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Spinal cord oligodendrocytes develop from ventrally derived progenitor cells that express PDGF alpha-receptors

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SUMMARY

Platelet-derived growth factor alpha-receptors (PDGFRα) are expressed by a subset of neuroepithelial cells in the ventral half of the embryonic day 14 (E14) rat spinal cord. The progeny of these cells subsequently proliferate and migrate into the dorsal parts of the cord after E16. Here, we show that E14 ventral cells are able to generate oligodendrocytes in culture but that dorsal cells acquire this ability only after E16, coinciding with the appearance of PDGFRα-immunoreactive cells in the starting population. PDGFRα-positive cells in optic nerve and spinal cord cultures co-labelled with antibody markers of oligodendrocyte progenitors. When PDGFRα-positive cells were purified from embryonic rat spinal cords by immunoselection and cultured in defined medium, they all differentiated into oligodendrocytes. Very few oligodendrocytes developed in cultures of embryonic spinal cord cells that had been depleted of PDGFRα-expressing cells by antibody-mediated complement lysis. These data demonstrate that all PDGFRα-positive cells in the embryonic rat spinal cord are oligodendrocyte progenitors and that most or all early-developing oligodendrocytes are derived from these ventrally-derived precursors.

Key words: PDGF receptor, oligodendrocyte, development, spinal cord, immunoselection, rat

INTRODUCTION

It is not known how the many different types of neurons and glia in the mature central nervous system (CNS) are generated from the neuroepithelial cells that line the lumen of the neural tube. We are addressing this question by focussing on the development of oligodendrocytes, the myelinating cells of the CNS, from their neuroepithelial precursors in the embryonic rat spinal cord. We recently presented evidence in support of the idea that the ventricular zone (VZ) of the neural tube is a mosaic of specialized neural precursors that express different sets of gene products and give rise to distinct subsets of differentiated neurons or glia during development (Yu et al., 1994). We identified a discrete microdomain in the ventral VZ of the embryonic day 14 (E14) rat spinal cord that we suggested might be devoted specifically to the production of oligodendrocyte progenitors (Pringle and Richardson, 1993; Yu et al., 1994). This specialized microdomain comprises a narrow, longitudinal ribbon of neuroepithelial cells that can be recognized in situ by several molecular markers characteristic of the oligodendrocyte lineage: 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP; Yu et al., 1994), antigens recognized by monoclonal antibody O4 (Ono et al., 1995), the platelet-derived growth factor alpha-receptor (PDGFRα; Pringle and Richardson, 1993; Yu et al., 1994; Nishiyama et al., 1996) and, possibly, the myelin proteolipid protein or a related isoform (PLP/DM-20; Timsit et al., 1995). Of these, PDGFRα seems to be a general marker, being expressed at an equivalent developmental stage and location in the VZ of the rat, mouse, chicken and *Xenopus* spinal cords (Pringle and Richardson, 1993; Pringle et al., 1996; N. Pringle, unpublished). After their first appearance at the ventricular surface of the E14 rat spinal cord, the PDGFRα$^+$ cells seem to proliferate and migrate away from the VZ, becoming widely distributed throughout the cross section of the cord and reaching the dorsal-most regions between E16 and E18 (Pringle and Richardson, 1993).

We describe experiments designed to test the idea that these PDGFRα$^+$ cells are oligodendrocyte precursors, and to determine the contribution they make to oligodendrogenesis in the spinal cord. We purified PDGFRα$^+$ cells from embryonic rat spinal cord by immunoselection and found that they all differentiate into oligodendrocytes when cultured under appropriate conditions in vitro. Very few oligodendrocytes developed in cultures of embryonic spinal cord cells that had been depleted of PDGFRα$^+$ precursors by antibody-mediated complement lysis. When dorsal and ventral E14 rat spinal cord cells were cultured separately, only ventral cultures contained PDGFRα$^+$ precursors and gave rise to differentiated oligodendrocytes. The ability of dorsal cells to generate oligodendrocytes was acquired after E16 in parallel with the appearance of PDGFRα$^+$ cells. These data demonstrate that PDGFRα$^+$ cells in the embryonic spinal cord are oligodendrocyte progenitors and suggest that most or all oligodendrocytes are generated from these ventrally derived precursors.

MATERIALS AND METHODS

Optic nerve cell cultures

The optic nerves of newborn or postnatal day 7 (P7; the day of birth is P0) Sprague-Dawley rat pups were dissected and dissociated as described previously (Miller et al., 1985). Approximately 2,000 cells were seeded in a 10 μl droplet on a 13 mm diameter poly-D-lysine-coated glass coverslip in modified Bottenstein and Sato's (BS) medium (Bottenstein and Sato, 1979) containing 10% FCS. After 30 minutes, 400 μl BS medium was added to dilute the FCS to 0.5%. Sometimes 10 ng/ml recombinant human PDGF-AA (Peprotech, New Jersey, USA) was added. BS medium is Dulbecco's modified Eagle's medium (DMEM) supplemented with transferrin (0.1 mg/ml), bovine serum albumin (0.1 mg/ml), progesterone (60 ng/ml), sodium selenite (40 ng/ml), thyroxine (40 ng/ml), triiodothyronine (30 ng/ml), putrescine (16 μg/ml) and insulin (5 μg/ml) (all from Sigma).

Embryonic spinal cord cell cultures

Sprague-Dawley rats from the University College London breeding colony were used throughout. The day of appearance of the vaginal plug was designated embryonic day zero (E0). Timed-mated females were killed by CO_2 asphyxiation and the embryos removed and killed by decapitation. The spinal cords were dissected away from surrounding tissue in Hepes-buffered minimal essential medium (MEM-H) and the meningeal membranes removed with watchmakers' forceps. The tissue was transferred to 2 ml Earle's balanced salt solution without calcium or magnesium (EBSS; Gibco-BRL) containing 0.0125% (w/v) trypsin (Boehringer Mannheim) and incubated at 37°C in 5% CO_2 for 30 minutes. The tissue was washed in DMEM containing 10% FCS (Gibco-BRL) to inhibit the trypsin, then transferred to fresh DMEM containing 10% FCS and 0.005% (w/v) DNase-I (Sigma). The tissue was immediately dissociated by gentle trituration with a Pasteur pipette. The resulting cell suspension was filtered through a 20 μm pore-diameter mesh and washed by centrifugation and re-suspension in DMEM containing 10% FCS. The number of live cells in a sample of the suspension was counted in a hemocytometer, using trypan blue (Sigma) exclusion as the criterion of viability. Cells were then plated on poly-D-lysine-coated 13 mm diameter glass coverslips in a 20 μl droplet. The cells were allowed to attach for 30 minutes at 37°C. 400 μl of BS medium was added, with or without 10 ng/ml PDGF-AA (Peprotech), and incubation continued at 37°C in 5% CO_2.

