in normal intact rat sciatic nerve during development (Fig. 4*A*) (data not shown). After nerve cut, TGFβ1 mRNA was strongly elevated at 1 d and, to a lesser extent, at 3 d, whereas levels of TGFβ3 mRNA were reduced in the distal stump as reported in the adult. This indicates that the TGFβ mRNAs expression profile after nerve cut is similar in newborn and adult nerves. We also measured levels of TGFβ1 protein in newborn, P2 contralateral control, and newborn nerve 2 d after transection by Western blot analysis. TGFβ1 protein is present in newborn and P2 nerve and is slightly elevated in newborn nerve 2 d after transection (Fig. 4*B*). Immunolabeling of sections prepared from transected nerves (4 d after transection) and contralateral control nerves with a different TGFβ1 antibody also showed an elevation of TGFβ1 protein with transection (data not shown). Thus TGFβ1 protein is present in the distal stump of transected nerves in newborn rats in agreement with the idea that it acts as a death signal under these circumstances.

Further tests of TGFβ involvement *in vivo* were performed by injecting TGFβ into nerves of newborn rats. Schwann cells in normal neonatal nerves can be presumed to be exposed to axon-associated NRGβ and to autocrine signals in the confined environment of the nerves, and we have suggested previously that Schwann cell survival at this stage of development depends on the combined input from these two sources (Meier et al., 1999). A simple prediction from the culture experiments (Fig. 3) was that such cells would be resistant to TGFβ. A different set of conditions exists in transected nerves. In the distal stump, transection is presumably accompanied by a loss of and reduction in exposure to axonal NRGβ distal to the cut while the cells continue to be exposed to autocrine signals. This situation leads to increased cell death, although most cells survive (Grinspan et al., 1996). The culture experiments (Fig. 2) predict that, under these conditions, Schwann cells *in vivo* would be sensitive to TGFβ killing. As outlined below, both of these expectations were fulfilled. We first confirmed that in normal newborn sciatic nerve there is a low level of cell death, as measured by the TUNEL assay (0.175 ± 0.02% of total cells; 12,000 nuclei per section were counted) and that at 24 hr after transection this is increased 10.7× (1.88 ± 0.15%) in the distal stump in agreement with previous results (Grinspan et al., 1996; Syroid et al., 1996) (Fig. 5, Table 1). Double immunolabeling of sections with S-100 antibody confirmed that dying cells were Schwann cells (data not shown). When three injections of TGFβ1 (each injection was 10 μl of a 400 ng/ml solution) were made into the relatively large intermuscular space that surrounds the sciatic nerve in the mid-thigh region of a newborn rat over 24 hr, no effect was seen; the number of TUNEL-positive nuclei remained at 0.l8%. In contrast, when identical TGFβ injections were performed during the 24 hr after nerve transection, the number of TUNEL-positive nuclei in the TGFβ-injected transected nerves was ~1.5 times higher (2.92 ± 0.09% vs 1.88 ± 0.15%) than in transected nerves injected with the control carrier solution only.

Last, a blocking pan-TGFβ antibody was injected into the area of the distal stump after nerve cut in newborn rats in two experiments (Fig. 5, Table 1). The antibody (250–500 ng/ml) was

Table 1. The effects of TGFβ, blocking TGFβ antibody, and nerve transection on Schwann cell death in neonatal nerves *in vivo*

Treatment	% TUNEL-positive nuclei	Total nuclei counted
Not transected PBS	0.175 ± 0.03	12,000 × 4
Not transected TGFβ	0.180 ± 0.21	12,000 × 3
Transected nerve PBS	1.88 ± 0.15	12,000 × 4
Transected nerve Pan TGFβ-antibody	0.71 ± 0.09	12,000 × 4
Transected nerve Control SM1.2 antibody	1.78 ± 0.08	12,000 × 2
Transected nerve TGFβ	2.92 ± 0.09	12,000 × 4

In every animal, the left sciatic nerve was operated and/or injected, and the right sciatic nerve was taken as control. Several sections were made from every treated sciatic nerve and observed under the microscope. One to two sections were randomly chosen for each animal. Approximately 12,000 nuclei were counted in each section. Two to four animals were used for each experiment.

injected three times during a 24 hr period after nerve cut as described above. Averaging the two experiments (two rats in each experiment) reveals that the blocking antibody reduced the number of apoptotic nuclei in the nerve by 62%. Identical injections of a control antibody SM 1.2 (280 ng/ml; see Materials and Methods) resulted in an insignificant (5%) reduction in the number of apoptotic nuclei (Table 1).

All of the above data are consistent with the possibility that Schwann cell death in transected neonatal nerves is not caused solely by loss of axon-associated NRGβ but is caused also by TGFβ present in the nerve.

Schwann cells acquire resistance to TGFβ killing as they differentiate

Two previously reported features of Schwann cell death *in vivo* gave us the opportunity to test further whether TGFβ acted in a manner expected of a death signal in transected nerves. The first of these is that the amount of cell death induced by cutting a nerve diminishes gradually after birth so that a point is reached some time between P5 and P20 after which transection no longer leads to the appearance of apoptotic nuclei in the distal stump (Grinspan et al., 1996). We therefore tested whether this loss of vulnerability to death *in vivo* was mirrored by loss of sensitivity to TGFβ-induced death in the cell culture assay. Schwann cells from rats of different ages were exposed to TGFβ for 24 hr using the same protocols as those used for newborn cells as described above. Under both low-density and high-density culture conditions on a polyornithine substrate, the death response to TGFβ decreased markedly with age, and by P8, Schwann cells were almost impervious to even a high dose of TGFβ (5 ng/ml; $p > 0.05$) (Fig. 6*A*). Using laminin-coated coverslips and Schwann cells from embryo day (E) 18, newborn, P8, and adult animals plated at 3000 cells, similar results were obtained (data not shown).

