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Sonic hedgehog signaling is required during the appearance of spinal cord oligodendrocyte precursors

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SUMMARY

Spinal cord oligodendrocyte precursors arise in the ventral ventricular zone as a result of local signals. Ectopic oligodendrocyte precursors can be induced by sonic hedgehog (Shh) in explants of chick dorsal spinal cord over an extended developmental period. The role of Shh during normal oligodendrocyte development is, however, unclear. Here we demonstrate that Shh is localized to the ventral spinal cord immediately prior to, and during the appearance of oligodendrocyte precursors. Continued expression of Shh is required for the appearance of spinal cord oligodendrocyte precursors as neutralization of Shh signaling both in vivo and in vitro during a defined developmental period blocked their emergence. The inhibition of oligodendrocyte precursor emergence in the absence of Shh signaling was not the result of inhibiting precursor cell proliferation, and the neutralization of Shh signaling after the emergence of oligodendrocyte precursors had no effect on the appearance of additional cells or their subsequent differentiation. Similar concentrations of Shh induce motor neurons and oligodendrocytes in dorsal spinal cord explants. However, in explants from early embryos the motor neuron lineage is preferentially expanded while in explants from older embryos the oligodendrocyte lineage is preferentially expanded.

Key words: Oligodendrocytes, Spinal cord, Sonic hedgehog, Cell signalling, Chick

INTRODUCTION

During development, the earliest cells of the oligodendrocyte lineage arise in the ventral ventricular zone of the spinal cord (Noll and Miller, 1993; Pringle and Richardson, 1993; Yu et al., 1994; Ono et al., 1995; Timsit et al., 1995). In the chick, oligodendrocyte precursors can be specifically identified beginning at around stage 29 (E6) by the monoclonal antibody (mAb) O4 (Ono et al., 1995, 1997a,b). These cells initially have a simple neuroepithelial morphology, and subsequently migrate dorsally and laterally to populate presumptive white matter (Ono et al., 1995). The origin of spinal cord oligodendrocyte precursors is influenced by local signals from the notochord, a mesodermal structure located ventral to the neural tube (Orentas and Miller, 1996; Pringle et al., 1996). For example, grafting an additional notochord dorsal to the spinal cord at stage 10 (E2) induces ectopic oligodendrocyte precursors adjacent to the transplanted notochord (Orentas and Miller, 1996; Pringle et al., 1996), and the addition of notochord to dorsal neural tube cultures results in the induction of oligodendrocytes (Trousse et al., 1995; Orentas and Miller, 1996; Pringle et al., 1996). The notochord is in fact required for the development of spinal cord oligodendrocytes. Inhibition of notochord development or ablation of the notochord early in development results in a failure of oligodendrocytes to appear in adjacent spinal cord (Pringle et al., 1996; Maier and Miller, 1997). The requirement for notochord is transient, since oligodendrocytes develop on a normal schedule in isolated cultures of spinal cord, separated from notochordal influences several days earlier (Warf et al., 1991).

One candidate molecule for mediating the effects of the notochord on oligodendrocyte development is Sonic hedgehog. During normal development, the notochord contributes to the development of ventral spinal cord cell types in part by secretion of sonic hedgehog (Shh) (Echelard et al., 1993; Roelink et al., 1994, 1995). Sonic hedgehog induces floor plate, motor neurons and interneurons in a dose-dependent manner (Roelink et al., 1995; Ericson et al., 1997); relatively high concentrations induce floor plate and motor neurons while slightly lower concentrations induce motor neurons but not floor plate (Roelink et al., 1995; Ericson et al., 1997). Oligodendrocytes can be induced in dorsal spinal cord by similar concentrations of Shh to those required for motor neurons (Pringle et al., 1996) indicating that Shh is sufficient for the ectopic induction of spinal cord oligodendrocytes (Poncet et al., 1996; Pringle et al., 1996). The notochord is not the only ventrally located source of Shh. The floor plate (Echelard et al., 1993; Roelink et al., 1994, 1995; Marti et al., 1995), and to a lesser extent motor neurons (Bitgood and McMahon, 1996), also produce Shh during specific stages of development.

The demonstration that similar concentrations of Shh induced

motor neurons and oligodendrocytes raises the possibility that the development of both cell types reflects the early induction of combined motor neuron-oligodendrocyte precursors (Pringle et al., 1996; Richardson et al., 1997). Consistent with this hypothesis, retrovirally mediated clonal analyses in chick spinal cord (Leber et al., 1990; Leber and Sanes, 1995) demonstrated clones containing both motor neurons and oligodendrocytes. Alternatively, Shh may induce different cell in the presence of additional environmental influences (Miller, 1996).

To determine the requirements for Shh signaling during the normal appearance of spinal cord oligodendrocyte precursors we have manipulated Shh signaling both in vitro and in vivo. We demonstrate that the initial appearance of spinal cord oligodendrocyte precursors is dependent on the local expression of Shh at the time these cells first appear. Inhibiting Shh signaling does not, however, affect oligodendrocyte precursor proliferation. We confirm that similar concentrations of Shh induce motor neurons and oligodendrocytes (Pringle et al., 1996) and demonstrate that the subsequent expansion of the specific cell populations is dependent on the age of the responding tissue. These data indicate that the continued local expression of Shh is essential for the initial appearance of spinal cord oligodendrocytes.

MATERIALS AND METHODS

Neural tube cultures

Fertilized White Leghorn chicken eggs (SPAFAS, Inc.) were incubated until the appropriate stage (Hamburger and Hamilton, 1951), the neural tubes isolated as previously described (Orentas and Miller, 1996) and they were then divided into dorsal and ventral regions. Explants were grown in collagen gels (3 mg/ml, Collaborative Research), in F12 basal medium supplemented with N2 (Bottenstein and Sato, 1979) and 10 ng/ml each of bFGF and PDGF. At appropriate intervals cultures were fixed and an average of 14 explants from 4 experiments assayed for each condition. Unless noted, O4+ explants contained greater than 20 immunoreactive cells while O4− explants contained no detectable immunoreactive cells. Dissociated cell cultures were established at a density of 2.5×10^5 cells/coverslip as previously described (Miller et al., 1997). The recombinant amino-terminal fragment of the rat-Shh protein (a gift from Dr Tom Jessell) was added as required. Function blocking anti-sonic hedgehog antibody (mAb 5E1) (Ericson et al., 1996) (Developmental hybridoma Bank) or control isotype-matched 7B11 antibody (Szigeti and Miller, 1993) was added at the time of plating (1:5 supe) and the total number of immunoreactive cells on duplicate coverslips counted in at least 3 experiments for a total of at least 6 coverslips under each condition. Data represent the mean ± standard deviation in all cases.

Immunohistochemistry

Oligodendrocyte precursors were identified by their expression of antigens recognized by the mAb O4 (Ono et al., 1995; Orentas and Miller, 1996). Neurons were identified by their expression of neuron-specific β-tubulin recognized by mAb TuJ1 or neurofilament (Moody et al., 1989; Lee et al., 1990; Easter et al., 1993). Motor neurons were identified by mAb 4D5 (supernatant 1:10) which identifies the Lim homeodomain genes Isl-1 and Isl-2 (Tsuchida et al., 1994) (Developmental Biology Hybridoma Bank). All antibody incubations were performed as previously described (Orentas and Miller, 1996) and the cells examined on a Nikon microscope under epifluorescence. Controls included omission of the primary antibody in which no specific labeling was seen. To specifically eliminate oligodendrocyte lineage cells, dissociated cells were sequentially incubated in mAb O4 supernatant and guinea pig complement as previously described (Fok-Seang and Miller, 1994).