Immunoselection

Immunoselection was carried out by a modification of published procedures (Barres et al., 1992; Collarini, 1995). Three 60 mm diameter Petri dishes were incubated overnight at 4°C with 2 ml anti-Ig antibody solution - two with 10 μg/ml goat-anti-mouse IgG (Jackson Immunoresearch, Pennsylvania) and one with 10 μg/ml goat anti-rabbit IgG (Jackson Immunoresearch) in 50 mM Tris pH 9. The dishes were washed three times with calcium- and magnesium-free phosphate-buffered saline (PBS) and incubated for several hours at room temperature with the appropriate antibodies for immunoselection (see below). The single-cell suspension of spinal cord cells (prepared as described above for spinal cord cultures) was washed by centrifugation and resuspension in MEM-H containing 0.5% FCS, passed over an uncoated Petri dish to remove macrophages, then sequentially over two dishes coated with a 1:10 dilution of RAN-2 hybridoma supernatant (Bartlett et al., 1981) to remove astrocytes and meningeal cells, then finally over a dish coated with a 1:200 dilution of anti-PDGFRα rabbit serum (Fretto et al., 1993). Each selection step was for 30 minutes at room temperature with occasional gentle swirling. The final dish was washed with MEM-H, then with EBSS, and the immunoselected PDGFRα^+ cells were removed with trypsin (0.125% [w/v] in EBSS), washed and resuspended in DMEM containing 10% FCS and plated at high density (1000 cells in a 3 μl droplet) on 6 mm diameter poly-D-lysine-coated glass coverslips in a 96-well tissue culture plate. The cells were allowed to settle for 30 minutes at 37°C before adding 35 μl BS-medium.

Antibody-mediated complement lysis

A suspension of E17 spinal cord cells was prepared as described above, except that FCS was omitted at all stages of the preparation to avoid non-specific serum-dependant complement lysis. The cell suspension was incubated simultaneously with rabbit complement (Cedar Lane Laboratories; diluted 1:12 in BS-medium) and A2B5 hybridoma supernatant diluted 1:5 in BS medium, at 37°C, 5% CO_2 for 45 minutes, with gentle inversion every 15 minutes. The cells were washed twice with DMEM and then once with DMEM containing 10% FCS. Approximately 40,000 viable cells (determined by trypan blue exclusion) were plated in a 20 μl droplet on a 13 mm diameter poly-D-lysine-coated glass coverslip. On the second day in vitro the complement treatment was repeated. The cells were incubated in antibody A2B5 (diluted 1:5 in BS medium) for 30 minutes at 37°C, then washed twice with DMEM. Rabbit complement (1:12 dilution in BS medium) was added for 30 minutes at 37°C, then the cells were washed twice with DMEM and incubated in fresh BS medium containing 0.5% FCS and 10 ng/ml PDGF-AA (Peprotech).

Immunocytochemistry

Cells on coverslips were lightly fixed in 2% (w/v) paraformaldehyde in PBS for 5 minutes at room temperature. The coverslips were washed three times in PBS, incubated in anti-PDGFRα rabbit serum (#3979) (Fretto et al., 1993; Nishiyama et al., 1996) diluted 1:100 in PBS for 30 minutes in a humid chamber at room temperature and rinsed three times in PBS. The cells were then incubated in goat anti-rabbit IgG (Jackson Immunoresearch) diluted 1:100 in PBS, for 30 minutes. Following this, the cells were incubated in one of the following primary antibodies: monoclonal antibody A2B5 (Eisenbarth et al., 1979), which labels a subset of gangliosides on the surface of O-2A progenitor cells (Raff et al., 1983); antibodies against the NG2 proteoglycan core protein (Stallcup and Beasley, 1987), which also label the surface of O-2A progenitors (Nishiyama et al., 1996), monoclonal antibody 04 (Sommer and Schachner, 1981), which labels sulphatide and other antigens on the surface of maturing O-2A progenitors (Bansal and Pfeiffer, 1992); monoclonal anti-galactocerebroside (GC; Ranscht et al., 1982), which reacts with an unidentified antigen in addition to GC (Bansal and Pfeiffer, 1992) on the surface of oligodendrocytes (Raff et al., 1978); monoclonal anti-glial fibrillary acidic protein (GFAP; clone GA-5, Sigma), an intermediate filament protein specific to astrocytes. Hybridoma cell supernatants were diluted 1:5 in PBS before use. The cells were post-fixed in 4% (w/v) paraformaldehyde in PBS and mounted under Citifluor (City University, UK). Labelled cells were viewed and photographed using a Zeiss Axioskop photomicroscope and Kodak T-Max 400 ASA film.

RESULTS

Characterization of PDGFRα^+-immunoreactive cells from rat optic nerve and spinal cord

Oligodendrocyte progenitor cells were first identified and characterized in cultures of perinatal rat optic nerve cells (Raff et al., 1983; for reviews see Raff, 1989; Pfeiffer et al., 1994). They were named O-2A progenitors because they can differentiate into either oligodendrocytes or type-2 astrocytes, depending on the culture conditions; in defined medium containing at most 0.5% fetal calf serum (FCS) they give rise to oligodendrocytes, whereas in the presence of 10% FCS they give rise to type-2 astrocytes (Raff et al., 1983). These cell types can be distinguished in rat optic nerve cultures by mor-

phology and by reactivity with a range of antibodies. O-2A progenitors have a simple process-bearing morphology, often bipolar, and express gangliosides recognized by monoclonal antibody A2B5 (Eisenbarth et al., 1979; Raff et al., 1983). Oligodendrocytes are complex, multi-process-bearing cells that label with anti-galactocerebroside (GC) (Raff et al., 1978; Bansal and Pfeiffer, 1992). Type-2 astrocytes label with A2B5 and anti-glial fibrillary acidic protein (GFAP) (Raff et al., 1983). In the later stages of their differentiation into oligodendrocytes, O-2A progenitors assume a more complex shape and start to express surface antigens recognized by monoclonal antibody O4 (Sommer and Schachner, 1981, 1982; Bansal et al., 1992). Progenitors at this (A2B5$^+$, O4$^+$) stage have been termed pro-oligodendrocytes (Pfeiffer et al., 1994). The O4 antigen continues to be expressed by GC$^+$ oligodendrocytes. Both (O4$^-$, A2B5$^+$) and (O4$^+$, A2B5$^+$) progenitor cells are proliferative whereas GC$^+$ oligodendrocytes are postmitotic (Small et al., 1987; Reynolds and Wilkin, 1991). Rat optic nerve cultures also contain type-1 astrocytes, which are not derived from O-2A progenitors and do not label with A2B5 or O4 (Raff et al., 1983). Optic nerve cultures do not contain any neurons.