The second *in vivo* finding that we explored *in vitro* is that transection-induced cell death in early postnatal nerves (P5) is to a large extent restricted to cells not involved in myelination (Grinspan et al., 1996). We tested whether this selective resistance of early myelinating cells to death in the nerve was reflected in selective resistance of such cells to TGFβ-induced cell death *in vitro*. Essentially, this involved repeating the experiment shown in Figure 6*A* using cells from P4 animals and one dose (5 ng/ml) of TGFβ1 only, but in this case analyzing the results for myelinating cells, and cells not involved in myelination, separately. Cells from P4 nerves were plated sparsely on polyornithine substrate, and at 3 hr (the reference point for the survival assay; see Materials and Methods), some cultures were immunolabeled with antibodies to MBP to identify cells that had started to myelinate. The MBP-negative cells were taken to be cells not yet engaged in myelination or cells destined to become nonmyelinating cells. Sister cultures were maintained with or without TGFβ (5 ng/ml) for 24 hr, fixed, and immunolabeled with MBP antibodies. It was striking that TGFβ had no effect on the number of MBP-positive cells in this assay, whereas it killed approximately two-thirds of the MBP-negative cells (Fig. 6*B*). Repetition of this experiment with nerves from P8 animals also revealed that no MBP-positive cells were killed by TGFβ (data not shown). Because TGFβ kills only a minimal number of P8 cells (Fig. 6*A*) and the percentage of MBP-positive cells at P8 was 52%, it follows that resistance to TGFβ spreads in this period to the whole Schwann cell population regardless of whether they are myelinating.

To test whether this was true *in vivo* for cells in the nerves of P8 rats, we transected nerves in P8 animals, and in other experiments we injected TGFβ, as described previously, in the area of the normal P8 sciatic nerve. No cell death was noted in normal P8 nerves, and neither injection of TGFβ nor transection led to the appearance of apoptotic nuclei in these experiments (data not shown). A single experiment was performed to determine whether the differentiation-related resistance to TGFβ killing was reversible. Cells from nerves of P8 rats were cultured in the presence or absence of TGFβ (5 ng/ml) for 5 d. At the end of this period, cell counts revealed that although 43% of cells in control cultures survived, <2% of TGFβ-treated cells were still alive. This was reminiscent of earlier findings that by 48 hr TGFβ had killed virtually all cells from nerves of newborn animals (above). Thus, whatever the process involved in imparting TGFβ resistance to older cells, it appears that the changes involved are reversible in culture.

Together these experiments indicate that resistance to TGFβ killing develops as one component of the mature phenotype of myelin-forming and nonmyelin-forming Schwann cells. The selective resistance of myelinating cells in early postnatal nerves may reflect the fact that differentiation along the myelin pathway starts before the maturation of nonmyelin-forming cells (Jessen and Mirsky, 1992). These relationships between vulnerability to TGFβ and Schwann cell differentiation seen in culture are similar to those between Schwann cell differentiation and death mechanisms in transected nerves.

TGFβ receptors ALK1, ALK2, and ALK5 are detectable during peripheral nerve development

Using semiquantitative RT-PCR we showed that the TGFβ type I receptors ALK1, ALK2, and ALK5 are expressed in E14 to adult nerve. ALK5, the major type I receptor involved in TGFβ signaling (Massague, 2000), was expressed at the same level throughout development. ALK2 was expressed at higher levels at early developmental stages and downregulated postnatally, whereas ALK1 was detectable at lower levels at E14 and E15, with higher levels at birth and postnatally (Fig. 7). To determine whether ALK5 protein was detectable, we used immunocytochemistry on newborn rat Schwann cells. All Schwann cells expressed the ALK5 receptor (data not shown).

