Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 9229–9234, August 1996
Neurobiology

Cell death in the Schwann cell lineage and its regulation by neuregulin

(apoptosis/glia/glial growth factor/nervous system)

DANIEL E. SYROID*, PETER R. MAYCOX*†, PATRICK G. BURROLA*, NAILI LIU‡, DUANZHI WEN‡, KUO-FEN LEE§, GREG LEMKE*¶, AND TREVOR J. KILPATRICK*‖

*Molecular Neurobiology Laboratory, §Clayton Foundation Laboratories for Peptide Biology, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, CA 92037; and ‡Amgen Inc., 1840 DeHavilland Road, Thousand Oaks, CA 91320

Communicated by Gerald D. Fischbach, Harvard Medical School, Boston, MA, May 14, 1996 (received for review March 15, 1996)

ABSTRACT **The development of Schwann cells, the myelin-forming glial cells of the vertebrate peripheral nervous system, involves a neonatal phase of proliferation in which cells migrate along and segregate newly formed axons. Withdrawal from the cell cycle, around postnatal days 2–4 in rodents, initiates terminal differentiation to the myelinating state. During this time, Schwann cell number is subject to stringent regulation such that within the first postnatal week, axons and myelinating Schwann cells attain the one-to-one relationship characteristic of the mature nerve. The mechanisms that underly this developmental control remain largely undefined. In this report, we examine the role of apoptosis in the determination of postnatal Schwann cell number. We find that Schwann cells isolated from postnatal day 3 rat sciatic nerve undergo apoptosis *in vitro* upon serum withdrawal and that Schwann cell death can be prevented by β forms of neuregulin (NRG-β) but not by fibroblast growth factor 2 or platelet-derived growth factors AA and BB. This NRG-β-mediated Schwann cell survival is apparently transduced through an ErbB2/ErbB3 receptor heterodimer. We also provide evidence that postnatal Schwann cells undergo developmentally regulated apoptosis *in vivo*. Together with other recent findings, these results suggest that Schwann cell apoptosis may play an important role in peripheral nerve development and that Schwann cell survival may be regulated by access to axonally derived NRG.**

Peripheral nerve development involves a symbiotic and dynamic interaction between axons and Schwann cells (1). When Schwann cell precursors first contact axons, they are actively dividing, flat, GAP43-positive cells. Their subsequent differentiation into committed Schwann cells, which in the rat occurs around embryonic days 16 and 17, is marked by the acquisition of a bipolar morphology and S100 immunoreactivity (2). At this stage, migrating cells extend processes, which penetrate between axons and segregate them into progressively smaller bundles. Schwann cell proliferation and segregation continue such that in the early neonate, each myelinated axon is wrapped by a single myelinating Schwann cell. Withdrawal from the cell cycle at postnatal days 2–4 (3) initiates terminal Schwann cell differentiation and the onset of myelination of a subset of large-diameter axons.

How Schwann cell numbers are regulated to produce the mature 1:1 relationship between axons and myelinating Schwann cells remains largely unexplored. While inhibition of proliferation is clearly involved, programmed cell death, which usually proceeds through a series of well-defined transitions in cellular morphology referred to as apoptosis (4), may also play a role. Indeed, it is well established that programmed cell death is a significant developmental event in both neuronal (5) and oligodendrocytic lineages (6), in which differentiating progenitor cells are susceptible to apoptosis upon exit from the cell cycle. More recently, Schwann cell precursors have been shown to undergo apoptotic cell death *in vitro* (7). If developmentally regulated apoptosis in the Schwann cell lineage were to play a significant role in establishing correct Schwann cell numbers in the mature nerve, it may be expected to function primarily as a postnatal event, subsequent to the cessation of neonatal Schwann cell proliferation and coincident with terminal differentiation. In this context, induction of Schwann cell death would serve to ensure that myelinating Schwann cell and axonal numbers were correctly matched by removing supernumerary Schwann cells.

Although apoptosis is an important mechanism by which cell numbers are regulated during development, it must be tightly controlled if it is to subserve this function efficiently. For developing neurons, this is largely accomplished by the positive selection of surviving cells through the competition for limiting target-derived trophic support (5, 8). Given the interdependency of Schwann cells and axons during peripheral nerve development (9), an axonally derived trophic factor could subserve an equivalent function for Schwann cells. One such factor that may regulate the survival of developing postnatal Schwann cells *in vivo* is glial growth factor. Glial growth factor, which was originally described as a mitogen for rat Schwann cells *in vitro* (10, 11), belongs to a group of related growth factors with diverse biological functions that are now known collectively as the neuregulins (NRG; for recent reviews on the NRGs and their receptors, see refs. 12 and 13). NRG is highly expressed by the neurons whose axons populate both developing and adult peripheral nerves, and this growth factor has been shown to mediate a variety of effects on glial cell development in culture (12–17). In particular, NRG has been demonstrated to prevent the apoptotic death of Schwann cell precursors *in vitro* (18).

The NRGs are encoded by a single gene, with alternative splicing resulting in the generation of at least 12 distinct membrane-bound or soluble molecules, all of which contain an epidermal growth factor (EGF)-like region in their extracellular domains. The various protein products can be subclassified into α and β species on the basis of sequence differences

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: NRG, neuregulin; NRG-β, NRG β isoform; EGF, epidermal growth factor; FGF-2, fibroblast growth factor 2; PDGF-AA and PDGF-BB, platelet-derived growth factors AA and BB; FCS, fetal calf serum; Fsk, forskolin; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP-fluorescein nick end-labeling.

