Regulation of oligodendrocyte differentiation: a role for retinoic acid in the spinal cord

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SUMMARY

During development, oligodendrocyte precursors undergo sequential stages of differentiation characterized by expression of distinct cell surface properties and proliferative responses. Although both PDGF and bFGF are mitogenic for these cells, the factors that regulate the progression of oligodendrocyte precursors through their differentiative program remain unclear.

One factor present in the embryonic spinal cord that may regulate differentiation of oligodendrocyte precursors is retinoic acid. Here we show that retinoic acid inhibits the maturation of embryonic spinal cord oligodendrocyte precursors in vitro at an early, highly motile stage of differentiation, characterized by the expression of A2B5 immunoreactivity. Basic FGF acts both as a mitogen and an inhibitor of spinal cord oligodendrocyte precursor maturation, but at a significantly later stage of differentiation, characterized by the expression of O4 immunoreactivity.

In the presence of RA both the mitogenic and differentiation inhibiting effects of bFGF are abolished, consistent with RA acting as an early regulator of oligodendrocyte differentiation. During embryonic development, oligodendrocyte precursors arise initially from a distinct group of cells at the ventral ventricular zone of the spinal cord. Myelination of the entire spinal cord is dependent on the migration of immature precursor cells to peripheral developing white matter. Since the embryonic spinal cord has the capacity to release relatively high levels of retinoids, we propose that RA inhibits oligodendrocyte differentiation during early embryonic development permitting their dispersal throughout the entire spinal cord.

Key words: oligodendrocyte, retinoic acid, spinal cord, PDGF, bFGF, mitogen

INTRODUCTION

Myelin, the fatty insulation surrounding vertebrate CNS axons is produced by a specific class of macroglial cells termed oligodendrocytes (Peters et al., 1990). During development, the proliferation and differentiation of oligodendrocytes from cells of the neural tube is closely regulated such that in any particular region of the CNS, mature oligodendrocytes appear at a specific stage of development. For example, in rat optic nerve such cells first appear around the day of birth (Skoff et al., 1976a,b), while in rat spinal cord they appear around embryonic day 17 (E17) (Warf et al., 1991).

During their cellular maturation, oligodendrocyte precursors go through a series of discrete differentiative steps which can be identified by the expression of particular cell surface epitopes and proliferative responses (Raff, 1989; Gard and Pfeiffer, 1990). In neonatal rat optic nerve cultures, oligodendrocytes develop from bipotential progenitor cells termed O-2A progenitors (Raff, 1989). These cells constitutively give rise to oligodendrocytes, but can be induced to differentiate in vitro into a distinct class of astrocytes by exposure to CNTF and basal lamina components (Hughes et al., 1988; Lillien et al., 1990). Initially, O-2A progenitors express tetrasialogangliosides recognized by the A2B5 antibody (Fredman et al., 1984; Eisenbarth et al., 1979). Subsequently, oligodendrocyte precursors begin to express antigens recognized by the monoclonal antibody O4 (Sommer and Schachner, 1981), which appears to bind to sulfatide, seminolipid and an unidentified antigen (Sommer and Schachner, 1981; Bansal et al., 1989). This intermediate stage of development is maintained for 2-3 days (Gard and Pfeiffer, 1990) before the expression of a third marker, galactocerebroside (GC) (Ranscht et al., 1982) a major myelin glycolipid. Anti-GC antibodies can be used to identify specifically oligodendrocytes as they mature in vitro (Raff et al., 1978).

Oligodendrocyte precursors proliferate in response to a number of different mitogens at particular stages of their differentation. A2B5-immunoreactive oligodendrocyte precursors proliferate in response to mitogens present in both astrocyte and neuronal conditioned medium (Gard and Pfeiffer, 1990), while O4-immunoreactive oligodendrocyte precursors proliferate in response to mitogens found only in neuronal conditioned medium (Gard and Pfeiffer, 1990). Furthermore, A2B5-immunoreactive precursors proliferate in response to...
platelet-derived growth factor (PDGF) (Richardson et al., 1988; Raff et al., 1988; Fok-Seang and Miller, 1993). In spinal cord cultures, however, this proliferative response is lost as cells acquire O4 immunoreactivity (Fok-Seang and Miller, 1993). In contrast, basic fibroblast growth factor (bFGF) promotes proliferation in both A2B5- and O4-immunoreactive precursors (Bogler et al., 1990; McKinnon et al., 1990; Fok-Seang and Miller, 1993). These observations suggest that the proliferative response of oligodendrocyte precursors is altered during the maturation step characterized by the acquisition of O4 immunoreactivity (Gard and Pfierrer, 1990; Fok-Seang and Miller, 1993).