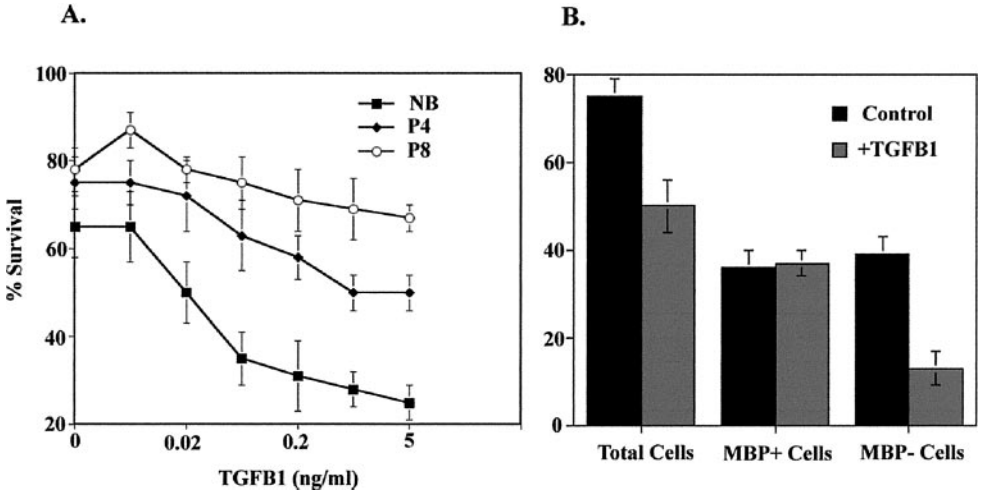

Figure 6. *In vitro* survival assays. The ability of TGFβ1 to cause apoptosis of Schwann cells is developmentally regulated. *A*, Survival of Schwann cells from newborn (*NB*), postnatal day 4 (*P4*), and P8 rats plated at high density (3000 cells per coverslip) with increasing concentrations of TGFβ1. Note that the ability of TGFβ to kill Schwann cells decreases as the cells get older. *B*, Survival assay for Schwann cells showing percentage of MBP-positive cells (*MBP+*) and MBP-negative cells (*MBP−*) from P4 animals in cultures treated with TGFβ1 (5 ng/ml) in a 24 hr assay. Note that the percentage survival of MBP-positive cells (cells that were myelinating *in vivo*) is relatively unaffected by TGFβ1.

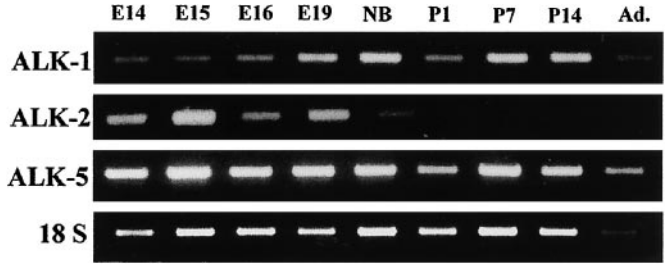

Figure 7. Expression of type I TGFβ receptors ALK1, ALK2, and ALK5 in sciatic nerve during development. Semiquantitative RT-PCR measurement of ALK1, ALK2, and ALK5 from E14 through to adult (*Ad.*) in rat sciatic nerve. Note marked downregulation of ALK2 mRNA in postnatal nerves.

Treatment of Schwann cells with TGFβ1 causes serine-63 phosphorylation of c-Jun and activation of AP1-dependent transcription

We now examined the intracellular mechanisms by which TGFβ induces cell death. Activation of the transcription factor c-Jun and induction of AP1-dependent transcription has been shown to be involved in apoptosis of several cell types, including fibroblasts (Bossy-Wetzel et al., 1997) and sympathetic neurons (Ham et al., 1995). Activation of c-Jun and AP1-dependent transcription requires phosphorylation of c-Jun on two N-terminal serine residues, ser-63 and -73 (Smeal et al., 1991). We therefore examined whether activation of c-Jun occurred in response to application of TGFβ1 to freshly isolated Schwann cells using an antibody that is specific for c-Jun when phosphorylated on the serine-63 residue (Watson et al., 1998). Schwann cells were plated at high density on laminin-coated glass coverslips in supplemented defined medium (see Materials and Methods). As a positive control, identical cultures were exposed to ultraviolet (UV) light, a procedure that has been shown previously to result in phosphorylation and activation of c-Jun (Devary et al., 1991; Derijard et al., 1994). One hour after addition of TGFβ1 (2 ng/ml), strong nuclear labeling was seen (Fig. 8*D*), indicating phosphorylation of c-Jun at serine-63. Similar labeling was seen 1 hr after UV irradiation (Fig. 8*F*). To determine whether levels of c-Jun protein and mRNA were unchanged during this time, we immunolabeled Schwann cells treated with TGFβ1 for 1 hr with antibodies to c-Jun. Levels of c-Jun were unchanged during this time in response to TGFβ1 treatment using several different serial dilutions of antibody (Fig. 8*G*,*H*) (data not shown). These results were confirmed at the mRNA level by semiquantitative RT-PCR of TGFβ1-treated and control cells (Fig. 8*I*); however, induction of c-Jun mRNA was observed 8 hr after TGFβ1 addition (data not shown), consistent with the previously reported induction of c-Jun itself by activated AP1 complexes (Stein et al., 1992; van Dam et al., 1995; Eilers et al., 1998). In support of activation of c-Jun in Schwann cells, we found that TGFβ1 caused an increase in cyclin D1 mRNA (Fig. 8*J*), a known transcriptional target for c-Jun-dependent transcription (Albanese et al., 1995; Bakiri et al., 2000).

To test functionally whether TGFβ1 activated c-Jun and stimulated the corresponding AP1-dependent transcriptional activity, we performed transient transfections into Schwann cells with an AP1-responsive CAT reporter construct (Bossy-Wetzel et al., 1997). Figure 8*K* shows that addition of TGFβ1 to Schwann cells resulted in a massive increase (~23-fold) in AP1-dependent transcription in Schwann cells, further demonstrating the link between TGFβ1 and activation of c-Jun/AP1 in Schwann cells.