†Present address: Department of Molecular Neuropathology, Smith–Kline Beecham Pharmaceuticals, Third Avenue, Harlow, Essex, CB11 3UG, United Kingdom.
¶To whom reprint requests should be addressed.
‖Present address: Neuroimmunology Laboratory, The Walter and Eliza Hall Institute of Medical Research, Royal Parade, Parkville 3052, Melbourne, Australia.

in the carboxyl-terminal region of the EGF-like domain. NRGs activate members of a subfamily of receptor tyrosine kinases that are related to the EGF receptor, a subfamily that also includes the ErbB2/Neu, ErbB3, and ErbB4 receptors (12, 13). Formation and activation of a functional NRG receptor is thought to involve heterodimerization between the signal transducer ErbB2 and either of two NRG binding components, ErbB3 or ErbB4, although ErbB4 on its own may be activated by NRG and elicit cellular responses.

In this paper, we establish that postnatal Schwann cells, both *in vitro* and *in vivo*, are susceptible to apoptotic cell death and, moreover, that Schwann cell death can be prevented by β forms of NRG (NRG-β). In contrast, other well-characterized Schwann cell mitogens, namely fibroblast growth factor 2 (FGF-2) and platelet-derived growth factor BB (PDGF-BB), fail to mediate a survival effect. Furthermore, NRG-induced survival activity in Schwann cells is shown to be potentially mediated through an ErbB2/ErbB3 heterodimeric receptor. These results suggest that programmed cell death in the Schwann cell lineage is initiated by a relative deficit in the provision of axonally synthesized NRG and provide a potential mechanism by which peripheral nerve modeling occurs in postnatal development.

MATERIALS AND METHODS

Cell Culture. Cultures of rat Schwann cells were prepared from postnatal day 3 sciatic nerve and purified to greater than 99.5% homogeneity essentially as described (19). Cells were plated on poly-L-lysine-coated (100 μg/ml; Sigma) 10-cm tissue culture Petri dishes (Falcon) and maintained in DMEM (GIBCO), 10% fetal calf serum (FCS; HyClone), 2 μM forskolin (Fsk; Calbiochem), and partially purified bovine glial growth factor, purified as described (11). Schwann cells grown on eight-well chamber slides (Lab-Tek) were plated on poly-L-lysine (100 μg/ml) and laminin (10 μg/ml; GIBCO). For survival assays, cells were dissociated by trypsinisation, washed once in DMEM containing 10% FCS to inactivate the trypsin, and then washed an additional five times in cold serum-free DMEM to remove serum. Cells were plated at 200–800 cells per well in microwell plates (Nunc), in either DMEM with growth factor or DMEM containing BSA (Sigma) as the negative control. In each experiment, the final concentration of BSA in the negative control was equivalent to that present in the test conditions. Assays were performed over a 72-hr period using multiple microwell plates, such that numbers of viable cells at each time point (0, 1, 2, and 3 days) were scored from six wells in a single plate using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) survival assay (20). Survival assays were carried out independently at least three times, except for conditions in which survival activity was not detected, in which case survival assays were carried out twice. The number of viable cells per condition was normalized as a fraction of the baseline or initial plating density (time 0) and expressed as a survival percentage.

Growth Factors. FGF-2, PDGF-AA, and PDGF-BB were from Boehringer Mannheim. Human recombinant β forms of NRG were obtained from Amgen. A full-length form of NRG-β1 [NRG-$\beta1_{(14-246)}$; amino acids 14–246] and two truncated forms [NRG-β, amino acids 177–228, and NRG-$\beta1_{(177-246)}$, amino acids 177–246] were used. BSA (0.1% final concentration) was added to all growth factors as a carrier protein.

MTT Survival Assay. MTT (Sigma) 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. The number of cells in the bottom of each well exhibiting a positive blue granular reaction product was assessed.

[^{3}H]Thymidine Incorporation Assay. Schwann cells (10,000 cells per well) were plated onto eight-well chamber slides in DMEM containing 10% FCS, Fsk (2 μM), and NRG-β (50 ng/ml) and grown for 24 hr. Cells were then washed three times in serum-deficient DMEM and incubated for 24 hr in either DMEM containing 10% FCS, Fsk (2 μM), and NRG-β (50 ng/ml), DMEM containing NRG-β only (50 ng/ml; using the full-length NRG-β1 and both truncated forms of the molecule), or DMEM containing BSA only. Each culture condition was performed in triplicate. [^{3}H]Thymidine (Amersham) was then added to all wells at a concentration of 0.03 μCi/ml (1 Ci = 37 GBq), and after a further 24 hr, the cells were fixed for 20 min at room temperature in 4% paraformaldehyde/PBS buffer (137 mM NaCl/2.7 mM KCl/4.3 mM $Na_2HPO_4 \cdot 7H_2O$/1.4 mM KH_2PO_4, pH 7.3).

Slides were washed for 5 min at room temperature in PBS; dehydrated through sequential washes for 2 min each in water and 70%, 95%, and 100% ethanol; and then finally air-dried at room temperature. The slides were then quickly dipped in NTB-2 emulsion (1:1 in water; Kodak) at 45°C, air-dried, and stored for 2 weeks at 4°C in the dark. Slides were developed in D-19 (Kodak) and fixed in Kodak fixer according to the manufacturer's specifications. Slides were counterstained in Mayer's hematoxylin (Sigma) for 5 min, washed in tap water for 5 min, and then mounted.