Because the O-2A lineage has been well characterized in rat optic nerve cultures, we chose this culture system to characterize our anti-PDGFRα serum, which was raised in a rabbit against the recombinant extracellular domain of human PDGFRα (Fretto et al., 1993). Hart et al. (1989) previously reported that the predominant cells in perinatal rat optic nerve cultures that labelled with ^{125}I-labelled PDGF-AA, which binds exclusively to PDGFRα, were O-2A progenitors and newly formed oligodendrocytes. We sought to confirm this by immunolabelling rat optic nerve cell cultures with our anti-PDGFRα. We cultured P7 optic nerve cells for 16 hours in defined medium containing 0.5% FCS and 10 ng/ml PDGF-AA, which stimulates proliferation and inhibits differentiation of oligodendrocyte progenitors (Richardson et al., 1988; Noble et al., 1988; Raff et al., 1988). We then fixed and double-immunolabelled the cells with anti-PDGFRα together with either A2B5, O4, monoclonal anti-GC or monoclonal anti-GFAP. A small proportion of PDGFRα$^+$ cells (less than 5%) were flat and fibroblast-like, and did not co-label with either A2B5 or anti-GFAP; these were almost certainly meningeal cells, which are known to express PDGFRα (Hart et al., 1989; Pringle et al., 1992) (not shown). The vast majority of PDGFRα$^+$ cells had small cell bodies and a few slender processes and co-labelled with A2B5 (Fig. 1A,B), which identifies them unambiguously as O-2A progenitors (Raff et al., 1983, see above). The PDGFRα labelling on these cells was punctate and distributed all over the cell surface including the processes (Fig. 1A,B). When the flat (PDGFRα$^+$, A2B5$^-$) cells were excluded from the analysis, we found that 100±1% of the remaining PDGFRα$^+$ process-bearing cells were A2B5$^+$ and, conversely, 100±1% of the A2B5$^+$ cells were PDGFRα$^+$. Therefore, in rat optic nerve cultures, all O-2A progenitors express PDGFRα and all PDGFRα$^+$ process-bearing cells are O-2A progenitors. In addition, 64±3% of the O4$^+$ cells were also PDGFRα$^+$. The (O4$^+$, PDGFRα$^+$) cells fell into two categories: those with immature morphology (i.e. few cell processes), which displayed relatively bright punctate PDGFRα immunoreactivity all over their surfaces (Fig. 1C,D) and those with more complex morphologies, which displayed

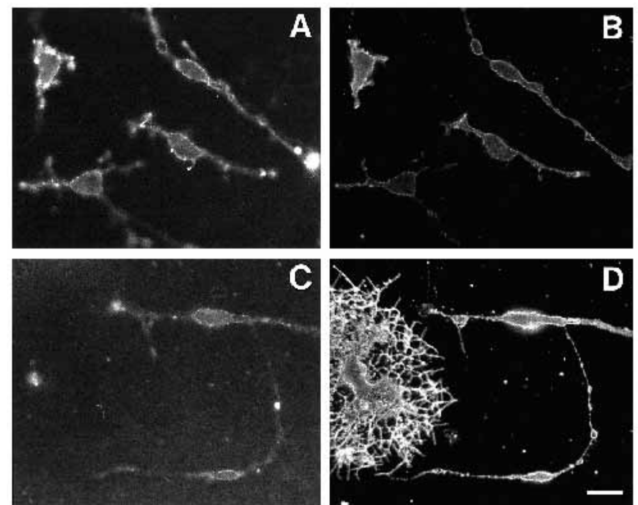

Fig. 1. Characterization of PDGFRα$^+$ cells in rat optic nerve cell cultures. P7 rat optic nerves were dissociated and cultured on glass coverslips in BS medium (see Materials and methods) containing 0.5% FCS and 10 ng/ml PDGF-AA for 16 hours, then fixed and stained with rabbit anti-human PDGFRα (A,C) and either monoclonal A2B5 (B) or monoclonal O4 (D). All PDGFRα$^+$ process-bearing cells also labelled with A2B5, and vice versa (A,B). A proportion (approx. 64%) of O4$^+$ cells also labelled with anti-PDGFRα (C, D). The (PDGFRα$^-$, O4$^+$) cells were almost certainly (O4$^+$, GC$^+$) oligodendrocytes (e.g. the large, mutiple-process-bearing cell in D). See Results for more information. Scale bar, 50 μm.

weak, punctate PDGFRα immunoreactivity on their cell bodies and some of their processes (not shown). The latter, weakly-staining cells were presumably recently formed (O4$^+$,GC$^+$, PDGFRα$^+$) oligodendrocytes, since we found that 6±4% of GC$^+$ oligodendrocytes in the cultures also were weakly labelled for PDGFRα (not shown). Therefore, PDGFRα immunoreactivity seems to be rapidly lost from O-2A progenitor cells after they start to differentiate into GC$^+$ oligodendrocytes.

Oligodendrogenic capacity originates in the ventral spinal cord, spreads dorsally and correlates with the presence of PDGFRα$^+$ cells

Warf et al. (1991) cultured dissociated cells from either ventral or dorsal halves of E14 rat spinal cords and found that oligodendrocytes developed in the ventral but not the dorsal cultures. When they cultured ventral and dorsal spinal cord cells from embryos aged E16 and later, they found that oligodendrocytes developed in both the ventral and the dorsal cultures. Warf et al. (1991) concluded that oligodendrocyte precursors first arise in the ventral half of the E14 spinal cord and migrate into the dorsal half by E16. Our in situ hybridization studies have revealed that there is a focus of PDGFRα$^+$ cells at the ventricular surface in the ventral half of the E14 rat spinal cord, approximately 15% of the distance from the floor plate towards the roof plate (Pringle and Richardson, 1993; Yu et al., 1994). After E14, these cells appear to proliferate and migrate away from the ventricular surface in all directions so that, at E16 and later, PDGFRα$^+$ cells are distributed widely throughout the ventral and dorsal halves of the cord, although at E16 they are still predomi-

nantly ventral (Pringle and Richardson, 1993). Thus, the spatiotemporal distribution of PDGFRα$^+$ cells closely matches that predicted for oligodendrocyte precursors by Warf et al. (1991).