The number of motor neurons and oligodendrocyte precursors in the presence and absence of Shh were quantified in explant cultures either by directly counting under a 20× objective or on frozen sections prepared as previously described (Tsuchida et al., 1994; Ericson et al., 1996). For double labeling, explants were fixed in 4% paraformadehyde and labeled with mAb O4 (Orentas and Miller, 1996). Explants were serially sectioned and the sections labeled with mAb 4D5 as previously described (Orentas and Miller, 1996). In all cases, GC+ oligodendrocytes only developed in tissues containing O4+ cells (Ono et al., 1995). The total number of immunoreactive cells was determined from at least 5 different explants in each condition and the data pooled. Results are expressed as the mean ± standard deviation in each case.

Cell proliferation assays

To determine whether Shh is mitogenic for chick oligodendrocyte precursors, a BrdU incorporation assay was used (Robinson and Miller, 1996). Dissociated cell cultures were grown in the presence of anti-Shh neutralizing antibody or exogenously added Shh and the number of O4+ cells and O4+/BrdU+ cells determined. BrdU (10 μM) was added for the last 18 hours of culture and the cells labeled as previously described (Robinson and Miller, 1996). The number of O4+ cells and the proportion of O4+ cells that had incorporated BrdU were assayed from at least 6 different cultures taken from 2 different preparations. The data represent the mean ± standard deviation.

In vivo localization and inhibition of Shh signaling

All tissue for in vivo analysis was taken from similar (thoracic) levels in all animals studied. Sonic hedgehog was localized by labeling with mAb 5E1 (1:3) in transverse frozen sections of stage 17, 26 and 29 embryos. Controls included elimination of primary antibody or substitution with a monoclonal antibody of similar class in which no specific staining was observed. To transiently eliminate Shh signaling in stage 17-20 embryos, 2×10^6 5E1 hybridoma cells were grown on the chorioallantoic membrane of developing chicks. Controls included either addition of similar quantities of medium (30 μl) or addition of similar numbers of control hybridoma cells (mAb 7B11) (Szigeti and Miller, 1993) secreting an anti-astrocyte antibody. Neither control was significantly different from normal. Animals were screened for gross developmental defects, hybridoma cells and the presence of hybridoma-derived antibody by labeling with second antibody (Robinson and Miller, 1996). In older animals, approximately 300 μl of 10× concentrated anti-Shh hybridoma supernatant was injected into the subblastemal space adjacent to the embryo. As above, controls included saline and 7B11 antibody injection. To determine the temporal requirements for Shh signaling in more detail, anti-Shh was added to cultures of chick spinal cords taken at different ages. The cultures were grown until equivalent age (stage 36) and the number of O4+ cells that developed compared between experimental and control cultures. The data represents the relative numbers of O4+ cells in each culture from three coverslips from at least two independent cultures.

Cholinesterase staining

Frozen sections of fixed spinal cord were washed twice and incubated in a visualization medium containing 4 mM acetlythiocholine, 10 mM glycine, 2 mM cupric sulfate and 50 mM sodium acetate, known to provide specific localization in avian systems (Katz and Karten, 1983). After a 1- to 2-hour incubation in the dark, sections were thoroughly rinsed in distilled water, dehydrated through graded alcohols and mounted in permount. The number of cholinesterase-positive lateral motor neurons/section of spinal cord was determined by counting under 20× magnification on at least 10 different sections from each animal and from at least three treated animals, and the data pooled.

Fig. 1. Sonic hedgehog supports the appearance of oligodendrocyte precursors in chick dorsal spinal cord explants. Explants derived from either the ventral (A) or dorsal (B,C) neural tube were isolated from stage 12 chick embryos, grown in a collagen gel matrix, and labeled for O4+ oligodendrocyte precursors after 6 days. (A) In ventrally derived explants, oligodendrocyte precursors develop as tightly clustered loci within the explant. (B) No O4+ oligodendrocyte precursors develop in dorsal control explants. By contrast, in the presence of 2.5 nM Shh (C) large numbers of O4+ cells are present in dorsal explants after 6 days in vitro. In both ventral control cultures and Shh-induced dorsal cultures, O4+ cells were more prevalent in certain regions of the explant, and many cells had an elongated morphology (arrow) similar to migrating cells in the intact spinal cord. Bar, 100 µm.

RESULTS

Oligodendrocytes are induced in dorsal spinal cord explants by Shh

To determine when oligodendrocyte precursors appear in response to Shh signaling, dorsal explants were grown in the presence of Shh and the appearance of O4+ oligodendrocyte precursors compared with that in ventral explants. In stage 12 (E2) ventral explants, O4+ oligodendrocyte precursors appeared after 5 days and were abundant after 6 days in vitro in more than 80% (15/17) of the explants (Fig. 1A). The O4+ cells were typically clustered in the explants, probably reflecting residual neural tube cytoarchitecture. At the periphery of the clusters many cells had the elongated morphology of motile precursors (Fig. 1A, arrow), while in regions of high density many cells were multi-processed and resembled more mature pro-oligodendroblasts. In the absence of Shh no E2 dorsal spinal cord explants contained O4+ cells after 6 days (0/15) (Fig. 1B). By contrast, in the presence of 2.5 nM Shh, O4+ oligodendrocyte precursors were first detectable in dorsal explants after 5 days in vitro, and more than 40% (7/17) of explants contained large numbers of O4+ cells after 6 days in vitro (Fig. 1C). The induced ectopic oligodendrocyte precursors developed in small clusters within the dorsal explants and had a morphology similar to O4+ cells in ventral neural tube explants. These data are consistent with previous studies demonstrating the induction of oligodendrocytes by Shh in stage 12 dorsal neural tube (Poncet et al., 1996; Pringle et al., 1996) and demonstrate that the ectopically induced, and endogenous oligodendrocyte precursors appear at similar developmental stages.

