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Oligodendrocyte Population Dynamics and the Role of PDGF In Vivo

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Summary

Oligodendrocyte progenitors originate near the floor plate of the spinal cord, then proliferate and migrate throughout the cord before giving rise to oligodendrocytes. Progenitor cell proliferation stops before birth because the cell cycle slows down, linked to an increase in differentiation and death. Experiments with transgenic mice show that platelet-derived growth factor (PDGF) drives progenitor cell division and suggest that slowing of and exit from the cycle reflects a decline in PDGF signaling. Overexpressing PDGF induces hyperproliferation of progenitor cells and excessive, ectopic production of oligodendrocytes. However, the superfluous oligodendrocytes die at an immature stage of differentiation, leaving a normal complement of myelin-forming cells. Therefore, cell survival controls override proliferation controls for determining the final number and distribution of mature oligodendrocytes.

Introduction

Oligodendrocytes are generated during embryonic and early postnatal life by differentiation of proliferative migratory glial progenitor cells known as O-2A progenitors (Raff et al., 1983; for reviews see Raff, 1989; Pfeiffer et al., 1994). In the embryonic spinal cord, the first O-2A progenitors are generated in a specialized part of the ventral neuroepithelium and subsequently proliferate and disperse evenly throughout the cord (reviewed by Miller, 1996; Richardson et al., 1997). Subsequently, postmitotic oligodendrocytes appear first in the ventral axon tracts and then in the dorsal and lateral tracts (developing white matter) (Jordan et al., 1989; Yu et al., 1994; this paper). It is not known how oligodendrocyte number is controlled during development nor what causes them to accumulate selectively in axon tracts even though their progenitor cells are not concentrated there. It seems likely that signaling between neurons and oligodendrocytes and/or their progenitors must play a role in the establishment and maintenance of their specific interactions (Levine, 1989; Barres and Raff, 1994; Burne et al., 1996). For example, it is reported that axons stimulate division of oligodendrocyte progenitor cells and that this depends on whether they are electrically active (Barres and Raff, 1993). Electrical activity might stimulate production or release of polypeptide mitogens either from the axons themselves or from other cells (e.g., astrocytes) in their vicinity (Barres and Raff, 1993). Axons are also thought to be required for oligodendrocyte survival (Barres et al., 1993).

A prime candidate for a neuron-derived mitogen is platelet-derived growth factor (PDGF), which is expressed by many neurons throughout the developing CNS (Yeh et al., 1991; Sasahara et al., 1991) and is known to be a potent mitogen for O-2A progenitors in vitro (Noble et al., 1988; Richardson et al., 1988; Levine, 1989). To test the role of PDGF in vivo, we examined PDGF knockout mice. There was a severe reduction in the number of O-2A progenitors and oligodendrocytes in the spinal cords of mice lacking PDGF-A, but not in mice lacking PDGF-B, implicating PDGF-AA homodimers in the control of progenitor cell proliferation in vivo. We also generated transgenic mice that overexpress PDGF-A in neurons, inducing hyperproliferation of oligodendrocyte progenitors and increasing their numbers up to 7-fold. This resulted in secondary overproduction of oligodendrocytes, many of which were abnormally located in gray matter. However, the extra, ectopic oligodendrocytes were all eliminated by programmed cell death at an immature stage of development so that the final number and distribution of mature oligodendrocytes was completely normal. These data demonstrate the dominance of cell survival controls over cell proliferation controls for determining the final number and spatial arrangement of postmitotic oligodendrocytes. Our data also suggest that the time at which myelinating oligodendrocytes start to appear in axon tracts might depend on the timed appearance of survival signals, not timed differentiation per se.

Results

Terminology

In this paper we have used the term "proliferation" to mean increase in number, not as a synonym for "divide." It is possible for progenitor cells to stop proliferating

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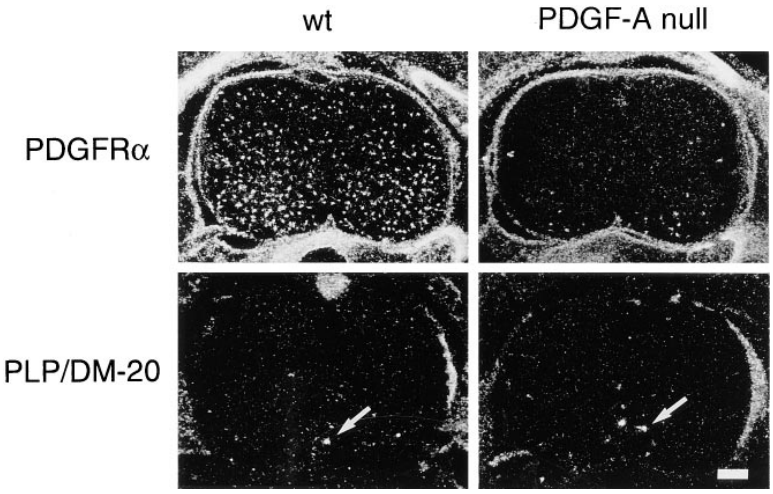


Figure 1. PDGF-A Is Required for Normal Proliferation of Oligodendrocyte Progenitors in the Spinal Cord

Sections were cut through the upper thoracic spinal cords of E17 homozygous *PDGF-A* knockout mice or wild-type littermates. These were hybridized with a ^{35}S-labeled RNA probe against *PDGFR*α, autoradiographed, and photographed under dark-field illumination. Each white dot within the spinal cord represents a cluster of silver grains overlying a single progenitor cell. In *PDGF-A*-null cords (top right) there were less than 5% of the normal number of progenitors compared to wild type (top left). The lack of progenitor cells in *PDGF-A*-null mice was not caused by premature differentiation into oligodendrocytes because there were very few cells positive for the myelin *PLP* mRNA (e.g., arrows) in either *PDGF-A*-null (bottom right) or wild-type cords (bottom left) at this age. The prominent structure lying above the spinal cord in the bottom-left panel is a cross-section through a blood vessel, which gives a false positive signal due to light scattering by red blood cells. Scale bar = 100 μm.

while continuing to divide if half of the newly formed cells are lost from the population each generation by differentiation and/or death. We define "differentiation" as a lengthy process starting at the moment progenitor cells exit the cell cycle, continuing until the oligodendrocytes myelinate axons. Therefore, if a progenitor cell exits the division cycle into G0 and immediately dies, we regard that as death of a newly formed oligodendrocyte, even if the postmitotic cell did not survive long enough to express recognizable oligodendrocyte markers such as GC or PLP/DM-20.

PDGF-AA Homodimers Are Required for Proliferation of O-2A Progenitor Cells in the Spinal Cord

Active PDGF consists of homodimers of A and B chains (AA, BB, AB), all of which can bind to and activate PDGFRα on O-2A progenitor cells (Heldin et al., 1988; Hart et al., 1989; Pringle et al., 1989). Many studies have shown that PDGF can influence oligodendrocyte development both in vitro and in vivo, but as yet there has been no confirmation that PDGF is necessary in vivo. We therefore investigated early development of the oligodendrocyte lineage in transgenic mice with targeted disruptions of the *PDGF-A* or *PDGF-B* gene.

Newborn *PDGF-A*$^{-/-}$ mice are outwardly normal but fail to thrive after birth. Most die within a few days of birth, but rare individuals survive for as long as three weeks, eventually dying of pulmonary failure (Boström et al., 1996). We visualized O-2A progenitor cells in spinal cords of *PDGF-A*$^{-/-}$ embryos and neonates and their wild-type littermates by in situ hybridization with a probe against *PDGFR*α mRNA. We and others have shown that PDGFRα marks O-2A progenitors in the perinatal rodent optic nerve (Hart et al., 1989), spinal cord (Yu et al., 1994; Hall et al., 1996; Nishiyama et al., 1996) and brain (Ellison and de Vellis, 1994). For example, when PDGFRα$^+$ cells are immunoselected from embryonic day 17 (E17) rat spinal cords and cultured in defined medium, they all give rise to oligodendrocytes, and very few oligodendrocytes develop in cultures of spinal cord cells that have been depleted of PDGFRα$^+$ cells by antibody-mediated complement lysis (Hall et al., 1996). Note that *PDGFR*α is rapidly downregulated after progenitors stop dividing and start to differentiate (Hart et al., 1989; Hall et al., 1996). Therefore, the *PDGFR*α expression pattern reflects the distribution of O-2A progenitors, not differentiated oligodendrocytes. *PDGFR*α is also expressed by cells in meningeal membranes and by many tissues outside the CNS (Orr-Urtreger and Lonai, 1992).