We repeated the cell culture experiments of Warf et al. (1991), to test the prediction that development of oligodendrocytes in cultures of dorsal or ventral cells depends on the presence of PDGFRα$^+$ precursor cells in the starting cell population. We bisected E14, E16 or E18 rat spinal cords longitudinally into dorsal and ventral halves, dissociated the cells and cultured them on glass coverslips in defined medium containing 0.5% FCS. Previous studies have shown that, under these conditions, cell cultures of embryonic rat brain (Abney et al., 1981), optic nerve (Raff et al., 1985) or spinal cord (Warf et al., 1991) first give rise to oligodendrocytes on or shortly before the equivalent of the day of birth (E21/P0), coinciding with the first appearance of significant numbers of oligodendrocytes in vivo (Abney et al., 1981; Miller et al., 1985; Jordan et al., 1989; Warf et al., 1991). Thus, cultures of E14 spinal cord cells generate substantial numbers of oligodendrocytes after 3-4 days (equivalent of E18), E16 cultures after 1-2 days and so on (Warf et al., 1991; our own unpublished data). Therefore, in the first series of experiments we fixed and labelled our spinal cord cultures after 16 hours with anti-PDGFRα and A2B5 to visualize presumptive oligodendrocyte progenitors, or with anti-GC on the equivalent of the day of birth to visualize oligodendrocytes. The results of these experiments are depicted in Fig. 2 and Table 1. No (PDGFRα$^+$, A2B5$^+$) process-bearing cells were present in E14 dorsal cultures after 16 hours, and no GC$^+$ oligodendrocytes developed in parallel cultures during the next 7 days in vitro (Table 1; Fig. 2A,B,E,F). Small numbers of (PDGFRα$^+$, A2B5$^+$) process-bearing cells were present in E14 ventral cultures, and many oligodendrocytes developed within 7 days (Table 1; Fig. 2C,D,G,H). Significant numbers of (PDGFRα$^+$, A2B5$^+$) process-bearing cells were present in cultures of both ventral and dorsal cells from E16 and E18 spinal cords after 16 hours in vitro, and many oligodendrocytes developed in these cultures by the equivalent of the day of birth (Table 1). These data confirm the findings of Warf et al. (1991) and in addition show that the presence of (PDGFRα$^+$, A2B5$^+$) process-bearing cells in the starting cell population correlates with the ability of the cultures to give rise to oligodendrocytes in the longer term. In all these spinal cord cultures, as in optic nerve cultures, there were small numbers of (PDGFRα$^+$, A2B5$^-$) flat meningeal cells, which

Table 1. PDGFRα$^+$ progenitors and GC$^+$ progenitors in cultures of rat spinal cord cells

	PDGFRα$^+$ cells per coverslip after 16 hours in vitro		GC$^+$ oligodendrocytes per coverslip at equivalent of P0	
Age	Dorsal	Ventral	Dorsal	Ventral
E14	0	44±9	0	1387±245
E16	46±3	212±18	368±71	1908±204
E18	1360±90	1865±516	262±170	323±55

E14 rat spinal cords were dissected into dorsal and ventral halves and cultured separately on glass coverslips as described in Materials and methods. After 16 hours the cells were fixed and labelled with anti-PDGFRα to visualize presumptive oligodendrocyte progenitors. Parallel coverslips were cultured longer, until the equivalent of the day of birth (i.e. E14 + 7DIV, E16 + 5DIV, E18 + 3DIV; DIV means days in vitro), then fixed and labelled with anti-GC to visualize oligodendrocytes. PDGFRα$^+$ cells were present initially, and oligodendrocytes developed subsequently, in all cultures except cultures of E14 dorsal cells. Tabulated are mean numbers of cells and standard deviations of three independent experiments conducted in duplicate or triplicate. There was not a strict correlation between the number of PDGFRα$^+$ cells in the starting population and the number of oligodendrocytes that developed in culture. For example, fewer oligodendrocytes developed in the E18 cultures than in the E16 cultures, despite the fact that there were more PDGFRα$^+$ cells initially present in the E18 cultures. Part of the reason for this is presumably that the E18 cells were cultured for a shorter time than the E16 cells (3 days rather than 5 days in vitro). However, our impression was that there was more cell damage and death caused during dissociation of E18 than E16 spinal cords, probably reflecting increased mechanical damage to neurons. Reduction in the number of neurons in the cultures might have affected the rate at which oligodendrocytes differentiated or or the proportion that survived in the cultures.

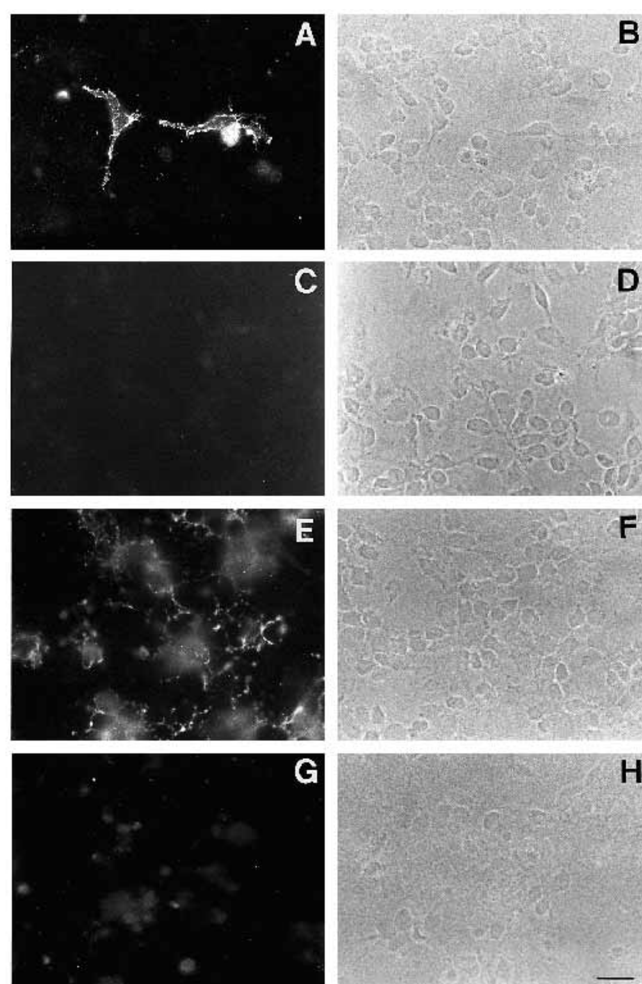

Fig. 2. PDGFRα$^+$ precursors and oligodendrocytes in cultures of E14 rat ventral and dorsal spinal cord cells. E14 cords were divided longitudinally into ventral and dorsal halves, dissociated and cultured on glass coverslips in BS medium containing 0.5% FCS with or without 10 ng/ml PDGF-AA. Cultures were immunolabelled with anti-PDGFRα (A-D) or anti-GC (E-H) and photographed under fluorescence or phase contrast optics. After 16 hours in vitro, ventral cultures contained small numbers of PDGFRα$^+$ cells (A,B) but dorsal cultures did not (C,D). After 7 days in cultures, many GC$^+$ oligodendrocytes had developed in ventral cultures (E,F) but few or none developed in dorsal cultures (G,H). See Table 1 and Fig. 3 for further information. Scale bar, 50 μm.

we omitted from the analysis. In both dorsal and ventral E14 cultures, unlike optic nerve cultures, there were also many (A2B5$^+$, PDGFRα$^-$) process-bearing cells after 16 hours in vitro (not shown). These presumably represent neural precursors of some sort; A2B5 is known to label many immature neurons, for example (Eisenbarth et al., 1979). Lower numbers of these cells were still present in E16 cultures, but they were very rare at E17 or later (see below), possibly because they differentiate and lose A2B5 immunoreactivity between E14 and E17.