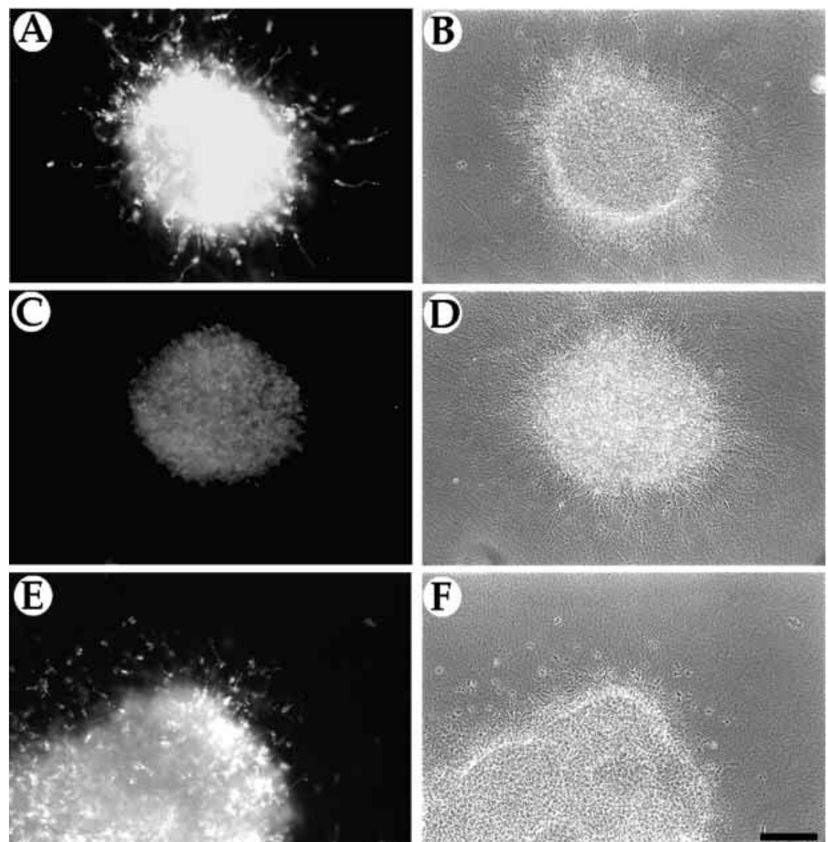

Fig. 2. Oligodendrocyte precursors develop in response to Shh in dorsal spinal cord from stage 24 embryos. (A,C,E) O4 labeling; (B,D,F) the corresponding phase images. In the majority of stage 24 ventral spinal cord explants (A,B), many O4+ oligodendrocyte precursors (A) are present after 6 days in vitro. By contrast in the majority of stage 24 dorsal spinal cord explants (C,D) there are no oligodendrocyte precursors (C) and only occasional O4+ cells were seen in any dorsal explant. In the presence of 2.5 nM Shh, (E,F), the majority of stage 24 dorsal explants contained large numbers of oligodendrocyte precursors (E). Bar, 100 µm.

Dorsal spinal cord developed oligodendrocytes in response to Shh for an defined developmental period

To determine the temporal regulation of Shh-induced oligodendrogenesis in dorsal spinal cord explants, spinal cord explants from stage 13, 15 and 24 embryos were incubated with 2.5 nM Shh and the appearance of O4+ oligodendrocyte precursors in dorsal and ventral explants compared. Approximately 50% (8/16) of stage 13 dorsal spinal cord explants contained oligodendrocyte precursors after 6 days in the presence of 2.5 nM Shh while none (0/15) contained oligodendrocyte precursors in the absence of Shh. Similarly, 33% (4/12) of stage 15 dorsal spinal cord explants contained oligodendrocyte precursors in the presence of Shh while there were none in controls (0/10). In both cases greater than 80% (13/15) of ventral explants at both ages contained O4+ cells. The first appearance of oligodendrocyte precursors in all cases occurred after approximately 4-5 days in vitro. The majority of ventral explants derived from stage 24 embryos contained O4+ cells after 6 days in vitro (Fig. 2A,B) while the majority of dorsal explants did not (Fig 2C,D) although occasional O4+ cells were found in some (21/79) dorsal explants. In the presence of 2.5 nM Shh, however, O4+ cells appeared after 2 days, and by 6 days the proportion of explants containing O4+ cells increased to greater than 90% (58/64). The number of O4+ cells in each explant also increased dramatically (Figs 2E,F, 3).

The ability of spinal cord cultures to develop oligodendrocytes in response to Shh was lost by stage 36. Oligodendrocyte lineage cells that normally develop in stage 36 cultures were eliminated by O4 mediated complement lysis (Ono et al., 1995) and the remaining cells grown in the presence of 2.5 nM Shh for 6 days. While large numbers of O4+ cells were present in complement-only treated control cultures, no O4+ cells developed in cultures treated with mAb O4 and complement in either the presence or absence of Shh (n=6). These data suggest there is a defined developmental interval in which dorsal spinal cord is competent to give rise to oligodendrocytes in response to Shh.

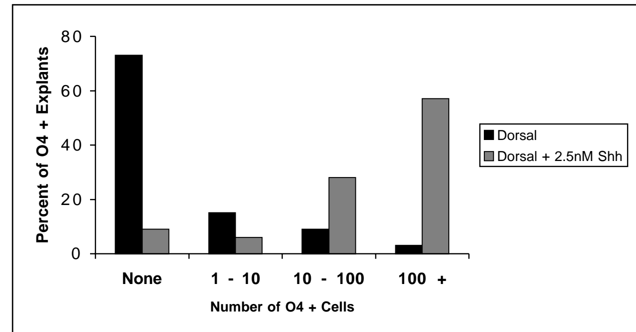

Fig. 3. Quantitation of the induction of oligodendrocyte precursors by 2.5 nM Shh in stage 24 dorsal spinal cord explants. In the absence of Shh, 73% (58/79) of explants contained no oligodendrocyte precursors, and less than 1% contained more than 100 oligodendrocyte precursors. By contrast in the presence of Shh greater than 90% of explants contained some O4+ cells and more than 55% of the explants contained greater than 100+ cells. The data represent the proportion of explants within each category taken from a total of 79 control and 64 experimental explants generated from 4 different preparations.

Shh was not required for O4+ oligodendrocyte precursor proliferation

The increase in oligodendrocyte progenitors in dorsal spinal cord cultures exposed to Shh could result from enhanced cell proliferation. To determine whether Shh was required for oligodendrocyte precursor proliferation, cultures of stages 26

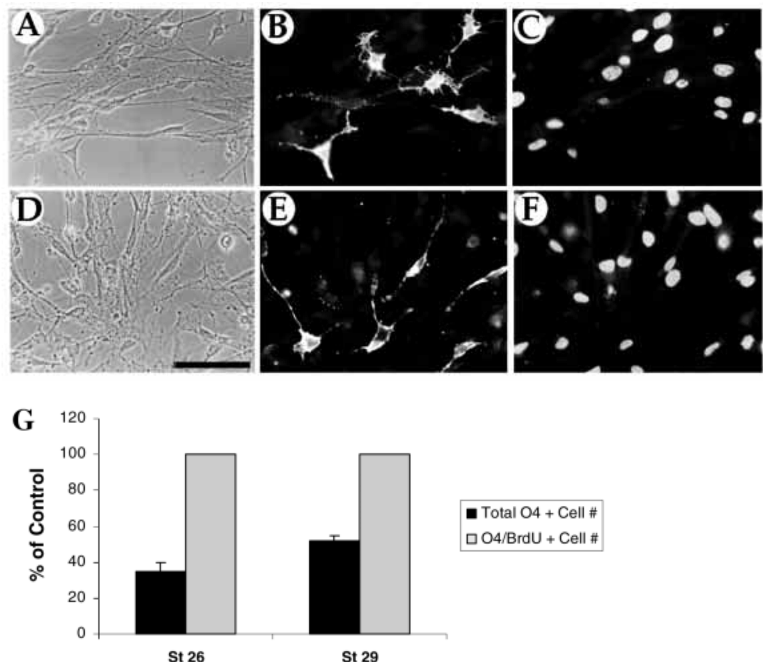

Fig. 4. Sonic hedgehog signaling is not required for the proliferation of chick spinal cord oligodendrocyte precursors. Dissociated cell cultures of stage 26 and 29 chick spinal cord were grown in the absence or presence of anti-Shh antibody for 3 days and labeled with BrdU for the last 18 hours. The proportion of BrdU labeled oligodendrocyte precursors and the total number of oligodendrocyte precursors that developed were compared. In stage 29 cultures in both the presence (A-C) and the absence of anti-Shh (D-F), similar proportions of O4+ cells (B,E) were BrdU+ (C,F), suggesting Shh is not required for oligodendrocyte proliferation, although the presence of the antibody appeared to induce a more process bearing morphology in O4+ cells. G. Quantitation of the total number and proportion of proliferating oligodendrocyte precursors that develop in stage 26 and 29 chick spinal cord cultures in the presence and absence of anti-Shh. After 3 days in vitro in the presence of anti-Shh the number of O4+ cells in stage 26 cultures was reduced to approximately 35% that of controls although the proportion of BrdU labeled cells was not different (65/64%). In stage 29 cultures in the presence of anti-Shh, the total number of O4+ cells was reduced to approximately 50% that of controls although the proportion of BrdU labeled cells was not different (60/60%). For total cell counts, the data represent the mean ± standard deviation of cells from 2 coverslips from at least 3 different preparations (n=6) while for the proportion of proliferating cells the data were pooled and the experimental and control means compared. Bar, 50 μm.