In *PDGF-A*-null spinal cords, the first O-2A progenitors appeared as normal at the luminal surface on E12.5, showing that PDGF-A is not required for initial specification of the oligodendrocyte lineage (data not shown). However, they did not proliferate normally after this; at all ages examined up to postnatal day 19 (P19) there were fewer than 10% of the normal number of O-2A progenitors in spinal cords of *PDGF-A*-null mice compared to their wild-type littermates. For example, we counted 236 ± 35 progenitors/section in E17 wild-type cords and 12 ± 8 in PDGF-A-null cords (mean ± SD of three sections from each of two embryos of each genotype) (Figure 1). We do not know whether the failure to proliferate in the absence of PDGF-A reflects a decrease in cell division or an increase in death or both. However, the lack of progenitors was not caused by premature generation of mature oligodendrocytes because in situ hybridization with probes for RNA transcripts coding for proteolipid protein (PLP; Figure 1) or myelin basic protein (MBP; data not shown) showed that there was very little mature oligodendrocyte production before birth in either wild-type or mutant spinal cords. The loss of PDGF-A in the knockouts does not cause general downregulation of *PDGFR*α mRNA because there was no change in the intensity of the *PDGFR*α signal in the many tissues that express this gene outside of the CNS. Also, longer exposures of the in situ autoradiographs failed to reveal any additional *PDGFR*α$^+$ progenitor cells in the knockout spinal cord.

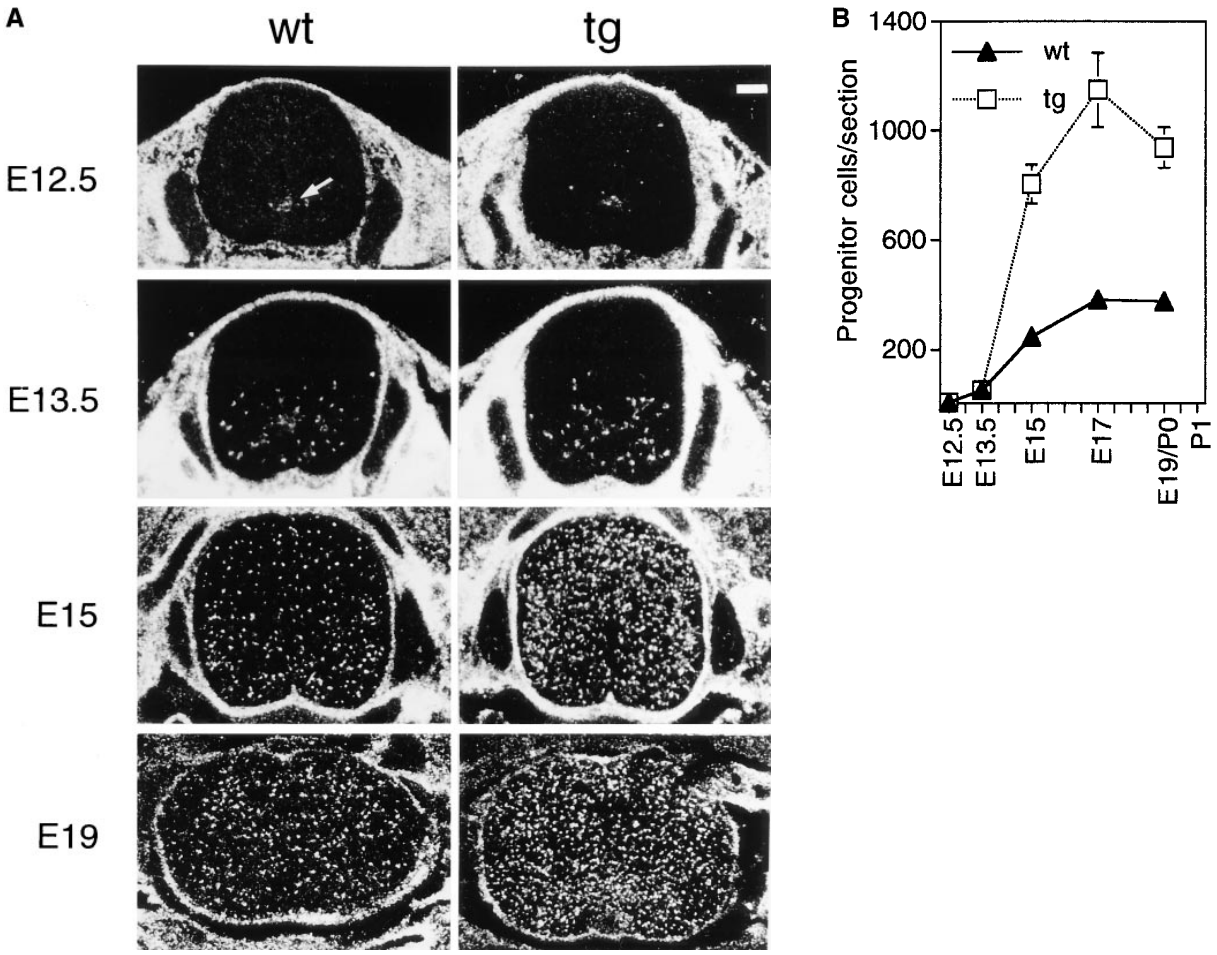


Figure 2. Proliferation of Oligodendrocyte Progenitors in Normal and Transgenic Spinal Cords Ceases after E15

(A) Sections through the upper thoracic spinal cord were subjected to in situ hybridization with a probe to *PDGFR*α as in Figure 1. In wild-type cords (left panels) the number of progenitor cells increased rapidly between E12.5 (arrow) and E15, but the rate of increase slowed down substantially after that, arresting between E15 and E17 (see [B]). In hemizygous *NSE-PDGF-A*$_S$ transgenic cords (right panels; see Figure 3), progenitor cell number was normal up until E13.5, but approximately three times the normal number of progenitors developed between E13.5 and E15, and this increase persisted until later times. Scale bar = 100 μm.

(B) Numbers of *PDGFR*α^+ progenitors in sections of spinal cords of hemizygous transgenic *NSE-PDGF-A*$_S$ mice (squares) and their wild-type littermates (triangles). Data is plotted as mean ± SD of counts of three or four sections from each of two animals of each age and genotype from separate litters. Cells stop proliferating between E15 and E17 in both wild-type and transgenic cords.

PDGF-B$^{-/-}$ mice have defective kidneys and capillary blood vessels; they are hemorrhagic and invariably die around birth (Leveén et al., 1994; Lindahl et al., 1997). In spinal cords of *PDGF-B*$^{-/-}$ mice, the number and distribution of O-2A progenitors was normal up to and including the day of birth (data not shown). We conclude that PDGF-AA (not AB or BB) is crucial for driving proliferation of O-2A progenitors during normal development. A more complete description of the CNS phenotypes of PDGF-null mice will be presented elsewhere.

O-2A Progenitors Stop Proliferating Several Days before Birth

As a prelude to studies of PDGF-A overexpression in transgenic mice (see below), we counted the numbers of *PDGFR*α^+ O-2A progenitors in autoradiographs of sections through wild-type spinal cords at various stages of embryonic and early postnatal development. The number of cells per section increases rapidly between E12.5 and E15 (Figures 2A and 2B, black triangles). Then, the number of cells reaches a plateau between E15 and E17 that persists until at least P3 (Figure 2B, black triangles). Since there is normally very little oligodendrocyte production before birth (Figure 1), it follows that the number of O-2A progenitors must be held constant either by cessation of cell division or by a balance between division and death. Further experiments (see below) showed that the cell cycle slows down markedly and there is also an increase in cell death.

Proliferation Arrest Results from Slowing of the Cell Cycle Coupled to an Increase in Cell Death

We compared the rate of O-2A progenitor cell cycling in normal embryos before and after they had stopped

proliferating by bromodeoxyuridine (BrdU)-labeling experiments in vivo. In one series of experiments, we gave a single intraperitoneal injection of BrdU to the mother, removed the embryos 2 hr later, and determined the proportion of O-2A progenitors that had incorporated BrdU. We identified progenitors by dissociating spinal cord cells, culturing them overnight on coverslips in the presence of PDGF-AA, and labeling with antibodies against the chondroitin sulphate proteoglycan NG2, a surface marker of oligodendrocyte progenitors (Stallcup and Beasley, 1987; Nishiyama et al., 1996), together with anti-BrdU (Figure 3A). The proportion of BrdU-labeled O-2A progenitors was high (about 75%) at E13 but dropped steeply as the cell number increased, reaching a stable low value (about 20%) between E17 and P3 (Figure 3A, black triangles). This suggested that the cell cycle time increases substantially between E13 and E17. To confirm this interpretation and to determine whether the decrease in BrdU labeling results from slowing of division in the progenitor cell population as a whole or from segregation into rapidly and slowly dividing subpopulations, we performed cumulative BrdU-labeling experiments. Sequential BrdU injections were made into pregnant mothers at 4 hr intervals starting on E14 or E17, and the proportions of BrdU-labeled progenitors were determined at various times after the first injection as described above. At E14, essentially all of the progenitors could be labeled by sequential BrdU injections, demonstrating that all of the progenitors were actively engaged in the cell cycle (Figure 3B, left panel, black triangles). The BrdU labeling index increased in a linear fashion with time after first injection, providing evidence that the whole population of progenitors was cycling together at the same rate. The same conclusion—that all the progenitors were cycling at the same rate—was also true at E17 (Figure 3B, right panel, black triangles). However, it took longer for the BrdU labeling index to reach 100% at E17 than at E14, confirming that the cell cycle slows down between E14 and E17 (Figure 3B, compare left and right panels, black triangles). Thus, the cumulative BrdU labeling experiments confirm that the BrdU labeling index in the single-injection experiments is indicative of cell cycle length. Making certain reasonable assumptions, we can use the BrdU data to calculate approximate cell cycle times (see Nowakowski et al., 1989, and Experimental Procedures for details). We estimate that the cell cycle time increases from ~6 hr at E13 to ~15 hr at E14 and then to ~22 hr at E17 and later.