In late embryonic and early neonatal animals, oligodendrocyte precursors intrinsically possess all the information required for their subsequent differentiation. For example, the timing of oligodendrocyte development does not appear to depend on positional information in the intact CNS. Oligodendrocytes develop GC immunoreactivity on the same schedule in high density dissociated cell cultures derived from either brain or spinal cord as they do in the intact tissue (Abney et al., 1981; Williams et al., 1985; Warf et al., 1991). In addition, oligodendrocyte development does not appear to require the presence of other neural cell types. Single oligodendrocyte precursor cells grown in clonal isolation retain the capacity to differentiate into GC+ cells (Temple and Raff, 1985, 1986).

Although all the information necessary for oligodendrocyte differentiation to a GC+ state is present in the precursor population, the temporal progression through the different stages of maturation appears to be influenced by environmental factors. For example, while in high density spinal cord cultures oligodendrocyte precursors in vitro undergo differentiation according to the in vivo schedule, in the absence of sufficient mitogen (PDGF), oligodendrocyte precursors will differentiate prematurely (Raff et al., 1988). Furthermore, when oligodendrocyte precursors are grown in the presence of both PDGF and bFGF, precursor proliferation is stimulated, while their differentiation is delayed at a maturation stage prior to the acquisition of GC immunoreactivity (Bogler et al., 1990; McKinnon et al., 1990).

Oligodendrocyte precursors are strikingly motile (La Chapelle et al., 1984; Noble et al., 1988) and may be influenced by several different tissue environments during their early differentiation (Small et al., 1987; Curtis et al., 1988; Hardy and Reynolds, 1991). The migrational behavior of oligodendrocyte precursors in vitro is more pronounced during the early stages of their differentiation (Pfeffer et al., 1993; Warrington et al., 1993). Thus, while A2B5 and O4-immunoreactive oligodendrocyte precursors are highly motile in vitro (Temple and Raff, 1986), this motile behavior is significantly reduced both in vivo and in vitro as the cells acquire GC immunoreactivity (Curtis et al., 1988). During development of the rat optic nerve, oligodendrocyte precursors appear to originate in the brain and subsequently migrate into the nerve (Small et al., 1987). In the rat spinal cord, however, oligodendrocyte precursors appear to originate from a discrete cluster of cells at the ventral ventricular zone dorsal to the ventral midline (Warf et al., 1991; Pringle and Richardson, 1993; Noll and Miller, 1993) and subsequently migrate dorsally and laterally to the developing white matter (Warf et al., 1991; Noll and Miller, 1993). This ventral origin of oligodendrocyte precursors in the embryonic spinal cord is in close proximity to the notochord and floorplate, a group of specialized cells important in the morphogenetic development of the spinal cord (Van Straaten et al., 1989; Jessel and Dodd, 1992). Floorplate cells provide positional information involved in the control of cell patterning in the vertebrate central nervous system (Tessier-Levigne et al., 1988; Yamada et al., 1991; Ruiz i Altaba and Jessell, 1991) and have the capacity to synthesize relatively high levels of retinoic acid (RA) and a precursor for the related retinoid 3,4-dihydroretinoic acid (Wagner et al., 1990). Other regions of the embryonic spinal cord also contain higher levels of retinoids than more anterior regions of the CNS (Wagner et al., 1993; Mendelsohn et al., 1991) suggesting that RA levels are elevated in embryonic spinal cord. We have examined the influence of RA on oligodendrocyte differentiation in vitro under conditions that allow the differentiation of oligodendrocyte precursors on the same schedule as that observed in vivo (Warf et al., 1991). We show that when embryonic spinal cord cultures are grown in the presence of exogenous retinoic acid, oligodendrocyte precursor cell maturation is inhibited early in their differentiation. Removal of exogenous retinoic acid allowed the precursors to progress through their normal stages of differentiation.

Since in developing spinal cord oligodendrocytes originate from a discrete region of the ventral cord, the successful myelination of the entire cord depends critically on the subsequent migration and differentiation of these cells. As the capacity for migration is closely correlated with an immature phenotype (Temple and Raff, 1986; Pfeffer et al., 1993), we propose that retinoic acid present at relatively high levels in the embryonic spinal cord inhibits oligodendrocyte differentiation until the precursor cells have migrated away from their localized origin and uniformly populated the entire spinal cord.