Overexpression of dominant negative c-Jun inhibits TGFβ1-induced cell death of Schwann cells

Having identified that there is activation of c-Jun and AP1-dependent transcription in Schwann cells after TGFβ1 addition, we next investigated whether activity of c-Jun was required for induction of cell death by TGFβ1. These experiments were performed with Schwann cells at high density at which TGFβ1 will induce apoptosis as measured by combined Hoechst/TUNEL analysis. Using an adenoviral infection protocol (see Materials and Methods) we overexpressed a FLAG-tagged dominant negative c-Jun protein, FLAGΔ169-Jun. This lacks the transcriptional activation domain of the protein and inhibits c-Jun-dependent transcription (Ham et al., 1995). After infection of immunopanned Schwann cells from newborn animals with FLAGΔ169-Jun adenoviral supernatant, >90% of Schwann cells showed expression of the dominant negative c-Jun protein at time 0 before TGFβ1 addition (Fig. 9*A*,*B*). Adenovirus expressing the LacZ gene was used as a control in these experiments. Figure 9 shows that overexpression of the dominant negative c-Jun (FLAG169-Jun) significantly ($p < 0.01$ at 10 ng/ml TGFβ1) inhibits TGFβ1-induced cell death in this assay as compared with Schwann cells infected with the LacZ control. TGFβ1 (10 ng/ml) kills 46% of control cells (LacZ) over a 24 hr period when compared with 15% in Schwann cells infected with the dominant negative c-Jun construct (FLAG169-Jun). After 48 hr, TGFβ1-