Since the cell cycle slows down markedly but does not stop, it seems likely that in order for proliferation to stop, new cell production must be matched by cell differentiation and/or death. We looked for dying progenitor cells in sections of E15 and E17 spinal cord by in situ hybridization for *PDGFR*α combined with propidium iodide staining to visualize cell nuclei. At E15 we were unable to detect any *PDGFR*α^+ progenitor cells with pyknotic nuclei (i.e., undergoing apoptosis). However, we did detect small numbers of apoptotic progenitors at E17 (data not shown). As described later, we also detected small numbers of dying, immature oligodendrocytes in the wild-type cord at E17. Therefore, it seems

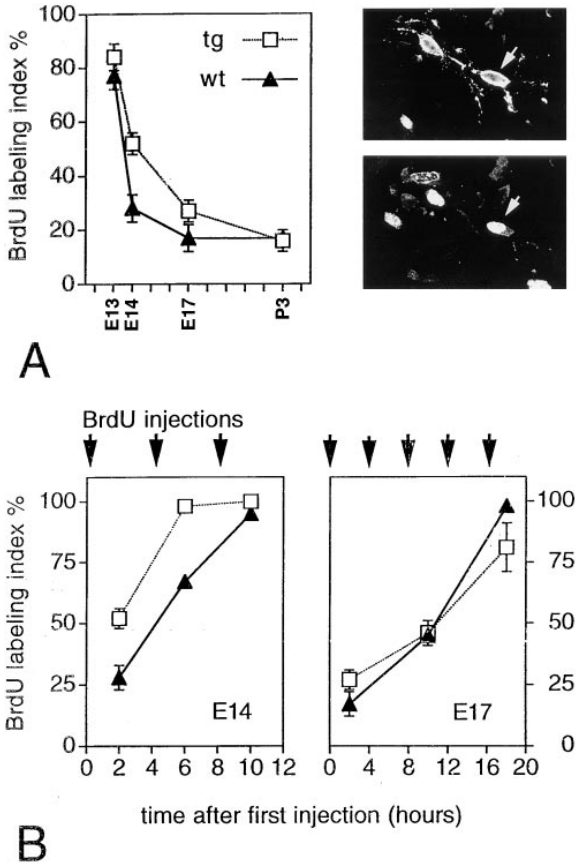


Figure 3. The Progenitor Cell Division Cycle Slows Down Markedly before Birth

(A) Pregnant female mice or young postnatal animals were given a single intraperitoneal injection of BrdU. The embryos or preweanlings were killed 2 hr later, and dissociated spinal cord cells were cultured on glass coverslips before immunolabeling with anti-NG2 proteoglycan (a marker of oligodendrocyte progenitors) and anti-BrdU (upper and lower micrographs, respectively). $BrdU^+$, $NG2^+$ double-positive progenitor cells (e.g. arrows) were counted and plotted as a proportion of the total $NG2^+$ progenitor population. The BrdU labeling index fell from ~80% to ~20% between E13 and E17 in both wild-type (triangles) and hemizygous transgenic *NSE-PDGF-*A_S animals (squares), corresponding to an increase in cell cycle time from ~6 hr to 22 hr (see Figure 3B and Experimental Procedures). Each data point represents the mean ± SD of at least two and up to six animals (each in triplicate) from two litters.

(B) Pregnant females were given sequential injections of BrdU at 4 hr intervals (vertical arrows) starting at E14 (left) or E17 (right), and the BrdU labeling indices of NG2-positive progenitor cells determined as for Figure 3A. Close to 100% of progenitor cells can be labeled with BrdU both in wild-type (triangles) and hemizygous transgenic *NSE-PDGF-*A_S embryos (squares) at both E14 and E17, indicating that all of the progenitors were actively cycling at these ages. The time taken for 100% of progenitors to label with BrdU was longer in wild-type embryos than transgenic embryos at E14 (left panel), indicating that the cell cycle is shorter in the transgenics at this age. The time taken for 100% of progenitors to label at E17 is longer than at E14, indicating that the cell cycle slows down after E14 in both wild types and transgenics. Moreover, the rate of BrdU incorporation (and hence the rate of cell division) is the same in wild types and transgenics at E17 (~22 hr; see text and Experimental Procedures for details). Each data point represents the mean ± SD from at least two and up to ten animals (each in triplicate) from one or two litters. Error bars are not shown where the SD is less than the size of the symbol.

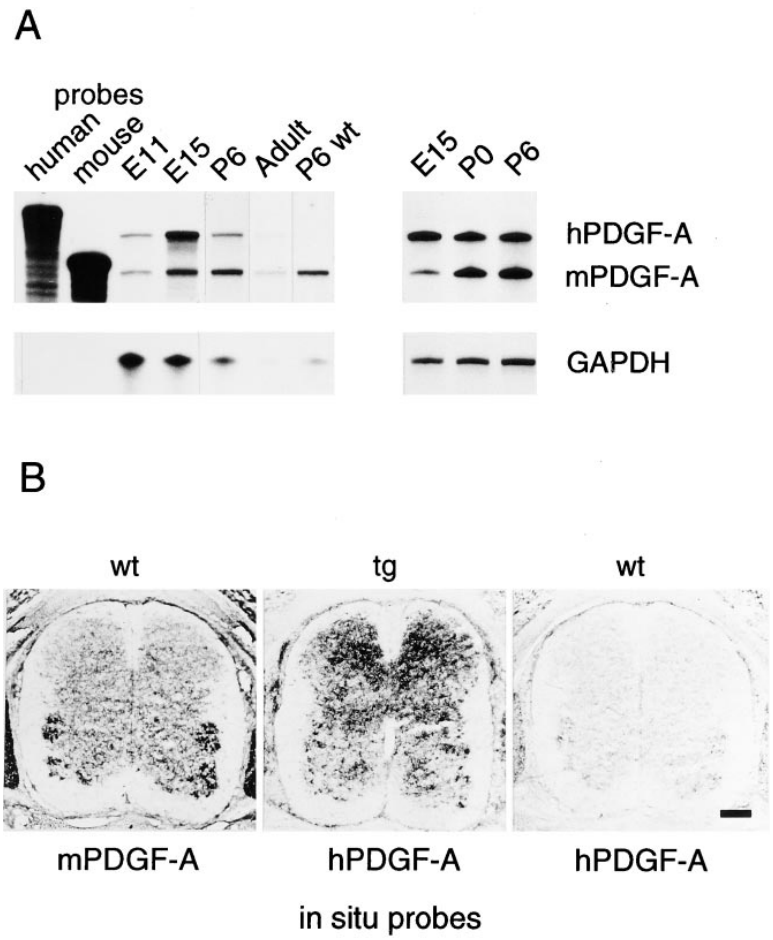


Figure 4. Expression of *PDGF-A* in Wild-Type and *NSE-PDGF-A*$_S$ Transgenic Spinal Cords

(A) RNase protection assays for *PDGF-A* transcript abundance in hemizygous *NSE-PDGF-A*$_S$ transgenic and wild-type spinal cords. Hybridization reactions contained either a mixture of probes for endogenous (mouse) and transgenic (human) *PDGF-A* or a probe for *GAPDH* as a control for RNA amounts. The undigested *PDGF* probes are shown on the left. Protected fragments corresponding to endogenous and transgene-derived *PDGF-A* transcripts can be distinguished (*mPDGF-A* and *hPDGF-A*, respectively). The human *PDGF-A* probe did not cross-hybridize to endogeneous mouse *PDGF-A* mRNA in a wild-type embryo (P6 wt); comparison of lanes P6 and P6 wt also demonstrates that the presence of the transgene did not affect expression of the endogenous *PDGF-A* gene. Both endogeneous and transgene-derived transcripts are expressed from before E11 until adulthood. The transgene was expressed at a roughly constant level from E15 to P6, whereas endogenous gene transcription increased between E15 and P0 but did not change much after that. The transgene was expressed at a similar level to endogeneous transcripts.