The simplest explanation for these results is that oligodendrocyte progenitors, possibly the (PDGFRα$^+$, A2B5$^+$) process-bearing cells described, arise first in the ventral half of the E14 spinal cord and migrate into the dorsal half by E16. However, we were concerned that oligodendrocyte progenitor cells might be missed in the E14 dorsal cultures if, for example, they were very infrequent in the E14 dorsal cord or if they were less able to survive in the dorsal cultures than the ventral cultures. We therefore repeated the E14 spinal cord cultures, plating the cells at a much higher density (75,000 instead of 5,000 cells per coverslip) and scoring the presence of (PDGFRα$^+$, A2B5$^+$) progenitor cells and GC$^+$ oligodendrocytes at more frequent intervals. We also cultured the cells for longer – until the equivalent of P5 or P7 – in case oligodendrocytes develop somewhat later in dorsal cultures than in ventral cultures. The outcome of these experiments was qualitatively the same as before. E14 ventral cultures contained a small number of PDGFRα$^+$ cells after overnight incubation, and these multiplied dramatically over the 12-day culture period (Fig. 3). Oligodendrocytes first appeared in these cultures 7 days after plating and increased in number thereafter. E14 dorsal cultures, by contrast, contained few or no PDGFRα$^+$ cells after overnight incubation, as expected, and usually no PDGFRα$^+$ cells were present in these cultures after the first week of culture (Fig. 3). In some cultures small numbers of PDGFRα$^+$ cells were visible after 1 week, and these usually occurred in isolated clusters, as if they had arisen by clonal expansion of one or a few cells in the starting cultures. We rarely found oligodendrocytes in the dorsal E14 cultures, even after 2 weeks in culture. Invariably, if no PDGFRα$^+$ cells were detected at the outset of a particular experiment (i.e. after 16 hours in culture), then no oligodendrocytes developed subsequently in the parallel cultures. The outcome of all these experiments was qualitatively the same whether PDGF-AA was added to the medium or not, although more PDGFRα$^+$ cells and oligodendrocytes developed in the presence of PDGF. In separate experiments we added basic fibroblast growth factor (10 ng/ml) or Sonic hedgehog (7×10^{-9} M) to the cultures, without changing the results.

Therefore, we have confirmed the central finding of Warf et al. (1991), that the ability of spinal cord cells to generate oligodendrocytes starts in the ventral cord at E14 and later progresses to the dorsal cord. Moreover, the presence or absence of PDGFRα-immunoreactive cells in spinal cord cultures correlates with the distribution of PDGFRα mRNA-positive cells visualized by in situ hybridization, and predicts the ability of the cultures to generate oligodendrocytes in vitro. The data is consistent with our hypothesis that PDGFRα$^+$ cells are the progenitors of oligodendrocytes, and that these cells begin life in the ventral region of the spinal cord and subsequently proliferate and migrate dorsally.

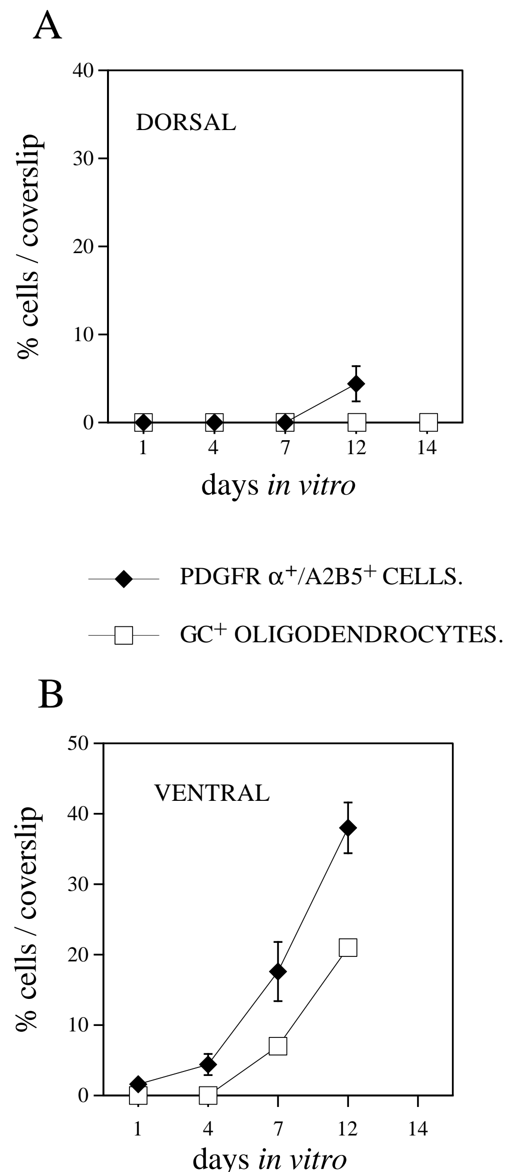

Fig. 3. Time course of appearance of PDGFRα$^+$ precursors and GC$^+$ oligodendrocytes in cultures of E14 dorsal or ventral spinal cord cultures. E14 spinal cords were divided into dorsal and ventral halves, dissociated and plated at high density on glass coverslips (75,000 cells per coverslip) in BS medium containing 0.5% FCS. After various culture periods, the cells were fixed and immunolabelled with anti-PDGFRα together with A2B5, or anti-GC antibodies. The cells were given a final wash in Hoechst 33258 (Sigma) to label all cell nuclei. The numbers of (PDGFRα$^+$, A2B5$^+$) process-bearing cells and GC$^+$ oligodendrocytes were counted (triplicate coverslips from two independent experiments) and expressed as a percentage of the total number of cells. Small numbers of PDGFRα$^+$ cells were present in ventral cultures at early times after plating, and these increased in number throughout the culture period. GC$^+$ oligodendrocytes first developed in ventral cultures between four and seven days after plating and increased in numbers thereafter. In dorsal cultures, no (PDGFRα$^+$, A2B5$^+$) cells were detected early on, although a few were detected at 7 days and later. These always appeared in tight clusters as though clonally derived from one or two cells in the starting population. GC$^+$ oligodendrocytes did not develop in the dorsal cultures, even after 14 days in vitro.