Cells that had four or more silver grains over their nuclei were considered to be [^{3}H]thymidine-positive. The experiment was performed three times, and on each occasion, the percentage of cells with positive nuclei from the three wells exposed to each culture condition was determined by assessing approximately 300 randomly chosen nuclei.

Detection of Apoptotic Cells. For the detection of apoptotic Schwann cells *in vitro*, Schwann cells grown on eight-well chamber slides were fixed for 5 min at room temperature in 4% paraformaldehyde/PBS. Cells were washed three times for 5 min in PBS, permeabilized by incubation for 5 min at room temperature in PBS containing 0.1% Triton X-100 (Sigma), and then washed as described above. Apoptotic cells were detected using the TUNEL technique (21) by incubating the cells for 1 hour at 37°C in terminal deoxynucleotidyltransferase-mediated dUTP-fluorescein nick end labeling (TUNEL) reaction mixture supplied in the *in situ* Cell Death Detection Kit, Fluorescein (Boehringer Mannheim). Cells were washed as above and then stained with Hoechst nuclear stain (1 μg/ml in PBS; Sigma) for 2 min at room temperature. Cells were washed again as above, and slides were mounted using Vectashield fluorescence mounting medium (Vector Laboratories).

For the detection of apoptotic Schwann cells *in vivo*, longitudinal sections of rat sciatic nerve were first fixed for 10 min in 4% paraformaldehyde/PBS. Sections were then washed two times for 5 min in PBS and digested with proteinase K (20 μg/ml in PBS; Sigma) for 10 min at room temperature. Sections were washed two times for 5 min in distilled water and fixed in 10% neutral buffered formalin (10% formaldehyde in PBS) for 10 min at room temperature. Sections were washed two times for 5 min in PBS and then post-fixed in ethanol/acetic acid (2:1) for 5 min at −20°C. Sections were washed two times for 5 min in PBS, and the TUNEL reaction was performed as described above except that reactions were allowed to proceed for 2 hr. Sections were washed four times for 5 min in PBS and stained with Hoechst nuclear stain as described for 5 min at room temperature. Sections were then washed three times for 5 min in PBS, and slides were mounted as described above.

Cryostat Sections of Sciatic Nerve. Postnatal day 3 rat sciatic nerves were dissected and fixed in 4% paraformaldehyde/PBS for 30 min. Nerves were first embedded in agar (2.5% in PBS; Sigma) and then incubated overnight in 20% sucrose/PBS at 4°C. Sciatic nerves were then embedded in Tissue-Tek OCT compound (Miles) and frozen in dry ice. Frozen longitudinal sciatic nerve sections (8 μm) were cut and collected onto

Vectabond-treated (Vector Laboratories) glass slides using a cryostat microtome and allowed to dry overnight at room temperature. Sections were stored at −70°C.

RESULTS

Postnatal Schwann Cells Undergo Apoptosis *in Vitro*. We first examined whether Schwann cells isolated from postnatal day 3 rat sciatic nerve undergo apoptosis *in vitro*, in serum-free conditions. Schwann cells were grown to 90% confluency, washed in serum-deficient DMEM, and then incubated for 48 hr in either the presence or the absence of serum (Fig. 1). Cells were assessed for the morphological features of apoptosis using phase contrast microscopy, Hoechst nuclear staining, and the TUNEL technique. Although cells maintained in the presence of serum appeared morphologically normal (Fig. 1*A*) with oval-shaped nuclei characteristic of Schwann cells grown *in vitro* (Fig. 1*B*), widespread Schwann cell death occurred upon serum withdrawal (see Fig. 2*A*). Cells became rounded, and a significant percentage were convoluted (Fig. 1*D*). Such cells displayed both nuclear fragmentation and chromatin condensation (denoted by arrows; Fig. 1*E*), both morphological features of apoptotic cell death (4). Moreover, the TUNEL technique established that both the fragmented nuclei and a subset of cells with morphologically normal nuclei served as substrates for the incorporation of dUTP (Fig. 1*F*). These observations suggest that such nuclei were at different stages in the apoptotic pathway but that DNA fragmentation, another distinguishing feature of apoptotic cell death, was well advanced in each dying cell. Cells maintained in the presence of serum did not incorporate dUTP (Fig. 1*C*). Despite the positive TUNEL reaction for Schwann cells grown in the absence of serum, we failed to identify DNA laddering (data not shown), similar to the previously reported inability to detect DNA laddering in oligodendrocytes undergoing apoptotic cell death (20). Death of Schwann cells subjected to serum withdrawal was partially inhibited over 24 hr (approximately 50%) when cells were cultured in the presence of the protein synthesis inhibitor cycloheximide (data not shown), suggesting that active metabolic processes were at least partially involved in mediating the observed Schwann cell death. Taken together, these results indicate that Schwann cell death was apoptotic rather than necrotic in nature.

Schwann Cell Death Is Inhibited by NRG. To assess the kinetics of Schwann cell death, cells were plated in serum-deficient DMEM in microwell plates at low cell density, and serial MTT incorporation assays were performed (Fig. 2). This study revealed an ongoing reduction in viable cell numbers over a 3-day period (BSA-negative control; Fig. 2*A*). These results are consistent with the morphological data above, which suggest that Schwann cell death was asynchronous in nature, a phenomenon that is also associated with apoptotic cell death (4). This was further corroborated by the observation that at any given time point there was significant variability in the degree to which individual cells incorporated and converted MTT into the chromogenic reaction product (data not shown).