and 29 chick spinal cords were grown in the presence of neutralizing anti-Shh for 3 days. The total number of O4+ oligodendrocyte precursors as well as the proportion of BrdU+/O4+ cells, was compared with controls. The total number of O4+ cells that developed was reduced to approximately 35% in cultures of stage 26 spinal cord grown in the presence of anti-Shh compared to controls, although the proportion of O4+ cells that incorporated BrdU was not significantly different from control (control 65%: experimental 64%) (Fig.4). Similar results were obtained in cultures derived from older explants. For example, in stage 29 cultures, after 3 days in vitro the total number of O4+ cells was approximately 50% that of controls while the proportion of cells that had incorporated BrdU were not significantly different from controls (Both 60%) (Fig.4). These data indicated that inhibiting Shh signaling did not prevent the proliferation of oligodendrocyte precursors and suggest that the increase in number of O4+ cells in the presence of the ligand reflected increased induction or survival of progenitor cells rather than proliferation of existing oligodendrocyte precursor cells.

Shh was present and necessary during normal development of spinal cord oligodendrocytes

The reduction in the number of oligodendrocyte precursors in spinal cord cultures exposed to anti-Shh suggests Shh is required for the normal appearance of spinal cord oligodendrocytes. Thus, Shh should be present in the ventral spinal cord prior to the time oligodendrocyte precursors first develop. The monoclonal antibody (mAb) 5E1 specifically recognizes Shh and blocks its function (Ericson et al., 1996). In embryos up to stage 24 anti-Shh antibody (5E1) labeled the notochord and floor plate. In older animals, at stages 24-26, cells in the dorsal region of the floor plate were strongly immunoreactive to 5E1 (Fig. 5A) as were adjacent regions of the ventral midline axon tracts. Lower levels of Shh remained detectable in ventral spinal cord until stage 34.

To determine whether Shh was necessary for oligodendrocyte development, Shh signaling was blocked by neutralizing anti-Shh antibody. Hybridoma cells secreting anti-Shh (Ericson et al., 1996) were added to the embryonic chorioallantoic membrane at stage 17 and animals allowed to develop until stage 33. The distribution of neutralizing antibody was detected by labeling sections of experimental animals with HRP-anti-mouse Ig (Fig. 5B). The distribution of neutralizing antibody was more widespread than the pattern of labeling seen on fixed tissue with 5E1 at younger ages. Neutralizing antibody was detected in cells at the periphery of the floor plate and in presumptive ventral white matter tracts (Fig. 5B). From 13 experimental animals that had detectable anti-Shh antibody all had dramatically lower numbers of O4+ cells in the spinal cord (Fig. 5D,F) compared to animals that

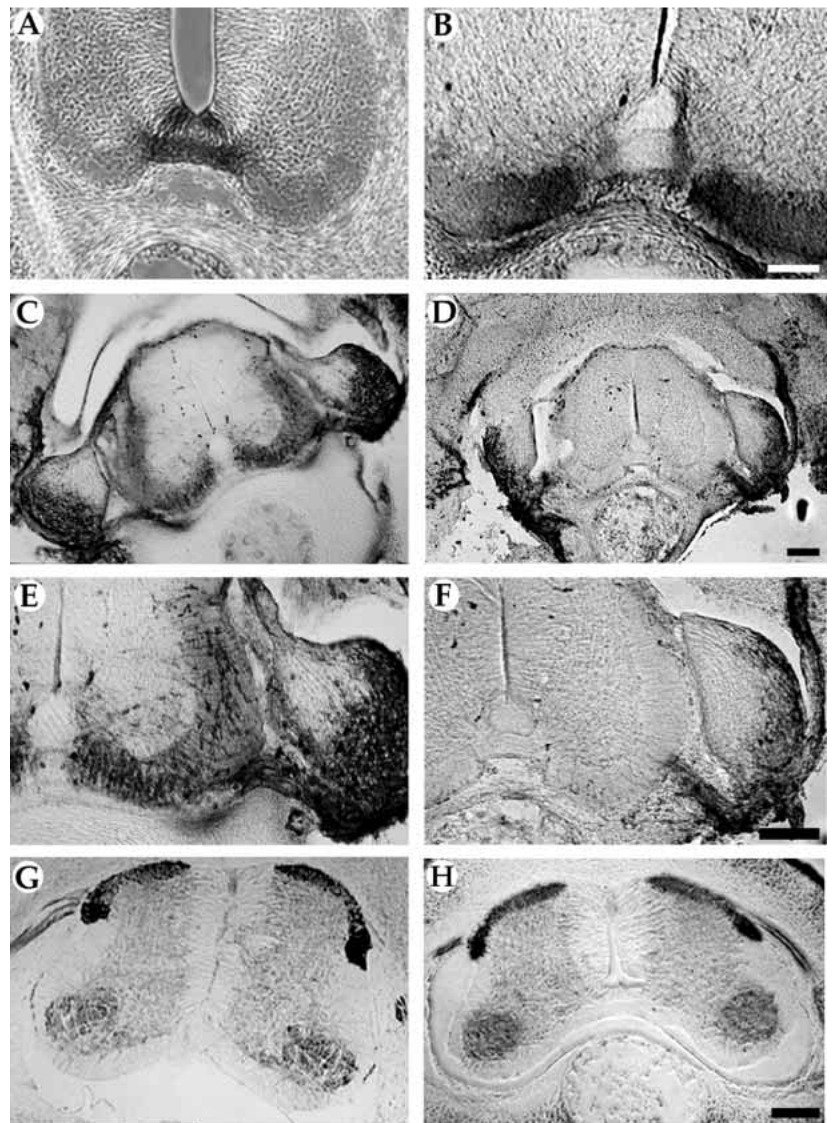

Fig. 5. Sonic hedgehog is present in the correct location, at the appropriate time and is required for the development of chick spinal cord oligodendrocyte precursors. (A) Labeling of transverse sections of stage 24 embryos with the anti-Shh antibody 5E1, demonstrates discrete localization of Shh in the floor plate and ventral midline white matter. (B) Labeling of spinal cord sections from experimental animals at stage 33 with HRP-conjugated second antibody alone reveals the distribution of anti-Shh antibody derived from hybridoma cells grown on the embryonic chorioallantoic membrane. Compared to the distribution of Shh in these older animals, the second antibody was more readily detectable and appeared more diffuse. (C,E) In control animals that received irrelevant hybridoma cells at stage 17, O4+ oligodendrocyte precursors are distributed throughout the ventral white matter of the spinal cord at stage 33. The Schwann cells in the adjacent ventral roots and dorsal root ganglia are also strongly labeled. (D,F) In experimental animals that received anti-Shh (5E1) hybridoma cells, oligodendrocytes and their precursors were completely absent from the spinal cord although O4+ Schwann cells were present in the adjoining ventral root and DRG. (G,H) Cholinesterase staining of control (G) and 5E1 exposed (H) spinal cords at stage 33 demonstrated that inhibiting Shh signaling from stage 17 did not dramatically disrupt the development of motor neurons even though the overall morphology of the spinal cord was altered. Bars: A and B, C and D E and F, 100 μm.

received control 7B11 (Szigeti and Miller, 1993) hybridoma cells (n=5) (Fig. 5C,E).