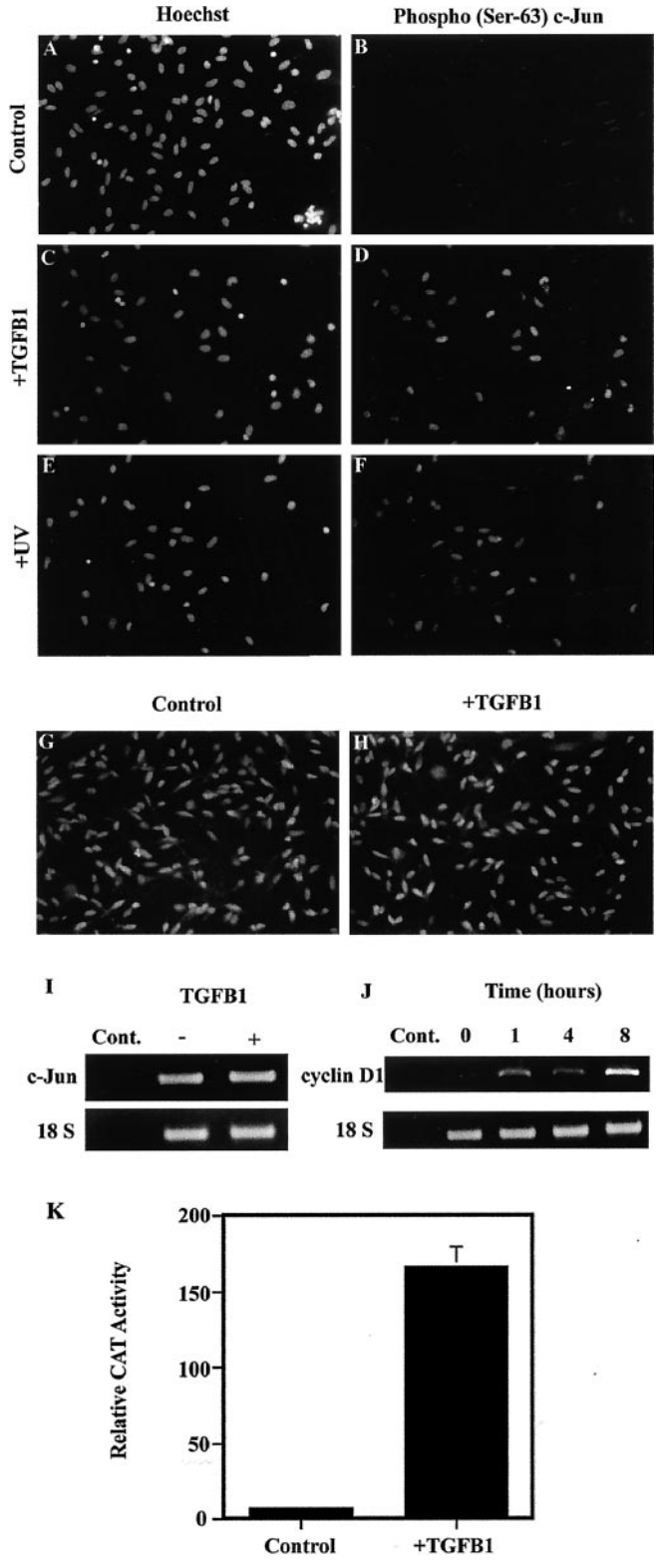

Figure 8. Addition of TGFβ1 to newborn rat Schwann cells *in vitro* causes serine-63 phosphorylation of c-Jun, activation of AP1-dependent transcriptional activity, and induction of cyclin D1 mRNA. *A*, *C*, and *E* show immunopanned newborn rat Schwann cells stained with Hoechst dye to reveal the nuclei, and *B*, *D*, and *F* show Schwann cells immunolabeled with antibody specific for the serine-63-phosphorylated form of c-Jun. *A* and *B* show control untreated cells with low levels of phosphorylated c-Jun. *C* and *D* show Schwann cells treated for 1 hr with TGFβ1 (2 ng/ml). Note substantial serine-63 phosphorylation of c-Jun. *E* and *F* are Schwann cells treated with UV light, a known activator of c-Jun phosphorylation, as positive control. *G* and *H* show immunolabeling in control (*G*) and TGFβ1-treated (*H*) (2 ng/ml for 1 hr) Schwann cells to show that c-Jun protein levels remain unaltered at this time point after TGFβ1 addition. c-Jun mRNA as assayed by semiquantitative RT-PCR (*I*) also remains unaltered at this time. *J* shows RT-PCR data demonstrating TGFβ1 (2 ng/ml) induction of cyclin D1 mRNA; amplification using primers specific for 18S rRNA indicates equal input of cDNA into PCR assays. *K* shows that TGFβ1 causes strong induction of AP1-dependent transcription in Schwann cells. Schwann cells were cotransfected with an AP1-dependent CAT reporter construct together with pCH110 lac-Z plasmid. Thirty hours after transfection in the absence (*Control*) or presence of 5 ng/ml of TGFβ1 (+*TGFB1*), lysates were assayed for CAT activity.

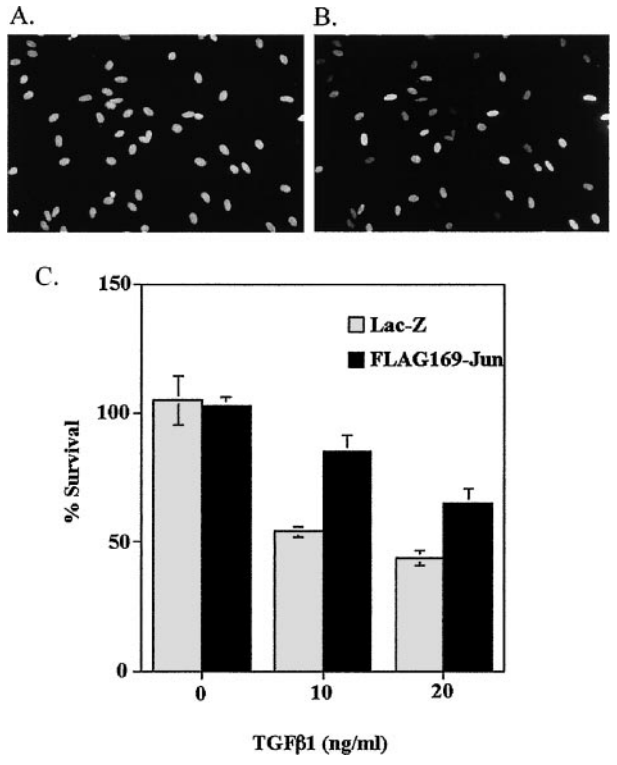

Figure 9. Overexpression of dominant negative c-Jun inhibits TGFβ1-induced apoptosis in Schwann cells. *A*, *B*, Immunolabeling of Schwann cells with anti-FLAG antibody 48 hr after addition of adenoviral supernatants before TGFβ1 addition shows expression of FLAG-tagged dominant negative c-Jun (FLAGΔ169-Jun) in >90% of Schwann cell nuclei. *A* shows Hoechst stain of nuclei; *B* shows immunofluorescence with FLAG antibody. *C*, *In vitro* survival assays of Schwann cells infected with control virus (*Lac-Z*) or with dominant negative c-Jun (*FLAG169-Jun*). The cells were exposed to medium alone or to medium containing 10 or 20 ng/ml TGFβ1 for 24 hr.

induced cell death in control cultures was further increased, whereas dominant negative c-Jun expression still exerted an inhibitory effect on death (data not shown). These experiments demonstrate a requirement for c-Jun activity in TGFβ1-induced cell death in Schwann cells.

To determine whether activation of c-Jun is sufficient to cause cell death, we used a construct expressing v-Jun, a constitutively active form of c-Jun. The transcriptional activity of v-Jun is independent of stress-activated protein kinase/Jun N-terminal kinase (SAPK/JNK) phosphorylation (Black et al., 1994) and has been shown to act as a strong transcriptional activator in a majority of systems (Bohmann and Tjian, 1989; Black et al., 1994;

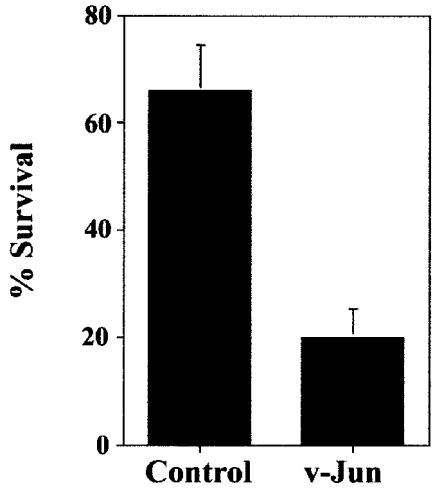

Figure 10. Overexpression of v-Jun causes apoptosis of Schwann cells in serum-free medium. Schwann cells infected with either LexA control (*Control*) or LexA-vJun (*v*-Jun) were plated at high density (5000 cells per coverslip). The medium of the cells was then changed to supplemented defined medium, and the number of surviving cells was counted after 48 hr.

Hartl and Bister, 1998; Huguier et al., 1998; Bader et al., 2000) (see however Gao et al., 1996; Kilbey et al., 1996). We found that when Schwann cells expressing v-Jun were cultured at high density in serum-free supplemented defined medium, the amount of cell death was strongly increased compared with control cells infected with vector alone (Fig. 10). Combined TUNEL/Hoechst analysis of these cultures, together with labeling with CM1 antibody for active caspase 3, established that cells in these cultures are dying by apoptosis (data not shown).