We next examined whether Schwann cell death could be inhibited by the administration of various growth factors previously implicated in playing a role in glial cell development *in vitro*. Three growth factors, namely FGF-2, PDGF-BB, and NRG-β1, are mitogenic for Schwann cells *in vitro*, an activity which is potentiated by the Fsk-mediated elevation of intracellular levels of cAMP (9, 16, 22–24). Moreover, FGF-2 and NRG-β1 can act as survival factors for Schwann cell precursors *in vitro* (7, 18), whereas PDGF-AA can mediate the survival of oligodendrocytes and their O-2A progenitors both *in vitro* and *in vivo* (20). As shown in Fig. 2*A*, FGF-2, PDGF-AA, and PDGF-BB could not effect the survival of Schwann cells plated in the absence of serum. However, NRG-β (a truncated form of NRG-β1 containing amino acids 177–228, essentially encompassing the β-type EGF-like domain) was able to maintain viable Schwann cell numbers at approximately 80% of the initial plating density over a 3-day period (Fig. 2*A*) and did so in a dose-dependent manner (data not shown). Another truncated form of NRG-β1 containing amino acids 177–246, NRG-$\beta1_{(177-246)}$, exerted a survival effect on Schwann cells similar to that derived from NRG-β, whereas the full-length molecule containing amino acids 14–246, NRG-$\beta1_{(14-246)}$, was slightly less efficient in potentiating Schwann cell survival (Fig. 2*B*). These observations are consistent with previous demonstrations that the NRG β-type EGF-like domain is sufficient to effect the survival of Schwann cell precursors *in vitro* (18)

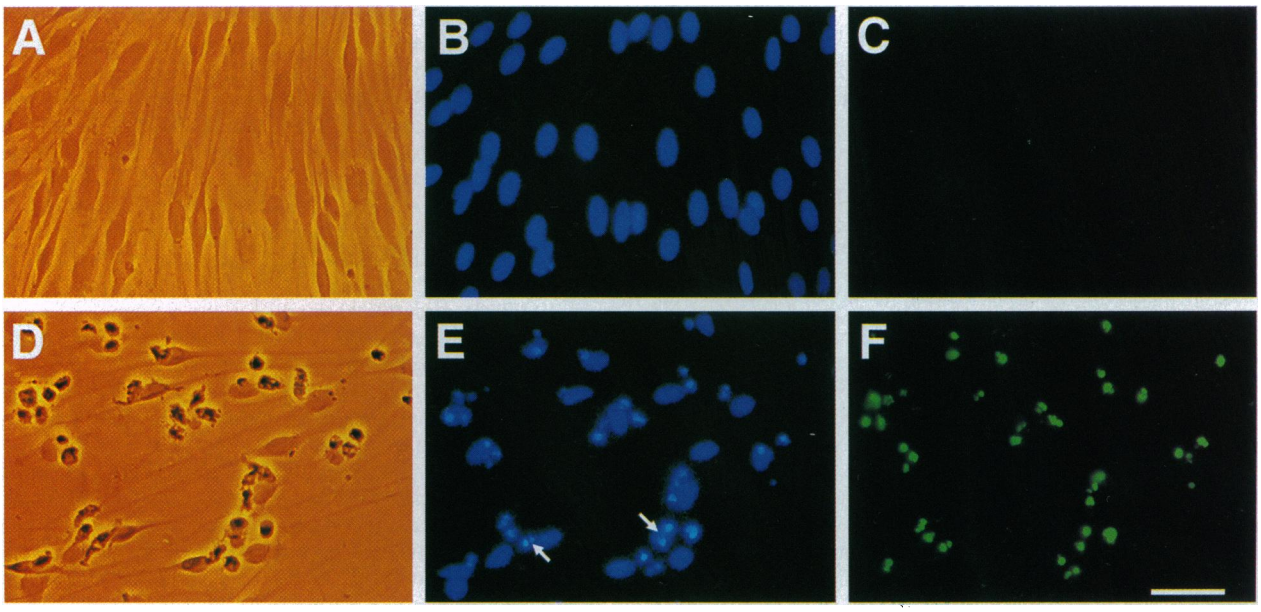

FIG. 1. Postnatal Schwann cells die by apoptosis *in vitro* upon serum withdrawal. Confluent cultures of rat Schwann cells in eight-well chamber slides were washed two times with serum-deficient DMEM and incubated for 48 hr in either the presence (*A–C*) or the absence (*D–F*) of serum. (*A* and *D*) Phase contrast microscopy showing cellular morphology. (*B* and *E*) Hoechst staining of cells showing nuclear morphology. (*C* and *F*) Detection of Schwann cells undergoing apoptosis using the TUNEL technique. Arrows denote nuclear fragmentation. (Bar = 10 μm.)