(B) Comparison of the spatial distributions of endogenous and transgene-derived *PDGF-A* transcripts. Sections through the spinal cords of E15 wild-type (wt) or hemizygous *NSE-PDGF-A*$_S$ transgenic (tg) mice were subjected to in situ hybridization with DIG-labeled probes for mouse or human *PDGF-A*. Cross-hybridization of the human probe to endogenous mouse transcripts was not a problem under our assay conditions (compare center and right panels). Both endogenous and transgene-derived *PDGF-A* transcripts are expressed widely throughout the spinal cord in many neurons. Endogenous *PDGF-A* is most strongly expressed in motor neuron pools in the ventral horns, while the transgene is expressed more strongly in the dorsal cord. Scale bar = 100 μm.

likely that the reason progenitor cells stop proliferating before birth is because the cell cycle slows down markedly, and this is accompanied by an increase in cell death.

Progenitor Cell Division Slows Down because PDGF Becomes Limiting

Why does the O-2A progenitor cell cycle slow down before birth? We thought it possible that PDGF or other mitogens might become limiting and cause cells to accumulate in G1, either because of a reduction in the concentration of extracellular mitogens or because of a decline in the sensitivity of the cells to mitogenic stimulation. If so, boosting the mitogen supply by overexpressing PDGF in transgenic mice might be expected to overcome slowing of the cycle. We therefore examined transgenic mice that overexpress PDGF-A in neurons under the control of the neuron-specific enolase (NSE) gene promoter (Forss-Petter et al., 1990).

There are two natural alternative-splice isoforms of PDGF-A: a short, freely diffusible form (PDGF-A$_S$) and a long form (PDGF-A$_L$) with a carboxy-terminal tail that binds to the extracellular matrix (e.g., Pollock and Richardson, 1992). Our transgenic mice express a Myc epitope–tagged version of human PDGF-A$_S$ (Pollock and Richardson, 1992). The generation of these mice has been described previously (Fruttiger et al., 1996). RNase protection assays showed that the transgene is expressed in the spinal cord from before E11 until adulthood (Figure 4A). The level of transgene-derived *PDGF-A*$_S$ mRNA was comparable to that of endogeneous *PDGF-A* after E15 (Figure 4A). RT–PCR also clearly revealed the presence of transgene-derived transcripts in addition to endogeneous *PDGF-A*$_S$ transcripts; *PDGF-A*$_L$ appeared not to be expressed in the spinal cord (data not shown) or retina (Fruttiger et al., 1996). In situ hybridization showed that transgene-derived human *PDGF-A*$_S$ mRNA, like endogeneous *PDGF-A*$_S$ mRNA (Yeh et al., 1991), is expressed widely throughout the embryonic spinal cord by neurons (Figure 4B). The overall distributions of transgene-derived and endogenous *PDGF-A* transcripts were therefore similar (Figure 4B). PDGF-A is also expressed in white matter astrocytes (Richardson et al., 1988; Pringle et al., 1989; Yeh et al., 1991), but these cells are

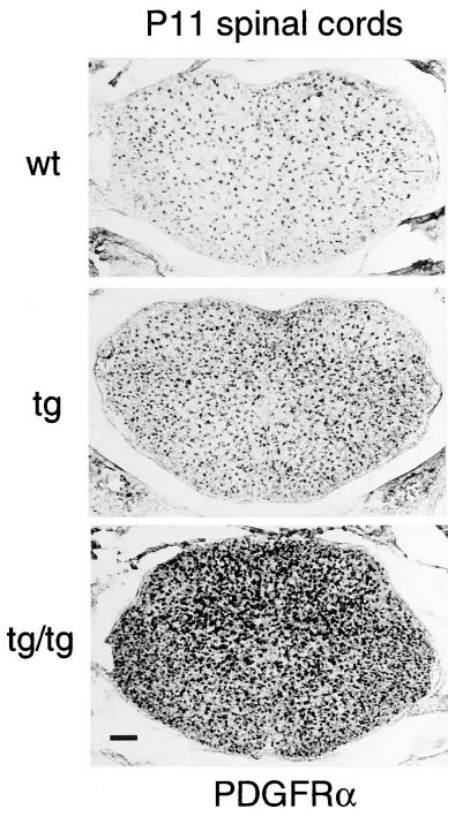


Figure 5. PDGF Dose Dependency of O-2A Progenitor Proliferation In Vivo

O-2A progenitors in P11 wild-type (top), hemizygous (middle), and homozygous (bottom) *NSE-PDGF-A*$_S$ transgenic spinal cords were visualized by in situ hybridization with a DIG-labeled probe for *PDGFR*α. There are approximately three times the normal number of progenitors in hemizygotes and seven times the normal number in homozygotes. Scale bar = 100 μm.

not generated in large numbers until after birth. Despite much effort, we were unable to detect transgene-derived PDGF-A polypeptides by immunohistochemistry in sections. This is probably because PDGF, a secreted molecule, does not build to detectable levels either inside cells or in the interstitial space.

We compared numbers of O-2A progenitors in hemizygous *NSE-PDGF-A*$_S$ transgenic mice and their wild-type littermates by in situ hybridization with a probe to *PDGFR*α as before (Figure 2). Up to E13.5, there was no noticeable difference in either the number or distribution of progenitor cells in the transgenics compared to their wild-type littermates. However, at E15 and after there was a marked increase in the number of progenitor cells in the transgenic cord (Figures 2A and 2B). The number of O-2A progenitors in transgenic spinal cords reached a plateau before birth just as in wild-types, except that there were more than three times the number of progenitor cells in the transgenics as in the wild-types at steady-state (805 $\pm$ 71 compared to 246 $\pm$ 18, data from four sections from each of two animals of each genotype) (Figure 2B). Following single injections of BrdU into transgenic mothers, we found that the BrdU labeling index of progenitors at E13 started at the same high level (75%–80%) as in wild-type embryos but fell after this, eventually declining to the same low value (about 20%) as in the wild types (Figure 3A, open squares). However, the decrease in labeling index was more gradual in transgenics than in wild types, so that there is a window of time between E13 and E17 when the progenitors divide more rapidly in the transgenics than in the wild types.

The fact that increasing the PDGF supply in the transgenics has no effect on the progenitor cell cycle at E13 (Figure 3A) but has a positive influence on the cycle at E14 (Figures 3A and 3B, left panel) demonstrates that in wild-type mice the PDGF supply is saturating for cell division at E13 but becomes limiting by E14. This suggests that the cell cycle normally slows down either because the extracellular PDGF concentration drops or because the cells' responsiveness to PDGF declines. Even in hemizygous transgenic mice, the supply of PDGF eventually becomes limiting because doubling the transgene copy number in homozygous transgenics increases proliferation still further and generates yet more cells at steady-state (around seven times normal in homozygotes compared to three times in hemizygotes) (Figure 5).

Excessive and Ectopic Production of Oligodendrocytes in *NSE-PDGF-A*$_S$ Transgenic Mice

To test whether the increased progenitor cell proliferation observed in *NSE-PDGF-A*$_S$ transgenic mice is matched by increased oligodendrocyte differentiation, we visualized postmitotic oligodendrocytes in sections of spinal cords from wild-type and hemizygous transgenic mice at different developmental stages by in situ hybridization with a probe for the myelin proteolipid protein PLP/DM-20. At E17, when there were very few *PLP/DM-20*-positive oligodendrocytes in the wild-type spinal cord, there were many oligodendrocytes in the transgenic cord (data not shown). Overproduction of oligodendrocytes was even more pronounced in E19 transgenic spinal cords (Figure 6). Many of the excess oligodendrocytes were abnormally located in the central gray matter, unlike oligodendrocytes in wild-type spinal cords that are mainly located in the developing white matter at all ages. However, within one week, at P6, the situation appeared to have resolved and the number and distribution of *PLP/DM-20*-expressing oligodendrocytes was normal (Figure 6). A similar picture emerged when sections were probed for *MBP* mRNA rather than *PLP/DM-20* mRNA (Figure 6).

We counted the numbers of *PLP/DM-20*-positive oligodendrocytes in sections of wild-type, hemizygous transgenic, and homozygous transgenic P6 spinal cords and compared these with the numbers of *PDGFR*α-positive progenitor cells in sections of E15 spinal cords. Despite the $\sim$7-fold variation in the number of oligodendrocyte progenitor cells, the number of mature oligodendrocytes did not change significantly (Figure 7). Thus, progenitor cell number and oligodendrocyte number are controlled independently of each other.