Specifically, in the presence of hybridoma cells secreting control (7B11) IgG the distribution of O4+ cells in the developing spinal cord was indistinguishable from normal. The first O4+ cells appeared in the ventral ventricular zone around stages 28-29 and by stage 32-34 these cells or their progeny were distributed in presumptive gray and white matter (Fig. 5C,E). In the presence of anti-Shh antibody, most regions of the spinal cord completely lacked O4+ cells (Fig. 5D,F). In some cases the inhibition of oligodendrocyte precursor development was less complete, but in all experimental animals (13/13) there was a dramatic reduction in the overall number of O4+ cells in the spinal cord.

The effects of anti-Shh antibody were cell-type specific. The development of O4+ Schwann cells in the ventral roots and dorsal root ganglia of the peripheral nervous system was not blocked by anti-Shh antibody (Fig. 5D,F). Furthermore, while addition of anti-Shh at stage 17 influenced spinal cord morphology, the general distribution of cholinesterase positive neurons (Roessmann and Friede, 1967; Navartnam and Lewis, 1970; Silver and Wolstencroft, 1971) was not dramatically affected by antibody treatment (Fig. 5G,H). These data suggest that spinal cord oligodendrogenesis may be directly dependent on Shh signaling.

The initial appearance of oligodendrocyte precursors is dependent on Shh

To accurately define when Shh was required for oligodendrocyte development, the number of oligodendrocyte precursors in spinal cord cultures derived from different ages was compared in the presence of control and anti-Shh neutralizing antibodies. In cultures derived from stage 20 embryos, addition of anti-Shh reduced the number of O4+ cells that developed after 3/4 days to approximately 10% of controls (Fig 6). Anti-Shh induced a similar reduction in O4+ cells in cultures up to stage 24 (Fig. 6). By contrast, in stage 26 cultures anti-Shh treatment reduced the number of O4+ cells by approximately 65% of control while at stage 29 there was a reduction of approximately 45% compared to controls (Fig. 6). By stage 31, anti-Shh treatment did not result in any significant reduction in the number of O4+ cells (Fig. 6). The antibody-induced inhibition of oligodendrocyte development was reversible and cell-type specific. Removal of the antibody from stage 26 cultures after 24 hours and addition of 5 nM Shh resulted in a recovery in the number of O4+ cells, while maintaining antibody exposure did not significantly affect the number of astrocytes or large neurofilament + neurons (Fig. 6). These data suggest there is a requirement for Shh signaling until stage 31 for the development of a full cohort of spinal cord oligodendrocytes.

To confirm the temporal requirements for Shh in the intact spinal cord, neutralizing antibody was added to embryos at stages 23, 25 and 27. Embryos were allowed to develop until stage 34 and the distribution of oligodendrocyte lineage cells was compared to age matched controls. In animals

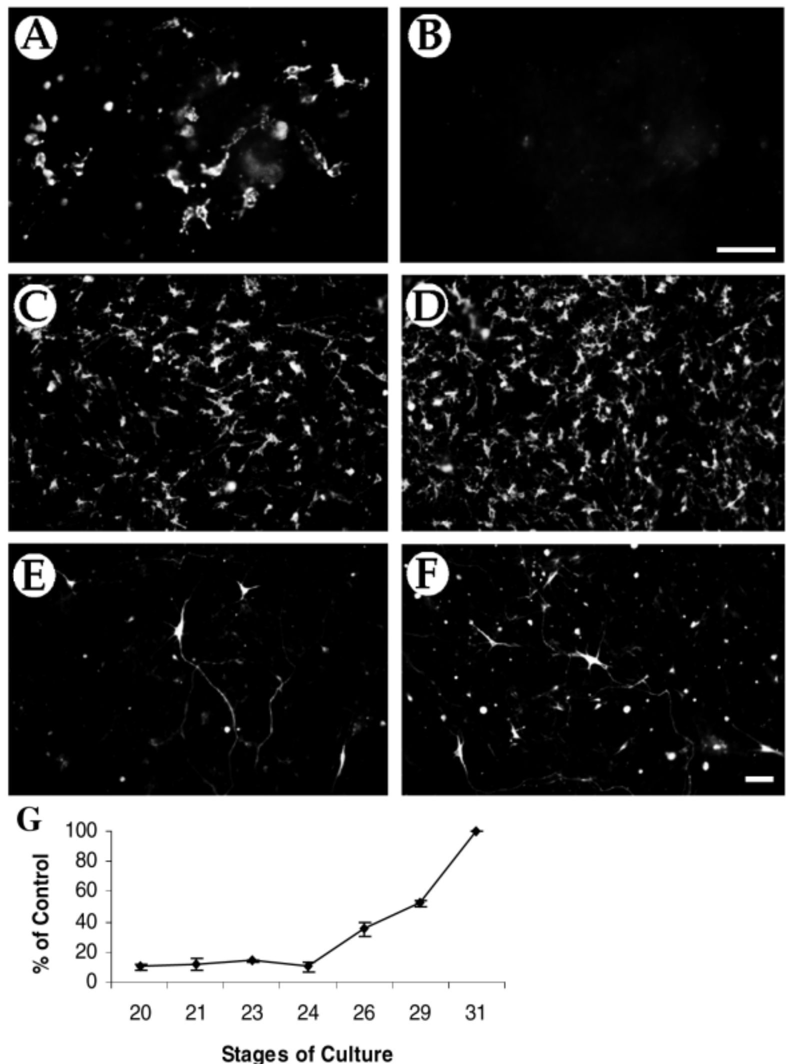

Fig. 6. Inhibition of Shh signaling blocks the appearance of oligodendrocyte precursors in a stage-dependent manner. The appearance of O4+ oligodendrocyte precursors in stage 26 spinal cord cultures after 3DIV is not affected by control (7B11) IgG (A), but is reduced by (5E1) anti-Shh IgG (B). In both cultures O4+ cells developed in clusters (A). The number of cells within a cluster was not dramatically affected by anti-Shh although the number of clusters was reduced. By contrast, the appearance of O4+ oligodendrocyte precursors in stage 31 spinal cord cultures after 3DIV is not affected by either control (7B11) IgG (C) or (5E1) anti-Shh IgG (D). (E,F) The appearance of NF+ large motor neurons in stage 26 spinal cord cultures after 3DIV is not dramatically affected by either control (7B11) IgG (E) or 5E1 anti-Shh antibody (F). (G) Quantitation of O4+ cell number. Parallel cultures of chick spinal cord cells were grown in the presence of control (7B11) or anti-Shh antibody and the number of O4+ cells that developed were determined at the equivalent of stage 36. Between stages 20-24 addition of anti-Shh reduced the number of O4+ cells to approximately 10% of controls. In stage 26 and stage 29 cultures addition of anti-Shh reduced the number of O4+ cells to approximately 35% and 50% of controls respectively. By stage 31 addition of anti-Shh had no effect of the number of O4+ cells compared to controls. The presence of control antibody had not effect on O4+ cell number compared to no-antibody controls. The data represents the mean ± s.d. from 3 randomly selected non-overlapping 20 fields from 3 coverslips taken from 2 separate experiments (n=6). Bars: A and B, C-F, 50 μm.