TGFβ activation of c-Jun occurs predominantly in MBP-negative cells

The previous experiment provided evidence that TGFβ kills Schwann cells from newborn nerves by activating the c-Jun pathway. We now tested whether a failure to activate this pathway could provide an explanation for the failure of TGFβ to kill more differentiated Schwann cells.

Immunopanned rat Schwann cells from P4 animals were plated at high density on laminin-coated coverslips in supplemented defined medium. As discussed in a previous section, approximately half of the Schwann cells in these cultures have started myelination as evidenced by expression of MBP, whereas the other half is less differentiated. One hour after TGFβ1 (2 ng/ml) addition, the cells were immunolabeled with antibodies against MBP and serine-63 phospho c-Jun. Figure 11 shows that TGFβ1 stimulated immunohistochemically detectable c-Jun phosphorylation in 31 ± 8.4% of MBP-negative cells but in only 5 ± 2.6% of MBP-positive cells. Furthermore, the immunohistochemical labeling was consistently stronger in the nuclei of the MBP-negative cells, indicating higher levels of phosphorylated c-Jun. This correlates well with our observation that MBP-positive Schwann cells are relatively resistant to induction of cell death by TGFβ1 and suggests that the inability of TGFβ to kill differentiating Schwann cells can be explained in part by a failure to activate c-Jun in these cells. Therefore, the molecular mechanism responsible for the differentiation-related immunity to TGFβ killing may lie upstream of c-Jun activation.

TGFβ-activated death pathways involve the interleukin-1β-converting enzyme-like protease pro-caspase 3

The interleukin-1β-converting enzyme (ICE) protease inhibitor Z-VAD.fmk was used to investigate the role of ICE-like proteases on Schwann cell death induced by TGFβ and by the absence of survival factors. This synthetic peptide inhibitor prevents other types of cell death by blocking irreversibly the activation of the ICE-like protease, pro-caspase-3 (Chow et al., 1995; Zhu et al., 1995; Slee et al., 1996). It is not clear that Z-VAD.fmk is specific for caspase-3 alone, and it might act similarly on the processing of other ICE-like proteases. It was found that Z-VAD.fmk exerted different effects on the two types of Schwann cell death: 100 μM Z-VAD.fmk did not affect death of cells in medium alone, whereas its effects on the TGFβ-mediated death were to increase survival by threefold, from 15 to 47% (Fig. 12). This effect was dose dependent, with 50 μM Z-VAD.fmk increasing survival twofold (data not shown). We also tested the effects of one proteasome inhibitor, lactacystin, a metabolite of streptomyces (Fenteany et al., 1995) on TGFβ-induced cell death. Like Z-VAD.fmk, lactacystin offered negligible protection against cell death caused by the absence of survival factors, whereas it completely blocked additional death induced by TGFβ in a dose-dependent way (data not shown).

These results implicate ICE-like proteases, in particular caspase-3, in the intracellular death pathways activated by TGFβ in Schwann cells. They also suggest that these pathways are to some extent different from those used when Schwann cells undergo cell death caused by the absence of survival-promoting factors.

DISCUSSION

Although developmental and trauma-induced cell death in the nervous system has classically been considered to be regulated by positive survival signals present in limiting amounts, it is apparent that in some situations cell death is in fact controlled by an interplay between survival factors and negative survival signals that actively induce cell death. The present work provides evidence that the survival of Schwann cells is in some circumstances regulated by such a dual mechanism involving the negative survival signal TGFβ, a family of growth factors that is expressed by Schwann cells and secreted by purified Schwann cells in a signal-poor environment *in vitro* in an inactive form (Stewart et al., 1995b). We pinpoint phosphorylation of c-Jun as a key downstream event in TGFβ-induced Schwann cell death. We also show that the ability of TGFβ to kill Schwann cells, like normal Schwann cell death *in vivo*, is under strong developmental regulation and provide evidence that the decreasing ability of TGFβ to kill older cells is caused by a decreasing ability of TGFβ to phosphorylate c-Jun in more differentiated cells.

c-Jun is strongly expressed in cultured Schwann cells and in Schwann cells in the distal stump of transected nerves, whereas expression levels in the cells of normal untransected newborn and adult nerves are low (De Felipe and Hunt, 1994; Stewart, 1995). We show here, using an antibody specific for the ser-63 phosphorylated and thus activated form, that TGFβ1 activates c-Jun in Schwann cells. In confirmation of this, we find that TGFβ1 will both activate transcription of an AP1 reporter gene and upregulate cyclin D1 mRNA, a known target of c-Jun [Wisdom et al. (1999) and references therein]. Activation of the c-Jun transcription factor is involved in cell death in a number of different cell types, including sympathetic neurons and fibroblasts (Ham et al.,