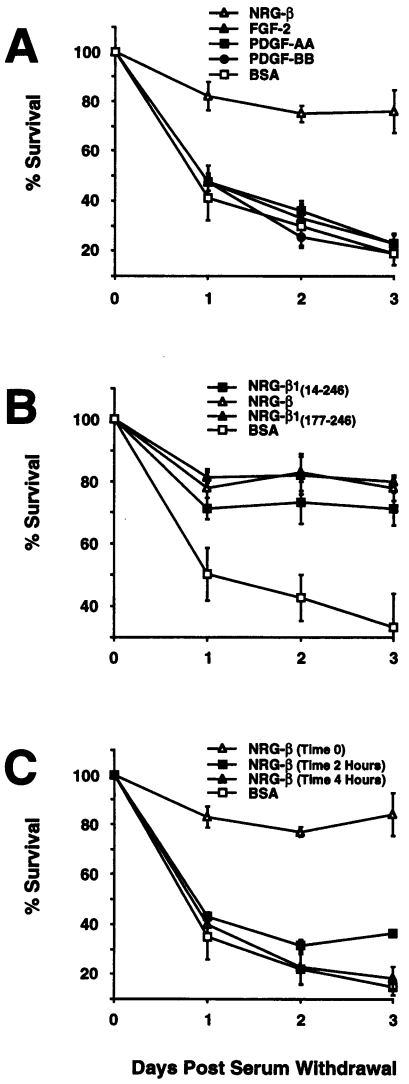

FIG. 2. NRG promotes the survival of Schwann cells *in vitro*. Rat Schwann cells were washed five times in serum-deficient DMEM and plated on multiple microwell plates either in the presence of the factor indicated or with BSA. Viable cells were identified and counted using an MTT incorporation assay at daily intervals over a 3-day period. (*A*) NRG-β could effect Schwann cell survival, whereas FGF-2, PDGF-AA, PDGF-BB, and BSA could not (factor concentration was 10 ng/ml). (*B*) Full-length NRG-β1$_{(14-246)}$ was less efficient in mediating Schwann cell survival than truncated NRG-β and NRG-β1$_{(177-246)}$ (factor concentration was 50 ng/ml). (*C*) NRG-β (10 ng/ml) could effect Schwann cell survival only when added coincident with cell plating (time 0), but not when added 2 or 4 hr after plating. Error bars represent the standard deviation from the mean in survival activity derived from three separate experiments. Conditions in which survival activity was not detected were carried out twice; error bars represent the range in survival activity.

and the proliferation of postnatal Schwann cells in culture (16), and support earlier work demonstrating that the EGF-like domain is both necessary and sufficient for NRG receptor activation *in vitro* (12, 13). Schwann cells cultured in serum-deficient DMEM containing NRG-β converted MTT into extremely high levels of the chromogenic reaction product, indicating that these Schwann cells were much more metabolically active than those cultured with the other factors or with BSA alone (data not shown). Cells cultured in DMEM containing NRG-β also displayed marked morphological differences in comparison with cultures containing the other factors, or control cultures containing BSA only. For example, cells cultured with NRG-β were elongated and bipolar (see Fig. 3*B*), whereas the majority of the few viable weakly MTT-positive cells in control cultures (data not shown) were rounded and did not display extended processes (see Fig. 3*C*).

Given that up to 3 days were required for a subset of the Schwann cells to lose MTT positivity and to become apoptotic in BSA-containing control cultures (Fig. 2*A*), we examined whether such cells could be rescued from cell death by the addition of NRG-β at various times after plating. Interestingly, administration of NRG-β 2 hr after plating was only able to effect the survival of a small subset of Schwann cells, whereas addition of NRG-β 4 hr after plating was entirely ineffective in down-regulating cell death (Fig. 2*C*). These results further support the notion that a genetic program is initiated during apoptotic cell death and demonstrate that Schwann cells are irreversibly committed to a cell death pathway within 2–4 hr of plating in the absence of serum.

NRG Is a Schwann Cell Survival Factor. To examine the possibility that NRG-β isoforms were acting as Schwann cell mitogens rather than as survival factors, [^{3}H]thymidine incorporation assays were performed. As shown in Fig. 3*A*, the vast majority of cells cultured under proliferative conditions actively incorporated [^{3}H]thymidine (89%; 946 nuclei). In contrast, only 0.03% of Schwann cells cultured in the presence of NRG-β alone incorporated [^{3}H]thymidine [Fig. 3*B*; 2922 nuclei (additional NRG-β1 isoforms not shown)], whereas no [^{3}H]thymidine incorporation was detected in negative control cultures containing BSA only (Fig. 3*C*; 758 nuclei). These results demonstrate that the NRG-β isoforms, in the absence of other cofactors, are Schwann cell survival factors *in vitro*.

ErbB2 and ErbB3 Are Expressed by Schwann Cells. To examine the expression profile of NRG receptors in rat Schwann cells, a Northern blot analysis was carried out. As indicated in Fig. 4, Schwann cells cultured in the presence of serum express both ErbB2 and ErbB3 NRG receptor mRNAs. However, expression of the ErbB4 receptor mRNA was not detected (data not shown). These results are in agreement with previous studies in which the expression profile of NRG receptors was examined in proliferating human Schwann cells *in vitro* (16). Interestingly, expression of the ErbB2 and ErbB3 receptors was maintained for both 4 and 24 hr after serum withdrawal (Fig. 4). The results suggest that the NRG-induced survival signal in postnatal Schwann cells is transduced through an ErbB2/ErbB3 heterodimeric receptor and that the temporal dependence in the ability of NRG-β to abrogate Schwann cell apoptosis subsequent to serum withdrawal (Fig. 2*C*) is not due to ErbB2 or ErbB3 down-regulation.