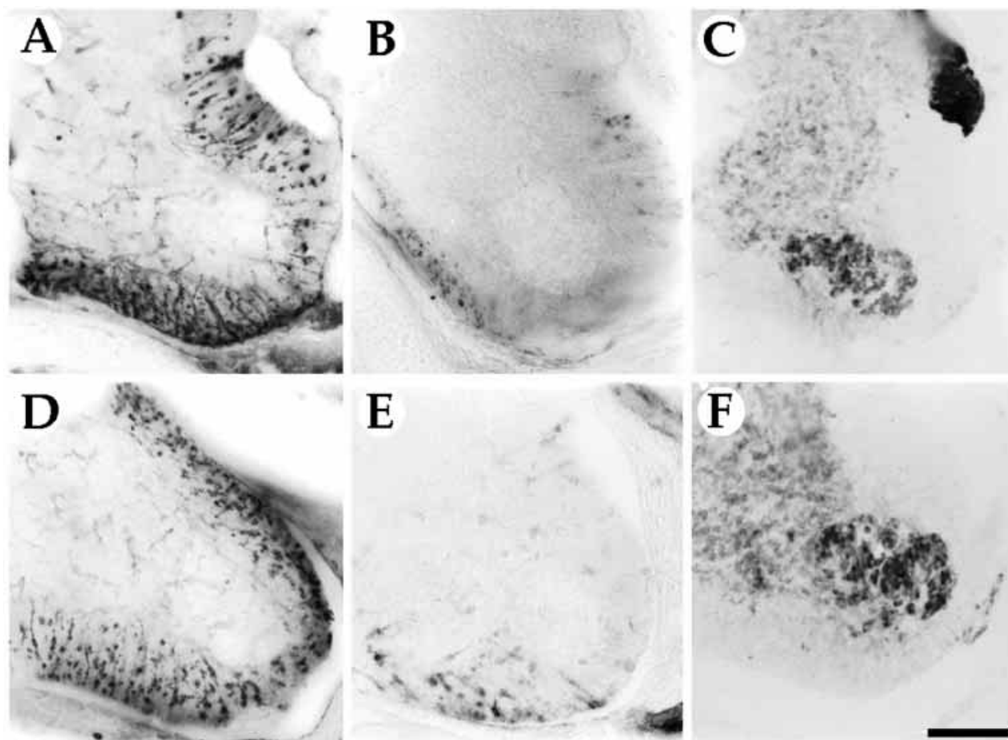

Fig. 7. Inhibiting Shh signaling in vivo at stage 23 (A-C) and 27 (D-F) blocks the emergence of spinal cord oligodendrocyte precursors but not motor neurons. (A) Embryos that received control antibody at stage 23 develop large numbers of O4+ oligodendrocytes in the spinal cord by stage 34. (B) Embryos that received anti-Shh antibody developed very few O4+ oligodendrocytes although an occasional O4+ cell was present in ventral and dorsal regions. (C) The pattern of motor neurons was not dramatically affected by the presence of anti-Shh. Similar results were seen in animals that received anti-Shh at stage 27. Control animals (D) had a normal distribution of oligodendrocyte precursors while animals treated with anti-Shh (E) demonstrated a dramatic reduction in the number of O4+ cells in the spinal cord. (**F**) As in the younger animals the pattern of motor neurons was not dramatically affected by anti-Shh. Bar, 100 μm.

that received antibody at stage 23 and retained detectable antibody at the time of death (*n*=3), few oligodendrocyte precursors were present in the spinal cord (Fig. 7A,B). Occasional O4+ cell bodies and processes were seen in dorsal and ventral white matter, while controls contained large numbers of O4+ cells. The number of oligodendrocyte precursors in animals exposed to anti-Shh at stage 25 (*n*=3) and 27 (*n*=6) was also significantly reduced from controls at stage 34 (Fig. 7B,E). The inhibition of cell development was restricted to oligodendrocytes. In all experimental animals, O4+ Schwann cells in the PNS were unaffected by the presence of the antibody. Furthermore, the total number of cholinesterase-positive neurons was not significantly affected in experimental animals (Fig. 7C,F). For example, in control animals there were approximately 30±8 cholinesterase reactive cells in the lateral motor columns. In animals exposed to antibody at stage 23 there were 34±10 and animals exposed to antibody at stage 27 contained 40±8 cholinesterase reactive cells in their lateral motor columns. These data are consistent with the in vitro studies and support the hypothesis that Shh signaling is required during the initial stages of spinal cord oligodendrogenesis.

Shh induction of oligodendrocyte precursors is temporally distinct from the induction of motor neurons

The finding that blocking Shh inhibits the development of oligodendrocyte precursors without significantly affecting the development of cholinesterase-positive neurons raises the possibility that the induction of oligodendrocyte precursors by Shh occurs after the induction of motor neurons. To compare the induction of oligodendrocytes and motor neurons by Shh, stage 12 and stage 24 dorsal explants were incubated with different concentrations of Shh and the development of motor neurons and oligodendrocyte precursors assayed at the equivalent of stage 36.

At both ages, in positive control assays, the majority of ventral spinal cord explants contained both motor neurons and oligodendrocytes.

In dorsal explants from stage 12 animals, greater than 90% of the explants contained no O4+ cells, while approximately 55% contained relatively small numbers of Isl+ motor neurons presumably derived from dorsal regions such as the column of Terni (Shanthini and Jessell, 1998) (Fig. 8A,B). Addition of Shh at concentrations below 0.6 nM did not induce any oligodendrocytes or additional motor neurons while higher concentrations induced both cell types (Fig. 8A,B) in none-overlapping populations (Fig. 9). The number of oligodendrocytes and motor neurons that developed were proportional to the concentration of Shh (Fig. 9). For example, in 0.6 nM Shh, 35% (5/14) explants contained up to ten O4+ cells and no explants contained more than ten O4+ cells (Fig. 8A). In 5 nM Shh 46% (6/13) explants contained up to ten O4+ cells and greater than 30% of explants contained greater than 10+ cells/explant (Fig. 8A). Similarly the number of Isl+ cells increased, depending on the concentration of Shh such that although less than 10% of explants contained between 10-100 O4+ cells at concentrations of Shh below 0.6 nM, greater than 43% of explants contained between 10-100 motor neurons in the presence of 5 nM Shh (Fig. 8B).