All PDGFRα⁺ process-bearing cells in embryonic rat spinal cord are O-2A progenitors

In situ hybridization studies show that there are increasing numbers of scattered cells that express PDGFRα mRNA in the rat spinal cord after E14 (Pringle and Richardson, 1993). We had previously suggested that these cells might be oligodendrocyte progenitors and the culture experiments described above are consistent with this. To test this idea directly, we immunoselected PDGFRα$^+$ cells from freshly prepared cell suspensions of E17 spinal cords (minus meningeal membranes) with the anti-PDGFRα antiserum described above. Briefly, the suspension was passed over uncoated plastic dishes to remove macrophages, then over a dish coated with monoclonal antibody RAN-2 to remove the majority of astrocytes and finally over a dish coated with anti-PDGFRα. The non-adherent cells were discarded from the final dish and the adherent cells were removed with trypsin and replated on glass coverslips in defined low-serum medium containing 10 ng/ml PDGF-AA (1000 cells in a 3 μl droplet). After overnight culture the immunoselected cells were at least 97% (PDGFRα$^+$, A2B5$^+$) and had a simple process-bearing morphology like optic nerve O-2A progenitors (Fig. 4A). Contaminating cells included small numbers of macrophages that adhere non-specifically to plastic and PDGFRα$^+$ meningeal cells that were not completely removed during the dissection. Excluding these contaminating cells, on the basis of antibody labelling or morphology, the remaining cells were >99% (PDGFRα$^+$, A2B5$^+$) process-bearing cells. All of these cells could also be labelled with antibodies against the NG2 chondroitin sulphate proteoglycan, another marker of O-2A lineage cells (not shown). They did not label with monoclonal antibody O4, anti-GC or anti-GFAP. When the immunoselected cells were washed free of PDGF-AA and cultured for an additional 24 hours in defined, low-serum medium without growth factors, they began to express the O4 antigen and to adopt a more highly branched morphology (not shown). After a further 24 hours in this medium, >95% of all the cells expressed GC and had the multipolar process-bearing morphology of oligodendrocytes (Fig. 4B). If, 24 hours after plating, the immunoselected cells were switched instead to medium containing 10% FCS for 72 hours, >95% of them developed into (GFAP$^+$, A2B5$^+$) type-2 astrocytes (Fig. 4C,D). Cell death was insignificant over the time course of these experiments (data not shown). Therefore, essentially all of the PDGFRα$^+$ cells in E17 rat spinal cord have the properties and differentiation potential expected of O-2A progenitor cells (Raff et al., 1983).

Most or all oligodendrocytes that develop in cultures of E17 rat spinal cord cells are generated from (PDGFRα⁺, A2B5⁺) progenitors

To ask whether all oligodendrocytes that develop in the rat spinal cord arise from PDGFRα$^+$ progenitor cells, or whether there might be a separate PDGFRα$^-$ oligodendrocyte lineage(s), we attempted to remove PDGFRα$^+$ progenitors from freshly prepared suspensions of E17 spinal cord cells by selective lysis with anti-PDGFRα and complement. Despite much effort, these attempts were unsuccessful; neither our anti-PDGFRα rabbit immunoglobulin nor a rat monoclonal antibody against the extracellular domain of mouse PDGFRα (a gift from N. Takakura and S.-I. Nishikawa, Kyoto University) seemed capable of fixing rabbit complement and killing the cells. However, we found that there was almost complete overlap between PDGFRα- and A2B5-immunolabelling among cells isolated from E17 spinal cord – out of 2,000 A2B5$^+$ process-bearing cells examined after 16 hours culture in defined, low-serum medium containing 10 ng/ml PDGF-AA, only 13 were not also PDGFRα$^+$. Conversely, almost all PDGFRα$^+$ cells in these cultures were also A2B5$^+$. The only exceptions to this rule were the (PDGFRα$^+$, A2B5$^-$) flat meningeal cells referred to above, which were present in variable but always very low numbers. Therefore, we were able to remove PDGFRα$^+$ process-bearing cells selectively from suspensions or cultures of spinal cord cells using antibody A2B5 and complement. We subjected E17 spinal cord cells to two rounds of A2B5-mediated complement lysis, first on the freshly prepared cell suspension before plating and again directly on the coverslip after 24 hours in vitro. Parallel control cultures were treated with A2B5 without complement, or with an anti-β-galactosidase monoclonal IgM together with complement, or with complement alone. After these treatments, the number of remaining (A2B5$^+$, PDGFRα$^+$) progenitors was

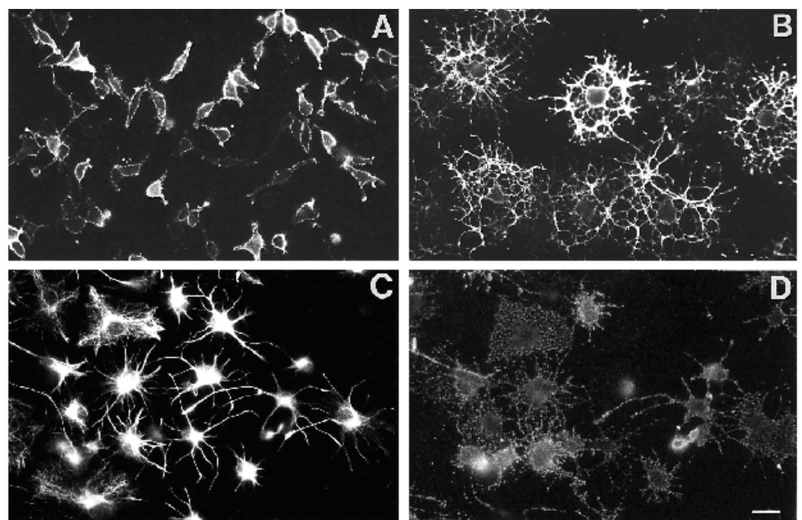

Fig. 4. PDGFRα$^+$ cells, immunoselected from embryonic rat spinal cords with an anti-PDGFRα antibody, differentiate into GC$^+$ oligodendrocytes or (GFAP$^+$, A2B5$^+$) astrocytes. Cell suspensions of E17 spinal cords were immunoselected as described in Materials and methods, and cultured on glass coverslips in BS medium containing 0.5% FCS and 10 ng/ml PDGF-AA. After 24 hours, the cells were fixed and immunolabelled with anti-PDGFRα, A2B5, anti-NG2, O4 or anti-GC. More than 99% of the process-bearing cells were (PDGFRα$^+$, A2B5$^+$, NG2$^+$, O4$^-$, GC$^-$). A shows these cells labelled with anti-PDGFRα. Some coverslips were transferred after 24 hours in culture into BS medium containing 0.5% FCS without PDGF, or into BS medium with 10% FCS. After culturing for a further 1-3 days, the cells were fixed and immunolabelled with anti-GC or double-labelled with anti-GFAP and A2B5. In the presence of 0.5% FCS, more than 99% of the cells became GC$^+$ oligodendrocytes (B). In the presence of 10% FCS, more than 99% of the cells became GFAP$^+$ astrocytes (C), the majority of which also labelled with A2B5 (D). Scale bar 50 μm.