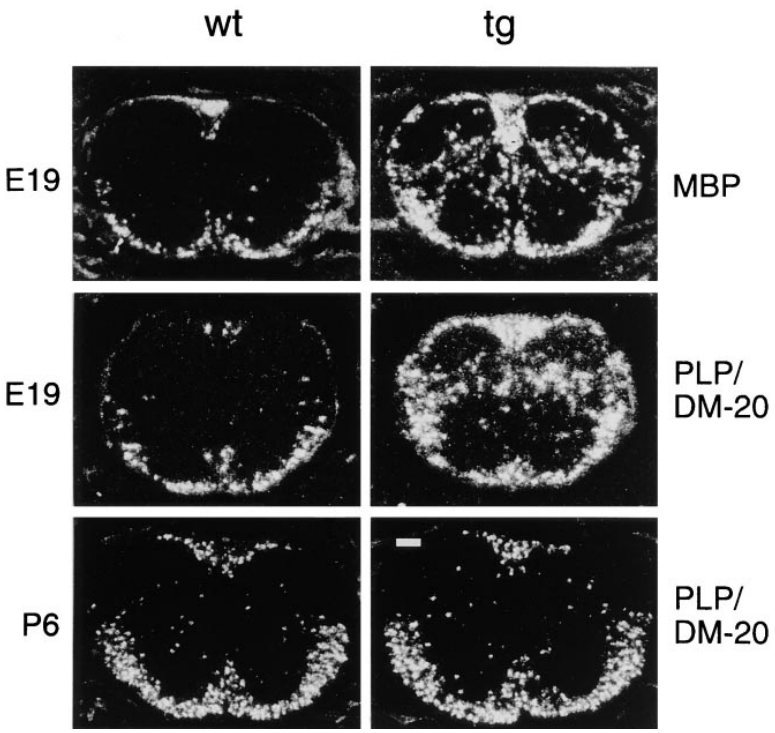


Figure 6. Excessive and Ectopic Production of Oligodendrocytes in *NSE-PDGF-A*$_S$ Spinal Cords

Oligodendrocytes were visualized in sections of spinal cord from wild-type (wt) and hemizygous transgenic (tg) mice by in situ hybridization with probes against *MBP* or *PLP/DM-20* and photographed under dark-field illumination. At E19, there are many more *MBP*-positive, *PLP/DM-20*-positive oligodendrocytes present in the transgenic spinal cord than in the wild-type cord. Many of the extra oligodendrocytes in the transgenics are found ectopically in the central gray matter of the cord. However, within one week, at P6, this situation resolves and the number and distribution of oligodendrocytes in the transgenic cord appears normal. Scale bar = 100 μm.

Elimination of Excess Oligodendrocytes by Programmed Cell Death

It seemed possible that the superfluous oligodendrocytes that developed before birth in the transgenic mice might be cleared by programmed cell death (PCD). To test this, we labeled E19 wild-type and transgenic spinal cord sections with anti-galactocerebroside (anti-GC) to mark oligodendrocytes and with propidium iodide to highlight pyknotic nuclei (Figure 8A). We counted the total number of pyknotic nuclei per section and the number of GC$^+$ oligodendrocytes with pyknotic nuclei. In wild-type spinal cord sections, we found a number of pyknotic GC$^-$ nuclei, possibly dying neurons, and a much smaller number of pyknotic GC$^+$ oligodendrocytes (Figure 8B). In contrast, there were large numbers of pyknotic GC$^+$ oligodendrocytes in the hemizygous transgenic spinal cords and an even greater number in the homozygous transgenic cords, although there was no increase in the number of pyknotic GC$^-$ cells in the transgenics (Figure 8B). Many, but not all, of the dying GC$^+$ oligodendrocytes were in the central gray matter, presumably corresponding to the ectopic MBP PLP/DM-20-positive oligodendrocytes that disappear between E19 and P6 (see Figure 6).

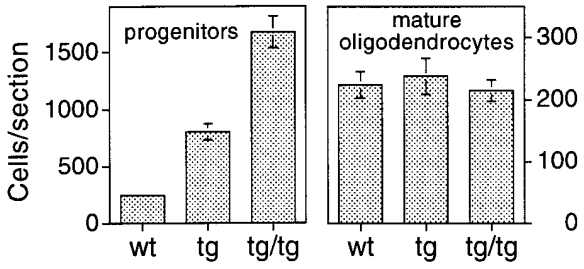
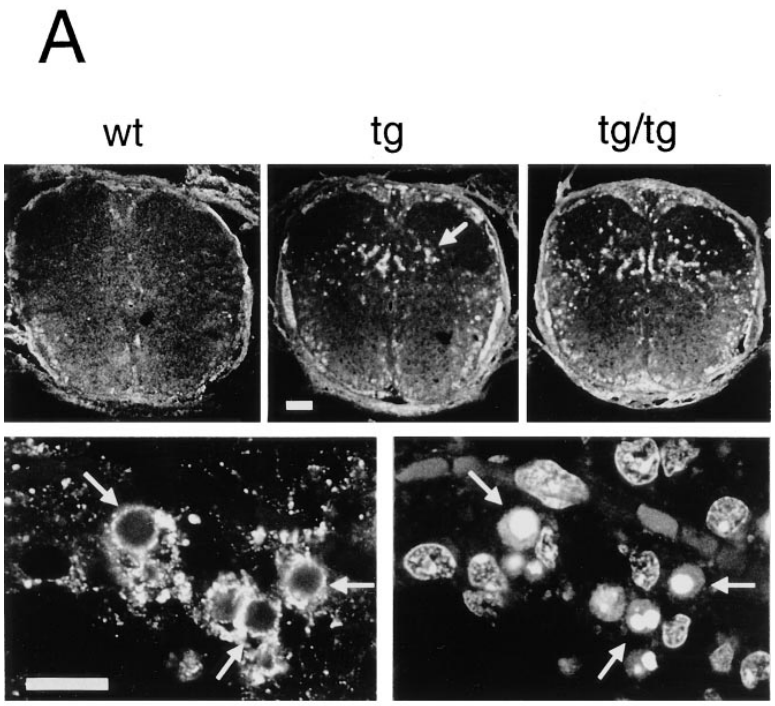


Figure 7. The Number of Oligodendrocytes Surviving Postnatally Is Independent of the Number of O-2A Progenitor Cells

Numbers of *PDGFR*α$^+$ progenitor cells in sections of wild-type (wt), hemizygous transgenic (tg), and homozygous transgenic (tg/tg) E15 spinal cords are displayed in comparison with the numbers of *PLP/DM-20*-positive oligodendrocytes in sections of P6 cords (mean ± SD of a total of 8–12 sections from 2 or 3 animals of each genotype). Despite the ~7-fold increase in the number of oligodendrocyte progenitor cells in homozygous transgenic animals, the number of surviving oligodendrocytes is completely normal.

Superfluous Oligodendrocytes Are Eliminated at a Distinct Immature Stage of Differentiation

Further examination revealed that there were two distinct categories of *PLP/DM-20*-expressing cells in both normal and transgenic spinal cords that could be distinguished on the basis of their in situ hybridization signal intensities. This was most obvious when pairs of E19 and P6 spinal cord sections were processed for in situ hybridization simultaneously under identical conditions and viewed under bright-field rather than dark-field illumination (Figure 9). It then became clear that the majority of the ectopic oligodendrocytes that appeared in the prenatal transgenic cords (see Figure 6) were of a type that express relatively low levels of *PLP/DM-20* transcripts compared to myelin-forming oligodendrocytes in the P6 cord (Figure 9). Small numbers of these faint *PLP/DM-20*-expressing cells could also be recognized in wild-type E19 cords. It seems likely that these cells in both wild-type and transgenic cords represent newly differentiated, immature oligodendrocytes that have not



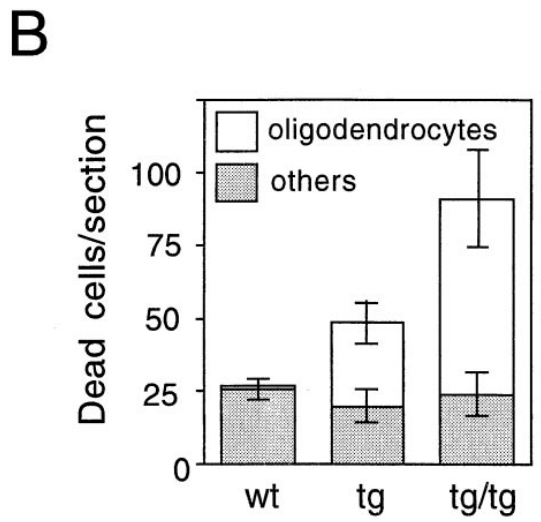


Figure 8. Superfluous Oligodendrocytes in *NSE-PDGF-A*$_S$ Transgenic Spinal Cords Are Eliminated by Programmed Cell Death

(A) Sections of wild-type (wt), hemizygous transgenic (tg), and homozygous transgenic (tg/tg) E19 spinal cords were immunolabeled with monoclonal anti-GC to visualize oligodendrocytes and counterstained with propidium iodide to reveal pyknotic nuclei characteristic of apoptotic cells. The lower two micrographs show a higher magnification view (left, anti-GC; right, propidium iodide) of the region indicated above by a single arrow. Double-labeled cells (dying oligodendrocytes) are indicated (arrows). Scale bars = 100 μm (top) and 20 μm (bottom).