Schwann Cell Death *in Vivo*. To determine whether Schwann cell apoptosis might play a role in postnatal peripheral nerve development *in vivo*, the TUNEL technique was adopted to identify apoptotic Schwann cells in longitudinal sections of postnatal day 3 rat sciatic nerves. Schwann cells can easily be identified in longitudinal sciatic nerve sections based on their typical elliptical nuclear morphology (Fig. 5 *A* and *C*). When numerous sections (n = 20; approximately 24,000 nuclei) were directly examined for the presence of apoptotic Schwann cells using the TUNEL technique, approximately 0.18% of the Schwann cells in these sciatic nerves were unambiguously undergoing apoptosis at the time of fixation. Two representative apoptotic Schwann cells detected using the TUNEL technique are shown (Fig. 5 *B* and *D*), whereas alterations in nuclear morphology associated with apoptotic cell death in these two cells are shown in the corresponding Hoechst stained nuclei (*A* and *C*, respectively). Note the more severe nuclear fragmentation (denoted by arrows) for the apoptotic Schwann cell in Fig. 5 *C* and *D*, suggesting that apoptosis for this Schwann cell is at a more advanced stage than that for the apoptotic Schwann cell in Fig. 5 *A* and *B*. In contrast to developing nerves, no apoptotic Schwann cells were detected in longitudinal sections of mature adult rat or mouse sciatic nerves [n = 17; approximately 25,000 nuclei (data not

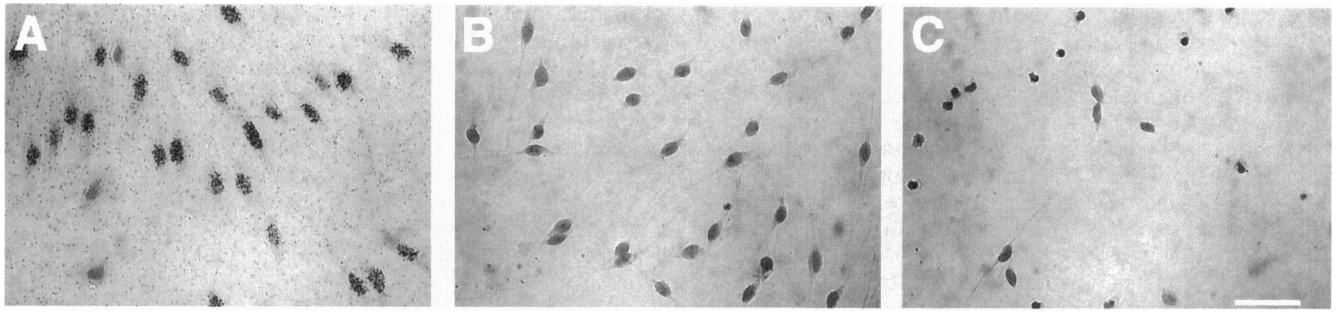

FIG. 3. NRG is a Schwann cell survival factor *in vitro*. Subconfluent cultures of Schwann cells were grown for 24 hr on eight-well chamber slides in the presence of serum, NRG-β (50 ng/ml) and Fsk (2 μM). Cells were washed three times in serum-deficient DMEM and incubated for 24 hr in DMEM containing either serum, NRG-β (50 ng/ml), and Fsk (2 μM) (*A*), NRG-β (50 ng/ml) (*B*), or BSA (*C*). [^{3}H]Thymidine (0.03 μCi/ml) was then added, and the cells were further incubated for 24 hr, after which the cells were fixed and [^{3}H]thymidine incorporation was assessed. (Bar = 10 μm.)

shown)]. The results demonstrate that postnatal rat Schwann cells undergo apoptotic cell death *in vivo* and suggest that this process may be a developmentally regulated rather than a stochastic event.

DISCUSSION

The results above demonstrate that postnatal Schwann cells, like their embryonic precursors (7, 18), are susceptible to apoptotic cell death *in vitro*. However, postnatal Schwann cells are not only morphologically distinct from their precursor cells but can also be distinguished on the basis of their *in vitro* behavior. Although postnatal Schwann cells remain viable but quiescent *in vitro* when cultured in the presence of serum but in the absence of exogenous factors, Schwann cell precursors undergo rapid apoptotic cell death in the absence of exogenous growth factor support (7). This may reflect the existence of an unidentified serum-derived survival factor to which only postnatal Schwann cells can respond or, alternatively, the synthesis of a factor by postnatal Schwann cells that then operates in an autocrine manner to potentiate their survival. Postnatal Schwann cells undergo apoptotic cell death *in vitro* only upon serum withdrawal, and cell death is asynchronous in nature, the majority of cells dying over 3 days (Fig. 2*A*). In contrast, Schwann cell precursors are more susceptible to apoptotic cell death *in vitro*, the majority of cells dying within 5–6 hr of plating (7). These observations are consistent with the existence of distinct molecular mechanisms for the induction of apoptotic cell death in the two Schwann cell populations or, alternatively, of the function of distinct downstream signaling pathways that ultimately converge in Schwann cell apoptosis. Multiple apoptotic signaling mechanisms may serve to modulate cellular responses to the variety of developmental cues that exist for these distinct Schwann cell populations.

Postnatal Schwann cells and their precursors can also be distinguished by their response to survival factors *in vitro*. Whereas NRG can mediate the survival of both cell types, FGF-2 can act as a survival factor only for Schwann cell precursors (Fig. 2*A*; ref. 7). That FGF-2 could not effect the survival of postnatal rat Schwann cells was somewhat surpris-