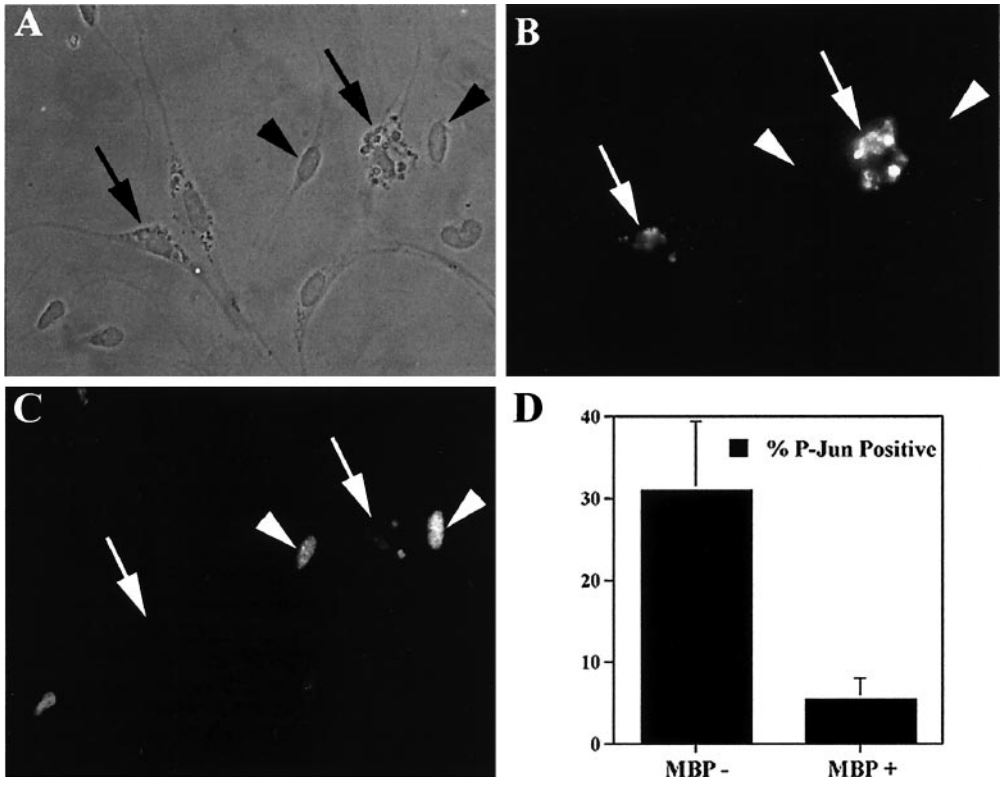

Figure 11. Phosphorylation of c-Jun in Schwann cells by TGFβ1 occurs mostly in MBP-negative cells. Immunopanned Schwann cells from P4 rats were treated for 1 hr with TGFβ1 (2 ng/ml) and double immunolabeled with antibodies to MBP (*B*) and ser-63 phospho c-Jun (*C*). After addition of TGFβ1, cells indicated by *arrowheads* in the phase-contrast micrograph (*A*) are shown to be negative for MBP (*B*) but positive for ser-63-phosphorylated c-Jun (*C*). The two MBP-positive cells in this field (*arrows*) are negative for phosphorylated c-Jun. Note the typical vesicular appearance of MBP in Schwann cells in culture after loss of axonal contact. *D* quantifies this effect, illustrating the percentage of MBP-negative (*MBP−*) and MBP-positive (*MBP+*) Schwann cells that are also positive for the phosphorylated serine-63 form of c-Jun in this assay.

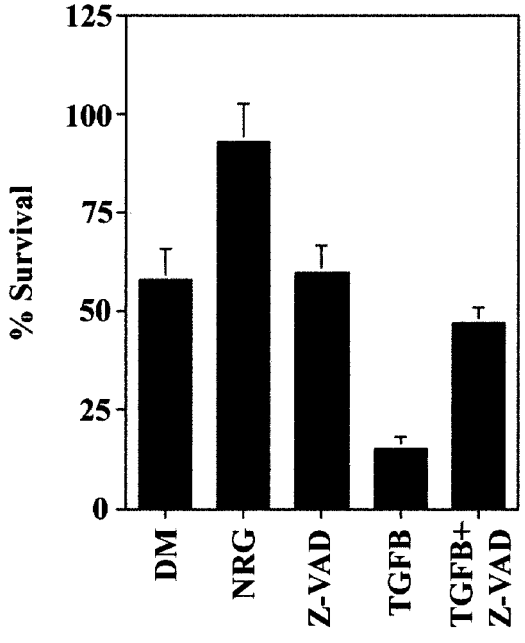

Figure 12. ICE protease inhibitor Z-VAD.fmk inhibits TGFβ1-induced apoptosis in Schwann cells. The graph shows 24 hr survival assays for immunopanned newborn rat Schwann cells plated at low density (300 cells per coverslip) on polyornithine-coated coverslips. Cells were maintained in defined medium alone (*DM*), defined medium supplemented with NRGβ (10 ng/ml) (*NRG*), Z-VAD.fmk (100 μM) (*Z-VAD*), TGFβ1 (2 ng/ml) (*TGFB*), or TGFβ1 plus Z-VAD.fmk (*TGFB+Z-VAD*).

1995; Bossy-Wetzel et al., 1997). Activation of c-Jun occurs by phosphorylation of two serine residues (ser-63 and ser-73) by members of the JNK family (Derijard et al., 1994; Minden et al., 1994; Eilers et al., 1998), and this phosphorylation event has been shown to correlate with transcriptional activation by the jun-containing AP1 complex (Smeal et al., 1991) and to be required for apoptosis (Watson et al., 1998). In addition, using an antibody specific for the active phosphorylated forms of SAPK/JNK, we have also recently shown that TGFβ strongly increases SAPK/JNK activity in Schwann cells (L. von Hertzen, unpublished observation). SAPK/JNK activation has also recently been implicated in apoptosis of Schwann cells after serum withdrawal (Cheng et al., 2001). We show that expression of a dominant negative c-Jun is sufficient to inhibit TGFβ1-induced cell death of Schwann cells. In addition to this, we have shown that expression of v-Jun, which is constitutively active independent of its phosphorylation status (Black et al., 1994; Clark and Gillespie, 1997), will induce cell death of Schwann cells when survival signals are removed, thus mimicking the effects of TGFβ application.