(B) The total number of cells with pynotic nuclei as well as the number of GC$^+$ oligodendrocytes with pyknotic nuclei were counted in 15 μm sections. There were many more GC$^+$ pyknotic cells (dying oligodendrocytes) in hemizygous transgenic cords (tg) than in wild-type cords and even more in homozygous transgenics (tg/tg). There were, however, no significant differences in the numbers of GC$^-$ pyknotic cells in the transgenics compared to wild type.

yet achieved their maximal levels of myelin gene expression, perhaps because they have not managed to associate with axons. It is these immature cells that are preferentially eliminated by PCD.

With appropriate autoradiographic exposure times (Figure 9), immature oligodendrocytes could also be detected in P6 wild-type and transgenic spinal cords and could be easily distinguished from more mature oligodendrocytes, mainly in white matter, that expressed markedly higher levels of myelin gene products. The immature oligodendrocytes were more numerous in P6 transgenic cords than in wild-type cords and seemed to be preferentially located in the central gray matter, as at earlier ages (Figure 9, arrows). A proportion of these immature oligodendrocytes in P6 transgenic cords also possessed pyknotic nuclei (data not shown), suggesting that oligodendrocytes are continually being overproduced and cut back by PCD throughout the embryonic and early postnatal period.

Discussion

PDGF-AA Drives Proliferation of O-2A Progenitors In Vivo

We investigated the control of proliferation of O-2A progenitor cells in the developing spinal cord. By analyzing transgenic knockout mice that lack either PDGF-A or -B chains, we showed that PDGF-AA homodimers are crucial for proliferation of these cells during normal development, although PDGF was not required for initial

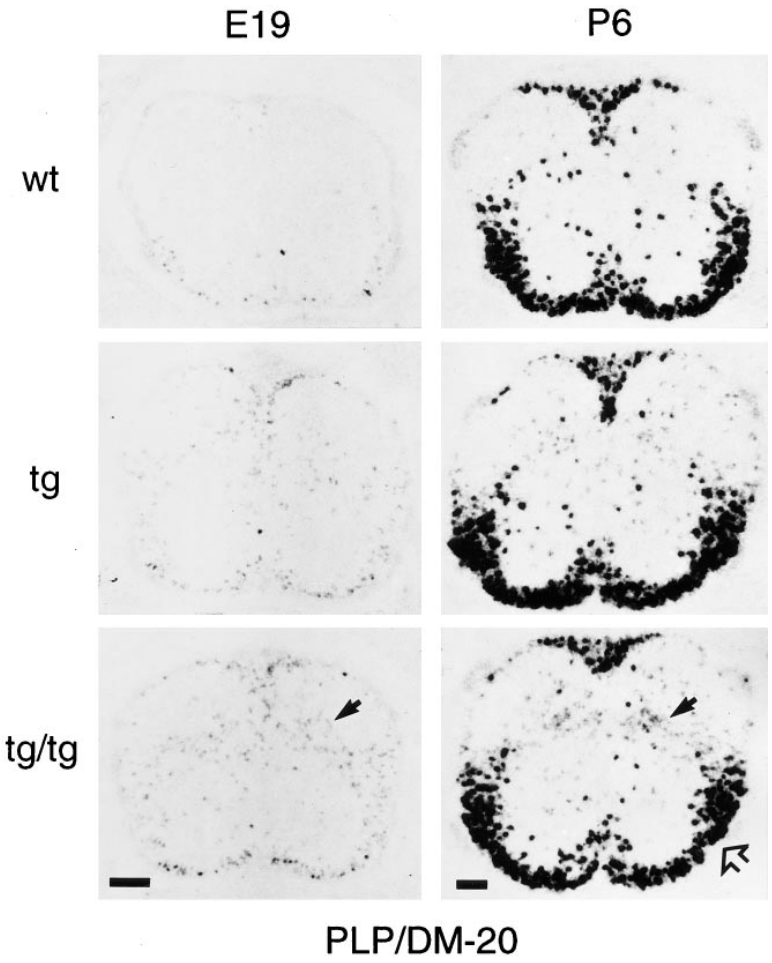


Figure 9. Excess Oligodendrocytes Die at an Early Stage of Differentiation

Sections of wild-type (wt), hemizygous transgenic (tg), and homozygous transgenic spinal cords (tg/tg) were subjected to in situ hybridization with a probe against *PLP/DM-20* mRNA and visualized by autoradiography (3 days exposure time) and bright-field microscopy. Sections from E19 (left column) and P6 (right column) spinal cords were processed and exposed in parallel under identical conditions so that the in situ signal intensities are directly comparable in all sections. There are two distinct populations of oligodendrocytes that can be distinguished on the basis of their *PLP/DM-20* signal intensity. This is most obvious in the transgenic spinal cords; oligodendrocytes in the ventral and dorsal white matter of the P6 cords express *PLP/DM-20* transcripts strongly (large arrowhead), whereas many oligodendrocytes in the central gray matter of the P6 cords as well as the majority of oligodendrocytes in the E19 cords express *PLP/DM-20* at a much lower level (small arrows). The latter faint cells probably represent young (i.e., recently formed) oligodendrocytes that have not yet accumulated maximal levels of myelin gene products. It is these faint oligodendrocytes mainly in the central regions of the transgenic cords that are eliminated by PCD (compare Figures 6 and 8). Small numbers of faint, newly formed oligodendrocytes can also be observed in wild-type spinal cords at both E19 and P6, arguing that continuous production and elimination of superfluous and ectopic oligodendrocytes is a normal feature of late embryonic and early postnatal development that is greatly exaggerated in the transgenic animals.

lineage specification. In contrast, PDGF-B seems to be unimportant for O-2A progenitor proliferation during development. PDGF-B is expressed by capillary endothelial cells in the CNS from early embryonic ages until adulthood (Mudhar et al., 1993; Lindahl et al., 1997); presumably, this PDGF-B is not available to O-2A progenitors. After birth, many neurons start to express low amounts of PDGF-B (Sasahara et al., 1991), but we have been unable to assess the role of PDGF-B in the postnatal CNS because the knockout mice die at birth.

Many previous studies have shown that PDGF stimulates O-2A progenitor proliferation in vitro (e.g., Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988; Levine, 1989); we have now shown that PDGF is crucial for proliferation in vivo. Note that our results do not imply that PDGF is the only growth factor that is important in vivo; PDGF might normally act in concert with other factors (e.g., glial growth factor or neurotrophin-3) (Barres et al., 1994; Canoll et al., 1996) whose combined action might be required for maximal cell proliferation in vivo.

Progenitor Cell Division Is Normally Limited by the PDGF Supply

We found that the O-2A progenitor cell cycle slows down markedly before birth in normal embryos, from ~6 hr to ~22 hr. This slowdown, together with an increase in cell death, results in the arrest of progenitor cell proliferation. Slowing of the cell cycle is a common feature of progenitor cell populations in the developing CNS. For example, Caviness and colleagues found that the cycle time of neural precursors in the mouse cerebral cortex lengthens from ~8 hr to ~24 hr between E11 and E17, with almost all variation falling within G1 (Takahashi et al., 1994, 1995).

Overexpressing PDGF in transgenic mice delayed deceleration of the O-2A progenitor cell cycle, causing a transient increase in the cell division rate compared to normal and an increase in the final number of progenitors at steady-state. This demonstrates that progenitor cell divisions are normally limited by the PDGF supply. There is evidence from in vitro studies that the cell cycle time of O-2A progenitors is dependent on the concentration of PDGF in the medium (Gao and Raff, 1997; P. van Heyningen and W. D. R., unpublished data). In addition, it is known that the duration of the cell cycle in fibroblast cultures is controlled by the concentration of mitogens, including PDGF (Shields and Smith, 1977; Brooks and Riddle, 1988).