Table 2. Antibody-mediated complement lysis of (A2B5$^+$, PDGFRα$^+$) cells greatly reduces the ability of embryonic rat spinal cord cultures to generate oligodendrocytes in vitro

	Complement only	A2B5 and complement
(PDGFRα$^+$, A2B5$^+$) cells per coverslip, after 48 hours in vitro	6300±913	199±48
GC$^+$ oligodendrocytes per coverslip after 4 days in vitro (≡ P0)	2831±663	113±44

E17 rat spinal cords were dissociated and the cells subjected to two rounds of incubation with antibody A2B5 plus complement, or with complement alone (see Materials and methods). The cultures were maintained in BS medium containing 0.5% FCS and 10 ng/ml PDGF-AA; 24 hours after the second round of complement treatment, the cells were fixed and immunolabelled with anti-PDGFRα and A2B5. Parallel coverslips were cultured longer, until the equivalent of the day of birth (total of 4 days in vitro), then fixed and immunolabelled with anti-GC to visualize oligodendrocytes. Tabulated are the numbers of (A2B5$^+$, PDGFRα$^+$) process-bearing cells or GC$^+$ oligodendrocytes in the cultures, means and standard deviations of at least three independent experiments conducted in duplicate or triplicate. Depleting the spinal cord cultures of (A2B5$^+$, PDGFRα$^+$) cells severely diminished the ability of the cultures to generate oligodendrocytes.

approximately 30-fold less than in any of the controls (Table 2; Fig. 5A-D). The depleted cell populations were maintained in defined, low-serum medium with or without PDGF-AA for a further 4 days, until the equivalent of the day of birth, to allow time for oligodendrocytes to develop in vitro. At the end of the culture period, the cells were fixed and stained with anti-GC to visualize oligodendrocytes. The cultures depleted of (A2B5$^+$, PDGFRα$^+$) process-bearing cells contained approximately 25-fold less GC$^+$ oligodendrocytes than either of the control cultures (Table 2; Fig. 5E-H). Therefore, the reduction in the number of oligodendrocytes approximately matched the reduction in the number of (A2B5$^+$, PDGFRα$^+$) precursors in the starting population.

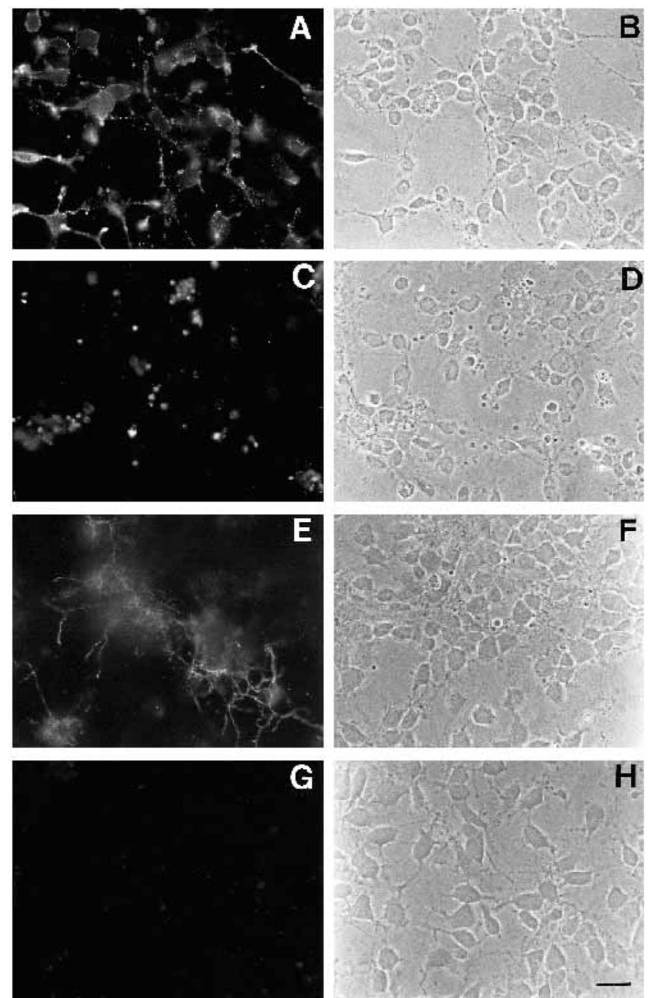

Fig. 5. Oligodendrocytes develop from (A2B5$^+$, PDGFRα$^+$) progenitor cells in mixed cell cultures of embryonic rat spinal cord. Cell suspensions from E17 rat spinal cords were treated with monoclonal antibody A2B5 and complement (C,D) or complement alone (A,B) as described in Materials and methods. At E17 almost all (>97%) A2B5$^+$ process-bearing cells in the spinal cord cultures are also PDGFRα$^+$, and vice versa. After 24 hours in culture the cells were fixed, immunolabelled with A2B5 and anti-PDGFRα and photographed in fluorescence and phase contrast optics. The complement-killing procedure removed the great majority (≥96%) of (A2B5$^+$, PDGFRα$^+$) cells from the cultures (A-D). Parallel ccoverslips were cultured for 4 days, until the equivalent of the day of birth, then the cells were fixed and immunolabelled with anti-GC. Very few GC$^+$ oligodendrocytes developed in the cultures depleted of (A2B5$^+$, PDGFRα$^+$) cells compared to control cultures treated with complement alone (E-H) (see Table 2 for further information). Scale bar, 50 μm.

DISCUSSION

The purpose of the experiments described in this paper was to test the idea that PDGFRα$^+$ cells in the embryonic rat spinal cord are oligodendrocyte progenitors, and to assess the contribution these PDGFRα$^+$ cells make to oligodendrogenesis in the cord. We found that essentially all PDGFRα$^+$ process-bearing cells in perinatal rat optic nerve or late embryonic spinal cord cultures co-labelled with antibody A2B5, an established marker of oligodendrocyte progenitors in optic nerve cultures. Conversely, nearly all A2B5$^+$ cells in cultures of optic nerve or spinal cord (after E17) co-labelled with anti-PDGFRα. PDGFRα$^+$ cells, immunoselected from suspensions of dissociated E17 spinal cord cells with an antibody against the extracellular domain of PDGFRα, all gave rise to GC$^+$ oligodendrocytes when cultured in defined medium containing less than 0.5% FCS, and into (A2B5$^+$, GFAP$^+$) type-2 astrocytes when cultured in the presence of 10% FCS. Therefore, in antigenic phenotype and differentiation potential, these cells closely resemble the O-2A progenitor cells originally identified as oligodendrocyte precursors in cultures of perinatal rat optic nerves. Very few oligodendrocytes developed in embryonic spinal cord cultures depleted of (A2B5$^+$, PDGFRα$^+$) cells by antibody-mediated complement lysis. Together, these data demonstrate that all PDGFRα$^+$ process-bearing cells in the late embryonic rat spinal cord are oligodendrocyte precursors and strongly suggest that most or all spinal cord oligodendrocytes develop from PDGFRα$^+$ precursors.

We have confirmed and extended the data of Warf et al.