In dorsal explants from stage 24 animals a similar induction of cell types was observed. Both cell types were induced by concentrations of Shh above 0.6 nM (Fig. 8). Compared to younger explants, however, there was a dramatic enhancement in the induction of oligodendrocytes and a reduction in the number of motor neurons induced. For example, while 0.6 nM Shh generated no explants that contained greater than ten O4+ cell at stage 12, at stage 24, 39% (9/32) of the explants

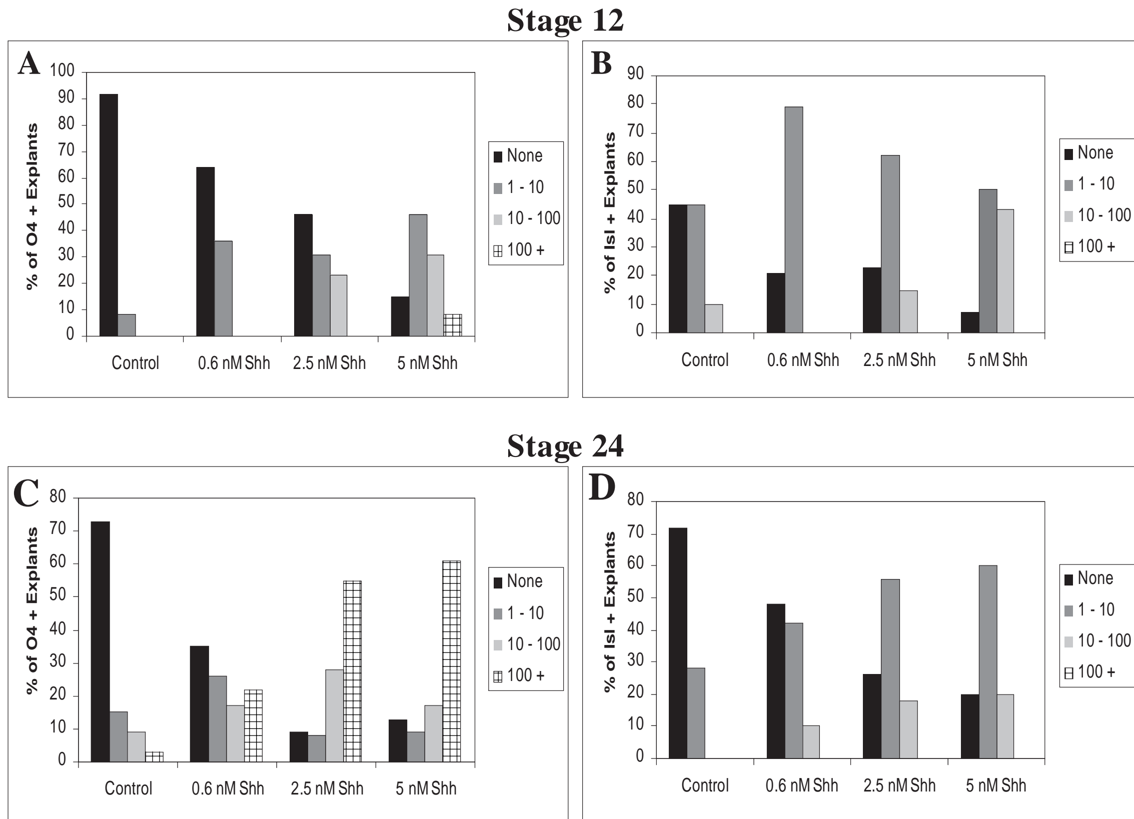

Fig. 8. The relative number of oligodendrocytes or motor neurons that develop in response to Shh is dose and age dependent. Addition of increasing concentrations of Shh to dorsal explants derived from stage 12 (A,B) and stage 24 (C,D) embryos resulted in increasing numbers of oligodendrocyte lineage cell (A,C) and Isl+ motor neurons (B,D). Note, that in stage 12-derived explants the increase in the number of oligodendrocytes (A) is relatively modest with less than 10% of explants (1/13) containing greater than 100 O4+ cells. By contrast in stage 24-derived explants (C) greater than 60% (14/23) explants contained more than 100 O4+ cells. The number of motor neurons was reduced in stage 24 (D) compared to stage 12 (B) explants. The data represent the proportion of explants in each category taken from a total of at least 13 different explants pooled from three different preparations.

contained more than ten 04+ cells and 22% (5/23) explants contained greater than 100 O4+ cells. Increasing concentrations of Shh resulted in increasing numbers of oligodendrocytes such that in 5 nM Shh more than 60% (14/23) explants contained more than 100 O4+ cells (compare Fig. 8A and C). Fewer motor neurons were induced in stage 24 explants than in stage 12 explants, especially at the higher concentrations of Shh. For example, 43% of stage 12 explants (6/14) but only 20% (4/19) of stage 25 explants contained more than 100 Isl+ cells in 5 nM (compare Fig. 8B and D). The induction of motor neurons and oligodendrocytes was closely linked. At both ages, a small number of explants contained motor neurons but no oligodendrocytes, however, greater than 98% of explants that contained oligodendrocytes also contained motor neurons. These data suggest that while similar concentrations of Shh induce both oligodendrocytes and motor neurons, the relative expansion of the different populations of cells depends on the age of the responding tissue.

DISCUSSION

In the developing chick spinal cord, oligodendrocytes initially arise in the ventral ventricular zone dorsal to the floor plate (Pringle and Richardson, 1993; Yu et al., 1994; Ono et al., 1995; Timsit et al., 1995). This localized origin depends in part on early influences from the notochord (Trousse et al., 1995; Orentas and Miller, 1996; Pringle et al., 1996). In vitro, the inductive activity of the notochord on dorsal spinal cord explants can be replaced by Shh (Poncet et al., 1996; Pringle et al., 1996) suggesting that Shh may contribute to the appearance of spinal cord oligodendrocyte precursors. Here we demonstrate that relatively low levels of Shh rapidly induce oligodendrocyte precursors in chick dorsal spinal cord cultures, suggesting Shh is required during the appearance of the oligodendrocyte lineage. Consistent with this hypothesis, in vivo Shh is specifically localized in regions of the spinal cord where oligodendrocyte precursors first arise prior to and during their appearance. Furthermore, inhibiting Shh signaling at stages prior to the appearance of oligodendrocyte precursors blocks their development both in vivo and in vitro, although it is not required for oligodendrocyte precursor proliferation. Similar concentrations of Shh induce oligodendrocytes and motor neurons although the relative expansion of the different cell populations depends on the age of the responding tissue. In young tissue, motor neurons and not oligodendrocytes are preferentially expanded while in older tissue oligodendrocytes and not motor neurons are preferentially expanded.

Several lines of evidence suggest that the development of

motor neurons and oligodendrocytes are closely linked. For example, both cell types arise in similar regions of the neural tube (Noll and Miller, 1993; Pringle and Richardson, 1993; Timsit et al., 1995; Pringle et al., 1996; Sun et al., 1998) and both cell types can be induced in dorsal explants by Shh (Poncet et al., 1996; Pringle et al., 1996) at similar concentrations (Pringle et al., 1996). Based on these observations it has been proposed that the role of Shh may be to induce a common motor neuron/oligodendrocyte precursor (Pringle et al., 1996; Richardson et al., 1997; Sun et al., 1998). Several additional lines of evidence support this hypothesis. Retroviral cell lineage studies demonstrated clones containing both motor neurons and glia, consistent with a lineage association between motor neurons and oligodendrocytes in the chick spinal cord (Leber et al., 1990; Leber and Sanes, 1991). Recent studies demonstrate changes in the location of oligodendrocyte precursors and in the timing of development of both motor neurons and oligodendrocytes in the *small eye* (Pax6-knockout) mutant (Sun et al., 1998), suggesting Pax6 dependent alteration in the location of the common precursor cell in the ventricular zone. In the current study, the finding that in virtually all dorsal explants that develop oligodendrocytes in response to Shh also contain motor neurons, provide additional support for the concept that motor neurons and oligodendrocytes are derived from a common precursor.