Another indication that PDGF might be in limiting supply during CNS development comes from the experiments of Gard and Pfeiffer (1993), who reported that

oligodendrocyte progenitors isolated directly from early postnatal rat cerebrum transiently reverted to a less mature state of differentiation when exposed to saturating concentrations of PDGF in vitro. This implies that the progenitors had been exposed to subsaturating concentrations of PDGF in vivo, prior to isolation.

Our finding that PDGF overexpression causes a transient increase in progenitor cell division rate seems inconsistent with a previous in vivo study (Barres et al., 1992a) in which exogenous PDGF-AA was delivered to the postnatal rat optic nerve. This resulted in increased numbers of immature oligodendrocytes but no apparent increase in progenitor cell division. It is questionable whether Barres et al. (1992a) would have detected a transient effect in their experiments. Moreover, it is likely that the cell cycle had already slowed down before PDGF was administered in the experiments of Barres et al. (1992a), and it is not known whether this is easily reversible. Intracellular changes that orchestrate slowing of the cell cycle (e.g., changes in the levels of cyclins and CDK inhibitors; Sherr and Roberts, 1995; Casacchio-Bonefil et al., 1997; Durand et al., 1997) might be self-reinforcing and put a permanent brake on the cycle.

Why should PDGF, which is initially present in saturating amounts, later become limiting for cell division? Possible explanations are that 1) the concentration of PDGF in the extracellular space falls, perhaps due to increased consumption by the increasing population of progenitors; 2) the intrinsic sensitivity of the progenitor cells to mitogenic stimulation by PDGF declines; or 3) the concentration of a PDGF antagonist or a general antimitogenic factor such as TGFβ rises. We are currently trying to distinguish among these possibilities.

Stochastic Exit from the Cell Cycle and Initiation of Oligodendrocyte Differentiation

Progenitor cell number stops increasing before birth despite the fact that they continue to divide about once a day. It follows that production of new progenitor cells must be balanced by cell differentiation, cell death, or both. We favor the idea that half of the newly formed progenitor cells exit from the cell cycle each generation and initiate oligodendrocyte differentiation. This is by analogy with the behavior of 3T3 fibroblasts growing in suboptimal concentrations of mitogens in vitro. When these cells were grown in 2% newborn calf serum, ~20% of the cells left the cycle (entered G0) each generation; in 1% serum, the proportion was closer to 30% (Brooks and Riddle, 1988). Because 3T3 cells are an immortal line of identical cells, the decision whether to exit the cell cycle or to continue cycling must be a stochastic one.

Since O-2A progenitors appear to experience subsaturating concentrations of mitogens in vivo (see above), it seems plausible that they should behave in an analogous manner to serum-deprived fibroblasts; that is, a fraction of the progenitors exits the division cycle each generation, enters G0, and starts to differentiate. At steady-state, when the number of progenitors is stable, this fraction would be exactly half. We believe for reasons given below that large numbers of oligodendrocytes cannot normally be detected before birth because most of them die and are cleared before they have the chance to express detectable amounts of myelin gene products.

Overproduction and Elimination of Immature Oligodendrocytes by Programmed Cell Death

Oligodendrocytes were generated in excess and at ectopic sites in the gray matter of *NSE-PDGF-A$_S$* transgenic spinal cords. Most of these ectopic oligodendrocytes were eliminated at an immature stage of differentiation by PCD, resulting in a completely normal number and distribution of myelinating oligodendrocytes by the end of the first postnatal week. Dying, immature oligodendrocytes could be detected in the gray matter of the transgenic spinal cord from before E17 until at least P6. Small numbers of dying, immature oligodendrocytes could also be detected in wild-type spinal cords throughout this period, suggesting that overproduction and elimination of immature oligodendrocytes in gray matter is a normal developmental process that is greatly exaggerated in the transgenic mice. Therefore, it appears likely that the reason mature myelinating oligodendrocytes normally accumulate selectively in white matter is not that progenitor cells differentiate preferentially in fiber tracts as recently suggested (Hardy and Friedrich, 1996); rather, they leave the cell cycle and start to differentiate in both gray and white matter, but their progeny mature and survive long-term predominantly within white matter.

It is worth remembering that the number of apoptotic cells that can be detected at any given moment depends not only on the rate of cell death (i.e., number of cells that die per unit time) but also on the rate at which dead or dying cells are cleared by phagocytosis. We suspect that both the rate of cell death *and* the clearance rate might increase in the transgenic cords because the number of apoptotic oligodendrocytes is increased about 10-fold in the hemizygous transgenics and 30-fold in the homozygotes, although the number of progenitor cells is increased only 3-fold and 7-fold, respectively. This suggests that normal clearance mechanisms might be overwhelmed by the increased number of dying oligodendrocytes in the transgenics. If clearance rates were increased nonuniformly across the transgenic cord, this alone could explain the uneven distribution of apoptotic oligodendrocytes that we observe without the need to invoke nonuniform oligodendrocyte differentiation and death.

Immature, dying oligodendrocytes were not confined to gray matter. There were also large numbers of these cells in the developing white matter of transgenic spinal cords and smaller numbers in wild-type cords. This is consistent with previous studies of developing fiber tracts. Barres et al. (1992a) showed that ~50% of all oligodendrocytes formed in the normal developing rat optic nerve are eliminated by PCD soon after they are formed. More recently, Trapp et al. (1997) showed that many oligodendrocytes in brain fiber tracts also die at an immature developmental stage when they express DM-20 but not yet PLP. We presume that the dying, faintly *PLP/DM-20*-positive oligodendrocytes that we

see in the spinal cord correspond to this ($DM20^+$, PLP^-) class of premyelinating oligodendrocytes, but this remains to be tested. As has previously been pointed out (Barres et al., 1994; Burne et al., 1996; Casacchio-Bonefil et al., 1997), axonal regulation of oligodendrocyte survival might be a simple strategy for ensuring that oligodendrocytes are matched to the surface of axons requiring to be ensheathed.

The Time of Appearance of Myelinating Oligodendrocytes in the Spinal Cord Depends on Timed Survival Cues, Not Timed Differentiation

It has been proposed that the first appearance of differentiated oligodendrocytes in vivo is timed by a progenitor cell–intrinsic clock that causes progenitors to exit the cell cycle and differentiate into oligodendrocytes after a predetermined period of time (Raff et al., 1985; Temple and Raff, 1986; Durand et al., 1997; Gao et al., 1997). This is suggested by the fact that O-2A progenitor cells from rat optic nerves do not proliferate indefinitely in vitro even in the presence of saturating concentrations of PDGF or other mitogens; the clonal progeny of a single optic nerve O-2A progenitor tend to stop dividing and differentiate together into oligodendrocytes after a maximum of about eight cell divisions (Temple and Raff, 1986). However, differentiation of O-2A progenitors from rat spinal cord or cerebral cortex seems not to be governed by a simple intracellular timer, because the clonal progeny of these cells do not necessarily differentiate synchronously into oligodendrocytes after a set period of cell division in vitro (Zhang and Miller, 1995; Ibarrola et al., 1996). Our in vivo data also seem inconsistent with a simple cell-intrinsic timing mechanism that determines when mature oligodendrocytes first appear in the spinal cord.

Dying, immature oligodendrocytes are present in the spinal cords of our *NSE-PDGF-A_S* transgenic mice for several days before myelin-forming oligodendrocytes start to appear around birth. Small numbers of dying, immature oligodendrocytes can also be detected in wild-type cords, suggesting that this is a normal feature of development that is greatly exaggerated in the transgenic mice, perhaps in part because clearance of dead cells is slower in the transgenics (see above). We know from the number of progenitor cells in a 10 μm section of wild-type late embryonic spinal cord ($\sim$230) and the calculated cell cycle time ($\sim$22 hr) that over 200 new progenitor cells must be generated each day per 10 μm section. Since progenitor cell number does not increase much after E15 and mature oligodendrocytes do not appear in significant numbers until after birth, we conclude that over 200 cells/day/section normally die during late embryonic development either as progenitor cells or newly differentiating oligodendrocytes. Only a very small proportion of these can be detected, presumably because the dead cells are cleared rapidly. Nevertheless, it seems possible that the reason more mature oligodendrocytes start to appear around birth is not that they start to differentiate (i.e., become postmitotic) at that time; rather, they are generated continuously for some time before birth but die for lack of survival cues. It is known that axons are required for long-term survival of oligodendrocytes (Barres and Raff, 1994), but why survival signals should start to appear specifically in ventral axon tracts around birth is a mystery.