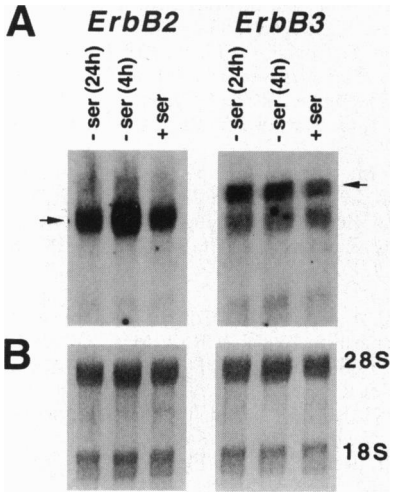

FIG. 4. NRG signals through an ErbB2/ErbB3 receptor heterodimer in mediating Schwann cell survival. Northern blots were prepared using total RNA (approximately 10 μg per lane) isolated from subconfluent Schwann cells either cultured in DMEM containing serum only (+Ser) or upon serum withdrawal (−Ser) for either 4 or 24 hr. (*A*) Both ErbB2 and ErbB3 are expressed in Schwann cells *in vitro* as indicated by arrows (transcript size approximately 4.8 and 6.2 kb, respectively). Moreover, expression was maintained upon serum withdrawal. cDNA fragments encompassing the extracellular domains of rat ErbB2 and human ErbB3 were used to probe blots, which were washed under high stringency conditions. Note that nonspecific trapping of ErbB3 probe was by 28S and 18S ribosomal RNA. (*B*) Methylene blue staining of 28S and 18S RNA after transfer.

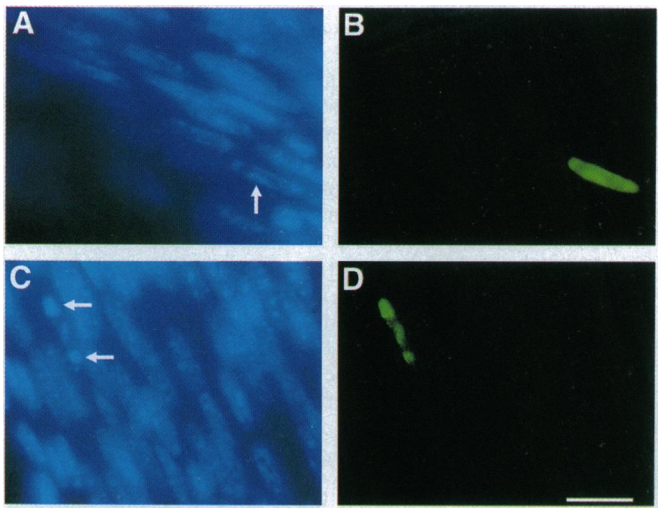

FIG. 5. Postnatal Schwann cells undergo apoptosis *in vivo*. Schwann cell apoptosis in the early rat neonate was examined *in situ* using 8-μm-thick cryostat frozen sections of postnatal day 3 sciatic nerve. (*A* and *C*) Hoechst staining of sciatic nerve showing typical elliptical Schwann cell nuclear morphology. (*B* and *D*) Detection of Schwann cells undergoing apoptosis using the TUNEL technique. Arrows denote nuclear fragmentation. Note the more severe fragmentation of the Schwann cell nucleus shown in *C* and *D*, indicating that apoptosis of this cell is at a more advanced stage than that of the apoptotic Schwann cell shown in *A* and *B*. Schwann cells undergoing apoptosis were not detected in the mature adult rodent sciatic nerve (data not shown). (Bar = 5 μm.)

ing given that FGF-2 by itself can be mitogenic for these cells (9, 22). Response to FGF-2 as a survival factor in postnatal Schwann cells may depend on coadministration of Fsk, which is known, through elevation of intracellular cAMP, to up-regulate growth factor receptor expression (25) and which is required for optimal stimulation of postnatal rat Schwann cell proliferation by FGF-2 (24). These same considerations apply to PDGF-BB, which also can act as a mitogen for postnatal rat Schwann cells (9, 22, 23), but whose mitogenic effect also depends on cAMP elevation (25).

Our results also demonstrate that postnatal Schwann cells in the rodent sciatic nerve undergo apoptotic cell death *in vivo* and suggest that Schwann cell apoptosis may be developmentally regulated. Cell death may represent a mechanism by which Schwann cell numbers are regulated during postnatal development and matched to the number of axons, such that the appropriate stoichiometry between axons and Schwann cells is established. Although the frequency of Schwann cell apoptosis in postnatal day 3 nerves at the time of fixation was low (approximately 0.18%), this rate of Schwann cell death appears to be maintained during early postnatal development, at least from postnatal days 2–6 (data not shown). During this period, the generation of new cells rapidly declines (3); hence, the cumulative proportion of apoptotic Schwann cells during early postnatal development is likely to be much greater and may be analogous to developmentally regulated neuronal apoptosis (5) and oligodendrocytic cell death (6), in which cumulative cell death approaches 50%. Indeed, the frequency of apoptotic oligodendrocytes at any given time during the peak period of oligodendrocyte cell death in the developing rat optic nerve (postnatal days 4–10) is only 0.25% (20). Although the relative importance of Schwann cell death in peripheral nerve development *in vivo* remains to be established, these results suggest that postnatal Schwann cell apoptosis may play a significant role in modeling of the peripheral nerve.

Differentiating Schwann cells may be induced to undergo apoptosis in response to either the loss of, or the failure to secure, limiting axonally derived trophic support, analogous to the mechanism by which correct neuronal numbers and appropriate connections are established during development (5, 8). Because NRG potentiates the survival of postnatal Schwann cells *in vitro* (Fig. 2), it is conceivable that Schwann cell survival *in vivo* depends upon the provision of axonally derived NRG. Furthermore, because at least some NRGs are known to be expressed as membrane-bound molecules, it is easy to envisage that Schwann cells that have lost axonal contact, and thus trophic support, would be susceptible to cell death. Strong evidence for this model is provided by recent studies in which the terminal Schwann cells associated with the motor endplates of the neonatal rodent neuromuscular junction have been observed to undergo apoptotic cell death after peripheral nerve transection, an event that is prevented by the exogenous provision of NRG (26). Given this observation, it would be of interest to examine whether exogenously supplied NRG could preclude apoptotic Schwann cell death in the developing nerve as well. It is conceivable that, in addition to a potential role in defining overt Schwann cell numbers during postnatal development, Schwann cell apoptosis may also be a means by which improperly positioned Schwann cells are removed from surrounding axons.