(1991), who reported that the ability of rat spinal cord cells to generate GC^+ oligodendrocytes in culture was acquired first by ventral cells at E14 and only later by dorsal cells. Their interpretation was that oligodendocyte precursors initially appeared in the ventral half of the cord and subsequently migrated dorsally. Subsequently, Fok-Seang and Miller (1994) presented evidence that oligodendrocytes in the spinal cord develop from $A2B5^+$ progenitor cells and that these progenitors originate in the ventral half of the cord. We agree with these conclusions and further characterize the oligodendrocyte precursors as the ventrally derived ($A2B5^+$, $PDGFR\alpha^+$) process-bearing cells described above. We were concerned that dorsally derived oligodendrocyte progenitors might be missed if they were less able to survive in dorsal cultures than in ventral cultures. We therefore increased the cell culture density from 5,000 to 75,000 cells per coverslip, reasoning that adequate amounts of locally produced survival factors were more likely to be produced in dense cultures. However, this did not alter the outcome of the experiments, i.e. the ability to generate oligodendrocytes was acquired first by ventral cells. This same trend is observed even in explant cultures of avian spinal cord (Trousse et al., 1995). Recently, we and others have demonstrated that specification of the oligodendrocyte lineage in the ventral spinal cord, like other ventral cell types such as floor plate cells or motor neurons (Yamada et al., 1991), depends on local signals from the notochord/floor plate complex (Trousse et al., 1995; Pringle et al., 1996; Orentas and Miller, 1996).

The idea that spinal cord oligodendrocytes are ventrally derived is not universally accepted. Cameron-Curry and Le Douarin (1995) recently described the results of chick-quail transplant studies which seem to show that oligodendrocytes can be generated all along the dorsal-ventral axis of the avian spinal cord. However, there are potential problems with the interpretation of their data which have to do with how one operationally defines a dorsal neural tube graft. This is discussed in detail elsewhere (Richardson et al., 1996). We ourselves have performed chick-quail neural tube grafts and conclude from these experiments, contrary to Cameron-Curry and Le Douarin (1995), that avian spinal cord oligodendrocytes originate exclusively in the ventral half of the cord (Pringle, N. P., Lumsden, A., Richardson, W. D. and Guthrie, S., unpublished data). Another recent report (Hardy and Friedrich, 1996) puts forward the idea that oligodendrocyte precursors are normally generated at many sites throughout the neuroepithelium. Their approach was to transplant fragments of hindbrain, spinal cord or dorsal telencephalon from a marked donor mouse to an unmarked recipient, and to look for the appearance of marked oligodendrocytes in the host. If donor-derived oligodendrocytes were detected in the chimeric animal, this was taken as evidence that the transplanted donor tissue included a source of oligodendrocyte precursors. Brain and spinal cord fragments were taken from the donors (transgenic mice that expressed a β-galactosidase reporter gene under the control of the myelin basic protein gene promoter) at ages from E10.5 to E14.5 and transferred into the brains of newborn wild-type recipients. Transplant-derived (β-galactosidase-positive) oligodendrocytes developed in the chimeric brains, regardless of whether or not the donor tissue contained $PDGFR\alpha^+$ progenitor cells at the time of transfer. While this experiment clearly demonstrates that neuroepithelial cells from embryonic dorsal telencephalon (and, by implication, perhaps also dorsal spinal cord) have the *potential* to generate oligodendrocyte lineage cells in a foreign environment (both heterotypic and heterochronic), it does not address the question of what the fate of the donor cells would have been if left undisturbed at their site of origin. It is frequently the case that the actual fates adopted by neuroepithelial precursors in situ are more restricted than the full range of fates they can possibly adopt when placed out of their normal context (e.g. Renfranz et al., 1991; Yamada et al., 1991; Simon et al., 1995; Purves and Lichtman, 1985).

It should be noted that all of the evidence we quote in favour of a strictly ventral origin for spinal cord oligodendrocytes applies only to the earlier stages of oligodendrogenesis, i.e. up to P7 in the experiments reported here. It remains possible that later-developing oligodendrocytes might be derived from different parts of the neural tube, though at present there is little reason to think so, at least for the spinal cord. Brain oligodendrocytes might well have more than one developmental origin, however. For example, there is a localized domain of $PDGFR\alpha^+$ precursors in the ventral diencephalon of the E13 rat that appears to be source of migratory cells that populate more dorsal regions of the forebrain including the cerebral cortex after E17 (Pringle and Richardson, 1993); by analogy with the spinal cord, it seems possible that these represent $PDGFR\alpha^+$ oligodendrocyte progenitors and recently we have obtained some evidence in support of this idea (Hall, A., Giese, N. A. and Richardson, W. D., unpublished data). However, there is also evidence that forebrain oligodendrocytes can develop postnatally from precursors in the subventricular zones underlying the lateral ventricles (Levison and Goldman, 1993; Levison et al., 1993; Zerlin et al., 1995).

There is evidence that some non-glial cells in the rodent CNS express PDGFRα. We previously described a PDGFRα mRNA expression domain in the dorsal rat spinal cord that we presumed to represent proliferating neural precursors (Pringle and Richardson, 1993). These cells start to express PDGFRα mRNA on or before E12 but expression declines by E14 and is undetectable shortly thereafter. We do not consistently observe this dorsal domain of PDGFRα expression by in situ hybridization, though we believe it to be real but weak. In the experiments described here, we never could detect PDGFRα-immunoreactive cells in dorsal E14 spinal cord cultures. Presumably the level of expression is too weak to be detected with our anti-PDGFRα antibody. Recently there was a report that motor neurons in the adult mouse spinal cord express PDGFRα mRNA (Vignais et al., 1995). We have never detected PDGFRα mRNA in motor neuron pools in the rat spinal cord by in situ hybridization. Neither was PDGFRα immunoreactivity reported in motor neurons in a recent immunohistochemical study of the developing rat spinal cord (Nishiyama et al., 1996). Perhaps PDGFRα expression is different in the mouse than in the rat, or is only activated in postnatal or adult motor neurons. Further experiments will clarify this issue. In any case, these considerations do not affect the conclusions of the present study.

The fact that PDGF is a potent mitogen (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988; Hunter and Bottenstein, 1990) and survival factor (Barres et al., 1992) for oligodendrocyte lineage cells in vitro implies that PDGF signalling might also be important for the development of these cells in

vivo. If so, and if most or all spinal cord oligodendrocytes develop from PDGFRα$^+$ precursors as our present study suggests, one would predict that PDGF or PDGFRα null mutants might be severely affected in oligodendrogenesis in the cord. In keeping with this prediction, we have found that there are only about 5% of the normal number of PDGFRα$^+$ progenitor cells in the in the spinal cords of PDGF-A knockout mice and a greatly reduced number of oligodendrocytes (Calver, A., Hall, A., Fruttiger, M., Yu, W.-P., Boström, H., Willetts, K., Heath, J. K., Betsholtz, C. and Richardson, W. D., unpublished data). This provides further indirect evidence that oligodendrogenesis in the spinal cord relies heavily on the PDGFRα$^+$ lineage that has been the subject of this investigation.

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