An alternative explanation for the stabilization of progenitor cell number before birth might be that half of the progenitors drop out of division each cycle and remain dormant as *PDGFRα*-negative, *PLP/DM-20*-negative cells until the appearance of oligodendrocyte maturation–inducing factors around birth. We think this is unlikely for three reasons. First, there is little evidence for the existence of a (*PDGFRα, PLP/DM-20*)-negative phenotypic stage in oligodendrocyte lineage progression. O-2A progenitors can be induced to differentiate into (*PDGFRα, PLP/DM-20*)-negative type-2 astrocytes in vitro, but these cells have not been identified in vivo (Fulton et al., 1991) and rather few astrocytes develop in the prenatal spinal cord in any case. Second, from the numbers quoted above we can calculate that a large number of these putative (*PDGFRα, PLP/DM-20*)-negative cells, $\sim$1,000 cells/10 μm section, would have to accumulate in the normal spinal cord before birth (and many more in the transgenics). This is more than the combined total of progenitors and mature oligodendrocytes in the P6 spinal cord ($\sim$200–250 of each; see Figure 7). Third, we can detect small numbers (around three per section) of both dying *PDGFRα^+* progenitor cells and dying, immature oligodendrocytes in the normal prenatal spinal cord; if their clearance time is similar to that estimated in the developing optic nerve ($\sim$1 hr; Barres et al., 1992a), then this alone could account for the majority of the missing oligodendrocyte lineage cells.

Population Dynamics of Oligodendrocyte Development: A Model

Most previous studies of oligodendrocyte development have been conducted in vitro. Our transgenic studies complement and extend those in vitro studies and provide some insights into the development of the oligodendrocyte lineage in vivo. On the basis of our findings, we can begin to construct a model of the population dynamics of oligodendrocyte development (see Figure 10). The essential features of this model are as follows: 1) numbers of progenitor cells and mature oligodendrocytes are controlled separately, progenitors by competition for limited supplies of PDGF and possibly other mitogens, oligodendrocytes by competition for limited supplies of survival factors. These ideas have been discussed before (e.g., Barres et al., 1992b; Burne et al., 1996) but gain strong support from the present study. 2) Exit from the cell cycle and initiation of oligodendrocyte differentiation is a stochastic process, depending on the probability per unit time of a progenitor cell's leaving the division cycle and entering G0 relative to its probability of entering the next S-phase. As mitogenic signaling intensity decreases and the cell cycle slows down, the likelihood of a cell dropping into G0 increases. Eventually, the number of cells that exits the cycle each generation becomes equal to the number that enters a new cycle, and net progenitor cell proliferation ceases. 3) The time of first appearance of mature oligodendrocytes in vivo does not reflect when oligodendrocytes are first

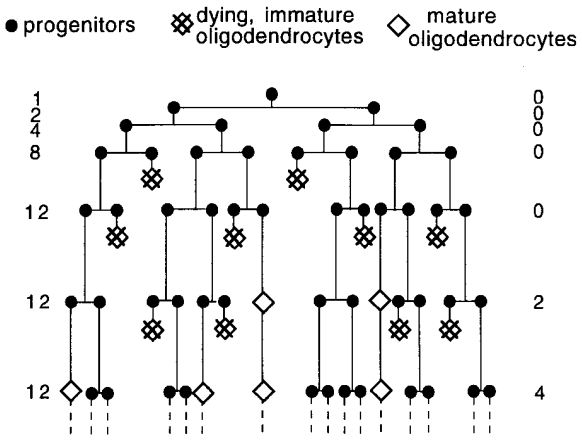


Figure 10. A Tentative Model of Spinal Cord Oligodendrocyte Development Derived from the Transgenic Studies Described in This Paper and Incorporating Ideas from the Previous Work of Ourselves and Others

Time runs from top to bottom (not to scale). Oligodendrocyte progenitors are specified in the ventral ventricular zone of the spinal cord and are first recognized by virtue of their expression of PDGFRα on E12.5 in the mouse (E14 in rat). This is a direct or indirect effect of signals, including Sonic hedgehog, from the notochord and/or floor plate (reviewed by Miller, 1996; Richardson et al., 1997). O-2A progenitors proliferate rapidly at first (6 hr cell cycle) in response to PDGF-AA and migrate throughout the spinal cord to become more or less evenly distributed in both gray and future white matter. As they increase in number, their division rate slows down (denoted by longer time lines), so that by E17 their division cycle stabilizes at around 24 hr. With the lengthening cell cycle, cells spend longer in G1 and the probability of their dropping out of cycle into G0 and differentiating into oligodendrocytes increases relative to the probability of their entering the next division cycle. When the number of progenitors that drops out of division each cycle matches the number that reenters S-phase, progenitor cell number reaches steady-state (i.e., proliferation ceases); this happens between E15 and E17 in wild-type mice. At first, the newly differentiating oligodendrocytes die for lack of survival factors. Starting around birth, they start to survive and accumulate, mainly in developing white matter. Numbers of progenitor cells (circles) and surviving oligodendrocytes (diamonds) are illustrative only. Dying, immature oligodendrocytes are denoted by a cross over the symbol.

generated but rather when they first start to mature and survive long-term due to the timed appearance of maturation/survival factors. Likewise, the distribution of oligodendrocytes depends on the distribution of survival and/or maturation factors, not localized differentiation per se.

This model attempts to combine ideas from our present in vivo study and many previous in vitro studies; we propose it tentatively as a focus for further debate and experimentation. The dynamics of O-2A progenitor cell proliferation and their conversion into differentiated oligodendrocytes is complex and much remains to be learned; however, a clear conclusion of the present study is that there is remarkable flexibility built into the mechanisms for regulating the number and arrangement of differentiated cells—progenitors can be overproduced more than 7-fold without overwhelming normal controls on oligodendrocyte number, which operate exclusively at the level of selective cell survival and death.

Experimental Procedures

Transgenic Mice

Production and genotyping of transgenic *NSE-PDGF-A*$_S$ mice has been described (Fruttiger et al., 1996). Transgene expression was analyzed by RNase protection essentially by the method of Melton et al. (1984). Protected RNA fragments were separated on a 6% (w/v) polyacrylamide sequencing gel and subjected to autoradiography.

Tissue Preparation and In Situ Hybridization

Our in situ hybridization procedures have been described (Pringle et al., 1996). For quantitative comparisons among different specimens, two or three spinal cords were aligned, frozen side by side, and sectioned simultaneously to standardize section thickness.

Cell Cycle Analysis In Vivo by BrdU Injection

Pregnant females or postnatal pups were injected intraperitoneally with 50 μg BrdU per gram of body weight, injected at 10 mg/ml in phosphate-buffered saline. This concentration of BrdU was previously found to be nontoxic for rapidly proliferating E14 cortical precursor cells in vivo (Nowakowski et al., 1989). For single-injection experiments, animals were killed 2 hr after injection, the spinal cords dissected, and dissociated cells cultured overnight before labeling with anti-NG2 and anti-BrdU antibodies. Cells from individual animals were cultured separately (three coverslips per animal) as described (Hall et al., 1996). Genotypes were determined retrospectively. The 2 hr chase period following injection was chosen because maximum labeling was achieved within this period. Reducing the chase period to 15 min and culturing the cells for only 3 hr did not qualitatively change the outcome of the single-injection experiments (data not shown). The cumulative labeling experiments consisted of consecutive injections at 4 hr intervals followed by a 2 hr chase before killing the animals.

We can make a rough estimate of the cell cycle time T_C from the cumulative labeling experiments (Nowakowski et al., 1989). In such experiments there is a linear increase in the proportion of cells labeled (labeling index, L) with increasing exposure to BrdU until all the cells in the growing fraction (in our case, 100%) are labeled. If the time taken to achieve maximum labeling is T and the length of S phase T_S, then $T_C = T + T_S$. For embryonic mouse cortical precursor cells, T_S is invariant and close to 4 hr (Takahashi et al., 1995). Assuming that T_S for O-2A progenitors is the same, then our experiments indicate that the cell cycle time T_C is ~14 hr at E14, increasing to ~22 hr at E17 (Figure 3B). In the single-injection experiments, the labeling index $L = T_S/T_C$, ignoring the small correction needed for the fact that the BrdU pulse is not instantaneous. From these data (Figure 3A) we can estimate that the cell cycle time is roughly 6 hr at E13, 15 hr at E14, and 24 hr at E17, consistent with the multiple injection experiments. Simply counting progenitors in sections of embryonic mouse spinal cord yields average cell cycle times of ~6 hr at E12.5–13.5 and ~16 hr at E13.5–15 (Figure 2), again consistent with the other estimates.

Antibody Labeling

Cryosections of fixed tissue or cells cultured on coverslips were immunolabeled by conventional procedures. Monoclonal antibody (N11.4) against the mouse and rat NG2 proteoglycan core protein were obtained from William Stallcup (Burnham Institute, La Jolla, CA). For BrdU labeling, monclonal BU209 (Magaud et al., 1989) was used. Monoclonal anti-GC was obtained from Martin Raff (University College London). Propidium iodide (Sigma) was used to identify pyknotic cell nuclei in sections.

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