Although growth factor withdrawal is almost certainly involved in the initiation of apoptotic Schwann cell death, it will be of great interest to identify the downstream molecules that mediate this process. Indeed, Schwann cells may afford a particularly useful system for characterization of the molecular mechanisms underlying programmed cell death in the nervous system in general. These cells are easily manipulated and expanded *in vitro*, unlike neurons, and can be maintained as primary cultures, unlike PC12 cells, which have been commonly adopted as an *in vitro* system for the study of neuronal cell death. Furthermore, the availability of Schwann cell-specific promoters renders the study of cell death in this lineage particularly amenable to molecular genetic approaches *in vivo* (27).

We thank Danny Ortuño, Catherine Jackson, and Darcie Baynes for technical assistance and Bob Hyman for kindly providing anti-Thy-1 antibodies. This work was supported by postdoctoral fellowships from the National Multiple Sclerosis Society (D.E.S.), the Boehringer-Ingelheim Funds and the Human Frontiers in Science Program Organisation (P.R.M.), the Howard Hughes Medical Institute and the Bushell Fellowship of the Royal Australasian College of Physicians (T.J.K.), and by a grant from the National Institutes of Health (G.L.).

1. Webster, H.deF. & Favilla, J. T. (1984) in *Peripheral Neuropathy*, eds. Dyck, P. J., Thomas, P. K., Lambert, E. H. & Bunge, R. (Saunders, Philadelphia), pp. 329–359.
2. Jessen, K. R. & Mirsky, R. (1992) *Curr. Opin. Neurobiol.* **2,** 575–581.
3. Brown, M. J. & Asbury, A. K. (1981) *Exp. Neurol.* **74,** 170–186.
4. Gerschenson, L. E. & Rotello, R. J. (1992) *FASEB J.* **6,** 2450–2455.
5. Oppenheim, R. W. (1991) *Annu. Rev. Neurosci.* **14,** 453–501.
6. Barres, B. A. & Raff, M. C. (1994) *Neuron* **12,** 935–942.
7. Jessen, K. R., Brennan, A., Morgan, L., Mirsky, R., Kent, A., Hashimoto, Y. & Gavrilovic, J. (1994) *Neuron* **12,** 509–527.
8. Barde, Y.-A. (1989) *Neuron* **2,** 1525–1534.
9. Eccleston, P. A. (1992) *Exp. Cell Res.* **199,** 1–9.
10. Raff, M. C., Abney, E., Brockes, J. P. & Hornby-Smith, A. (1978) *Cell* **15,** 813–822.
11. Lemke, G. E. & Brockes, J. P. (1984) *J. Neurosci.* **4,** 75–83.
12. Carraway, K. L., III & Burden, S. J. (1995) *Curr. Opin. Neurobiol.* **5,** 606–612.
13. Lemke, G. (1996) *Mol. Cell. Neurosci.* **7,** 247–262.
14. Pinkas-Kramarski, R., Eilam, R., Spiegler, O., Lavi, S., Liu, N., Chang, D., Wen, D., Schwartz, M. & Yarden, Y. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9387–9391.
15. Vartanian, T., Corfas, G., Li, Y., Fischbach, G. D. & Stefansson, K. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 11626–11630.
16. Levi, A. D., Bunge, R. P., Lofgren, J. A., Meima, L., Hefti, F., Nikolics, K. & Sliwkowski, M. X. (1995) *J. Neurosci.* **15,** 1329–1340.
17. Shah, N. M., Marchionni, M. A., Isaacs, I., Stroobant, P. & Anderson, D. J. (1994) *Cell* **77,** 349–360.
18. Dong, Z., Brennan, A., Liu, N., Yarden, Y., Lefkowitz, G., Mirsky, R. & Jessen, K. R. (1995) *Neuron* **15,** 585–596.
19. Brockes, J. P., Fields, K. L. & Raff, M. C. (1979) *Brain Res.* **165,** 105–118.
20. Barres, B. A., Hart, I. K., Coles, H. S., Burne, J. F., Voyvodic, J. T., Richardson, W. D. & Raff, M. C. (1992) *Cell* **70,** 31–46.
21. Gavrieli, Y., Sherman, Y. & Ben-Sasson, S. A. (1992) *J. Cell Biol.* **119,** 493–501.
22. Stewart, H. J. S., Eccleston, P. A., Jessen, K. R. & Mirsky, R. (1991) *J. Neurosci. Res.* **30,** 346–352.
23. Hardy, M., Reddy, U. R. & Pleasure, D. (1992) *J. Neurosci. Res.* **31,** 254–262.
24. Davis, J. B. & Stroobant, P. (1990) *J. Cell Biol.* **110,** 1353–1360.
25. Weinmaster, G. & Lemke, G. (1990) *EMBO J.* **9,** 915–920.
26. Trachtenberg, J. T. & Thompson, W. J. (1996) *Nature (London)* **379,** 174–177.
27. Messing, A., Behringer, R. R., Hammang, J. P., Palmiter, R. D., Brinster, R. L. & Lemke, G. (1992) *Neuron* **8,** 507–520.