The mechanisms regulating the timing of oligodendrocyte development in the spinal cord are not well understood. During normal development, the majority of motor neurons are generated before the appearance of oligodendrocytes (Nornes and Das, 1974; Altman and Bayer, 1984; Pringle and Richardson, 1993; Ono et al., 1995). It is currently unclear why, if both cells are derived from a common precursor, they are not generated at the same time. Sonic hedgehog is expressed by the notochord and floor plate during early development (Marti et al., 1995) (Roelink et al., 1994) several days before the first appearance of oligodendrocytes. One hypothesis is that an intrinsic developmental program in the common progenitor cell regulates the fate of the progeny (Sun et al., 1998). Thus, motor neurons are preferentially generated early in the lineage while oligodendrocytes are preferentially generated later in the lineage. An alternative model is that environmental signals combine with Shh to dictate the fate of the progeny and that these signals are temporally regulated. For example, motor neurons might provide a feedback loop that stimulates production of oligodendrocytes from the precursor. This simple lineage restricted model seems unlikely since oligodendrocytes develop in vitro in the absence of motor neurons (Sun et al., 1998). Signals from other neural cell types developing in the spinal cord may, however, provide signals that promote oligodendrocyte precursor expansion in a developmentally regulated manner. Consistent with this model, exposure of older dorsal explants to Shh generated greater numbers of oligodendrocytes than at younger ages but still generated some motor neurons. Likewise, in the developing chick spinal cord while most lateral motor neurons are generated between days 3 and 4, prior to the appearance of oligodendrocyte precursors, motor neurons in the medial region of the anterior horn are still being generated between days 5 and 6, coincident with oligodendrocyte precursors (Langman and Haden, 1970). These observations do not, however, rule out the alternative possibility of cellular heterogeneity within a common progenitor population.

Potential candidate molecules that might influence the early development of oligodendrocytes include members of the neuregulin family (Falls et al., 1993; Marchionni et al., 1993) which play important roles during later oligodendrocyte development (Vartanian et al., 1994; Canoll et al., 1996). Our preliminary studies suggest neuregulins are expressed in the ventral ventricular zone around the time that oligodendrocytes first develop and that they play a critical role in early development of spinal cord oligodendrocyte precursors (Vartanian et al., 1999). Clearly, if signals in addition to Shh are required for oligodendrocyte precursor appearance, they are not restricted to the ventral spinal cord since addition of Shh alone is sufficient to induce oligodendrocytes in dorsal spinal cord cultures.

The precise role of Shh in the specification of spinal cord

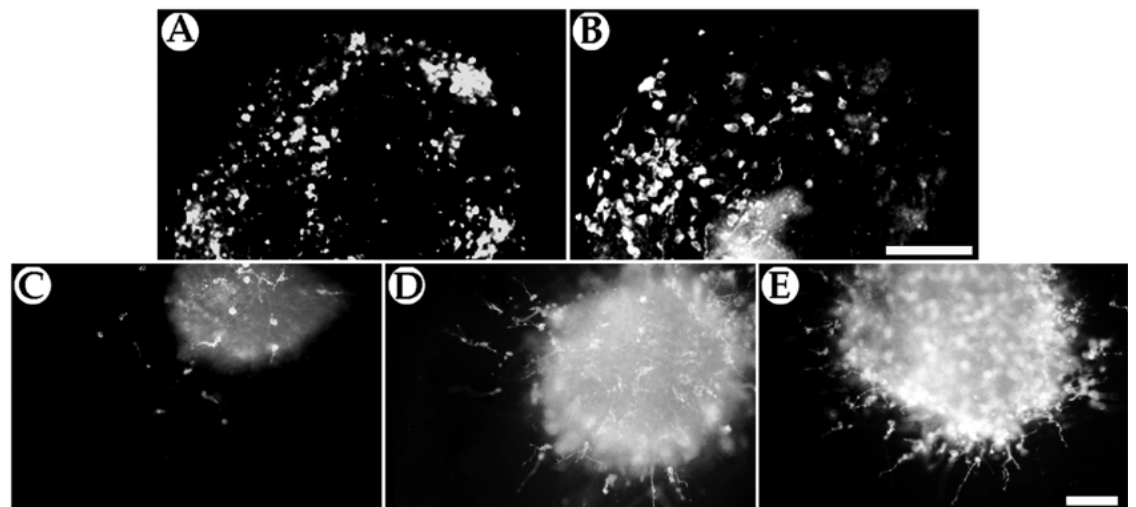

Fig. 9. Sonic hedgehog induced Isl-1/2 motor neurons (A) and O4+ oligodendrocytes (B) develop in non-overlapping cell populations in stage 12 dorsal spinal cord explants. The explant was supplemented with 5 nM Shh and labeled with mAbs 4D5 (A) to identify motor neurons and O4 (B) to identify oligodendrocyte lineage cells at the equivalent of stage 36. (C-E) The number of O4+ oligodendrocytes that develop in stage 24 chick dorsal spinal cord explants increases with the concentration of Shh: C, 0.6 nM; D, 2.5 nM; E, 5 nM.. Bars: A and B, C-E, 100 µm.

oligodendrocytes is unclear. The finding that Shh is required during the appearance of oligodendrocyte precursors argues strongly against it simply inducing a progenitor cell early in development that subsequently generates oligodendrocytes several days later in a Shh-independent fashion. Likewise, while Shh has been suggested to be a mitogen for neuronal precursors in vitro (Kalyani et al., 1998), our data indicate that it is not required for the proliferation of committed oligodendrocyte progenitors. Alternatively, Shh may be required for the survival of early precursors. Such a mechanism has been suggested in sclerotome formation where Shh supports the expression of Pax-1+ sclerotomal cells in the ventral somite (Fan and Tessier-Lavigne, 1994; Johnson et al., 1994; Fan et al., 1995; Munsterberg et al., 1995). In mice lacking Shh, however, low levels of sclerotome Pax-1 gene expression initially develop but were not sustained (Chiang et al., 1996), suggesting Shh is required for sclerotome survival. Finally, Shh may be required for the commitment of the early common MN-Ol progenitor and that these cells are continually recruited until a cohort of oligodendrocyte precursors have been generated, the cellular source exhausted, or the local concentration of Shh falls below the required threshold. Whatever the mechanism, the emergence of spinal cord oligodendrocyte precursors appears to be dependent on the expression of Shh during their initial appearance.

Oligodendrocyte precursors develop in more rostral regions of the CNS than the spinal cord (Pringle and Richardson, 1993; Ono et al., 1997a,b) and the cellular and molecular bases of their induction are unknown. While the notochord is capable of inducing oligodendrocytes in explants of dorsal spinal cord, it seems likely that during normal development the floor plate and not the notochord acts as the local source of spinal cord Shh (Ericson et al., 1996). For example, Shh is expressed by floorplate cells around the time that oligodendrocytes appear, and development of spinal cord oligodendrocytes becomes independent of the notochord several days before the earliest precursors are detectable (Warf et al., 1991; Fok-Seang and Miller, 1994; Orentas and Miller, 1996). Other regions of the CNS that lack an associated floor plate (Pringle and Richardson, 1993; Cameron-Currey and LeDouarin, 1995; Timsit et al., 1995; Ono et al., 1997a,b) have also been proposed to generate oligodendrocytes. Whether oligodendrocyte development in these regions is dependent on local signaling by Shh remains to be determined. Sonic hedgehog is widely distributed in specific populations of neurons throughout the CNS (Bitgood and McMahon, 1996) and is required for development of ventral forebrain neurons (Ericson et al., 1995). It is an attractive hypothesis that similar molecular mechanisms mediate the early development of oligodendrocyte precursors in all regions of the vertebrate CNS.

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