TGFβ1 has been shown to induce apoptosis in hepatoma cells, B-lymphocytes, epithelial cells, and some other cell types, and blocking antibodies to TGFβ have recently been shown to prevent developmentally regulated motor and neuronal cell death in chick (Chalaux et al., 1999; Schrantz et al., 1999; Shima et al., 1999; Krieglstein et al., 2000). Analysis of pathways involved in TGFβ1 signaling has identified activation of the JNK pathway by the TGFβ-activated kinase, TAK1 (Wang et al., 1997). Activation of TAK1 has also recently been linked to induction of apoptosis in eye development in *Drosophila* (Takatsu et al. 2000). In addition, TGFβs activate multiple pathways in different cell types, including activation of the SMAD family of proteins, which may cooperate with other transcription factors to elicit a cell-specific response to TGFβ stimulation (Massague and Wotton, 2000). SMAD3 and SMAD4 proteins have been shown to interact with jun/fos heterodimers, stimulating AP1-dependent transcription, demonstrating convergence of the JNK and SMAD pathways in

response to TGFβ (Zhang et al., 1998). Indeed, treatment of Schwann cells with TGFβ causes nuclear localization of SMAD4 (D. Parkinson, unpublished observation). The link seen in the present experiments between TGFβ stimulation, c-Jun phosphorylation, and cell death is of particular interest, because to our knowledge it has not been seen before in the same cell type.

We find that TGFβ-induced death is distinct from the cell death observed after the withdrawal of survival signals such as NRGβ or the autocrine survival mixture of IGF-2, NT3, and PDGF-BB (Grinspan et al., 1996; Syroid et al., 1996; Meier et al., 1999), because in Schwann cells the cell death induced by withdrawal of these growth factors is not inhibited by the general caspase inhibitor Z-VAD.fmk and apparently is not accompanied by phosphorylation of c-Jun (C. Meier, unpublished observation). In contrast, the cell death induced by TGFβ1 in Schwann cells requires the activity of caspases and is inhibited by the caspase inhibitor Z-VAD.fmk, in keeping with previous findings of caspase activation in cells by TGFβ (Schrantz et al., 1999; Shima et al., 1999). TGFβ-induced cell death is reduced but not prevented by autocrine survival signals or by NRGβ, although in combination these signals allow survival of TGFβ-treated cells. This requirement for a combination of survival signals may suggest a role for TGFβ-mediated death during the embryonic and neonatal phase of Schwann cell development when autocrine signals are less prominent than at later stages (Meier et al., 1999). TGFβ1 has also been shown to reduce levels of NT3 mRNA expression in Schwann cells (Cai et al., 1999). Because this is an important component of the autocrine survival factors produced by Schwann cells, it may be an additional mechanism by which TGFβ1 induces cell death (Meier et al., 1999). The resistance of Schwann cells to TGFβ-induced killing as the nerve matures is paralleled by a failure to phosphorylate c-Jun *in vitro*. Nevertheless, after some days in culture, presumably as they dedifferentiate, previously resistant cells become susceptible to TGFβ-induced killing, suggesting that under some circumstances, even in mature Schwann cells, TGFβ could play a role in cell death, particularly in combination with other factors such as TNF-α (Skoff et al., 1998).

Induction of apoptosis is often related to regulation of the Bcl-2 family of molecules, specifically an alteration in the balance between pro- and anti-apoptotic members of this group (Newton and Strasser, 1998). Upregulation of JNK activity involved in apoptosis of Schwann cells after serum deprivation is inhibited by Bcl-X(L) overexpression (Cheng et al., 2001). We have observed a transcriptional upregulation of the pro-apoptotic Bax mRNA by TGFβ1 in Schwann cells, and furthermore that Bax and p53 mRNAs are strongly downregulated during development in a manner that is inversely related to differentiation (data not shown). Regulation of such pro-apoptotic molecules may contribute to TGFβ1-induced apoptosis in Schwann cells and the altering susceptibility of cells to apoptosis during development.

The present experiments argue that one of the functions of TGFβ in peripheral nerves is to take part in negative survival regulation of developing Schwann cells. There is evidence that NGF/p75 signaling acts in a comparable manner, whereas positive survival signals in developing nerves are likely to include NRGβ, IGF-2, NT3, PDGF-BB, and LIF (for references, see introductory remarks). In addition to taking part in this network of survival-regulating signals, there is good evidence that TGFβ is capable of controlling Schwann cell proliferation and differentiation without necessarily inducing cell death (Mews and Meyer, 1993; Morgan et al., 1994; Einheber et al., 1995; Guenard et al., 1995). This shows clearly that the effects of TGFβ on Schwann cells are context dependent, a point illustrated in the present work in the interactions between TGFβ and NRGβ and autocrine signals. It will be some time before we are in a position to generate an integrated picture of the involvement of TGFβ in Schwann cell biology.

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