MATERIALS AND METHODS

Spinal cord dissociation and cell culture

Embryonic rat spinal cord cultures were prepared from the thoracic and lumbar regions of embryonic day (E) E14, E16 and E18 Sprague-Dawley rat embryos. After removal of the meninges, spinal cords were dissociated into single cells using trypsin and EDTA as previously described (Warf et al., 1991). Dissociated cells were resuspended in DMEM medium with 10% fetal bovine serum (FBS), plated onto 12 mm poly-L-lysine (0.1 mg/ml Sigma) coated coverslips at a density of 0.5×10^6 cells/coverslip for high density cultures. For low density cultures, only the ventral third of the spinal cord was used to enrich for oligodendrocyte precursors and the cells plated at a density of 0.5×10^3 cells/coverslip. Approximately 60% of cells in such cultures were oligodendrocyte precursors. All supplements were added to cultures at the time of plating. After 12 hours to allow cell attachment, cultures were switched to N2 medium (Bottenstein and Sato, 1979) containing 1% FBS and all supplements were replaced at that time. Each experiment consisted of sister cultures grown under five different conditions: (1) control without any supplements, (2) PDGF with 8 ng/ml PDGF, (human natural, AB heterodimer; #40113, Collaborative Biomedical Products), (3) PDGF and bFGF with 8 ng/ml PDGF+10 ng/ml bFGF, (bFGF-bovine pituitary; #40002, Collaborative Biomedical Products), (4) PDGF, bFGF and RA (8 ng PDGF/ml+10 ng bFGF/ml+1 μM all-trans retinoic acid/ml; (R)-2625, Sigma); and (5) PDGF and RA (8 ng PDGF/ml+1 μM all-trans retinoic acid). The concentrations of supplements were selected based on previous studies. For example, PDGF and bFGF concentrations are similar to those used...
in analysis of purified progenitor cells (Bogler et al., 1990; McKinnon et al., 1990) while RA concentrations are similar to those that cause anteroposterior transformations in the developing CNS (Durston et al., 1989). All supplements were replaced every day for the duration of the experiment and the complete medium was replaced every 2 or 3 days. Exogenous PDGF was added to all but control experiments to eliminate the possibility that any effects of RA were mediated indirectly through inhibiting the release of mitogen from other cells in the culture (Richardson et al., 1988; Yeh et al., 1991).

**Immunofluorescence labeling**

Cultures were fixed with 4% paraformaldehyde at 37°C for 3-5 minutes and rinsed briefly in DMEM. Antibody incubations were performed at room temperature for 30 minutes in DMEM with 50% normal goat serum (NGS) added. Sister cultures were incubated with monoclonal antibodies A2B5 (Eisenbarth et al., 1979; supernatant 1:1), O4 (Sommer and Schachner, 1981; supernatant 1:10) or anti-galactocerebroside (anti-GC, Ranscht et al., 1982; ascites fluid 1:200). After rinsing, cells were incubated for 30 minutes in either fluorescein-conjugated goat anti-mouse IgM, (for A2B5 and O4) or rhodamine-conjugated goat anti-mouse IgG (for anti-GC). Both secondary antibodies (Cappel) were diluted 1:50 in DMEM+50% NGS. Cultures were then rinsed and mounted in 80% glycerol/PBS with 5% propyl gallate added to prevent fluorochrome bleaching. In control cultures, deletion of the primary antibody or substitution with normal mouse serum resulted in the total absence of specific staining. Cells were viewed using a Nikon optiphot microscope equipped with epifluorescent illumination and photographed on Tri-X (400 ASA) film.

**Quantitative analysis**

To determine the number of immunoreactive cells that developed in each culture, the total number of labeled cells were counted in 10 consecutive fields under a ×20 objective using rhodamine or fluorescein filters. Counts were adjusted to reflect the area occupied by all cells on the coverslip. In all cases, the results represent the means ± s.d. of cell counts from at least three separate experiments. For each experiment, the cells in control and supplement containing cultures were derived from a single dissociation procedure, grown on similarly treated substrata under otherwise identical conditions and all supplements were added to individual cultures simultaneously to ensure consistency between cultures. Regardless of the initial age of the animals used to establish the culture, all quantitative analysis was performed on cultures at an equivalent age of the first day after birth (P1), and 4 days (P5) later in recovery experiments. Previous studies indicated that under these conditions reproducible numbers of oligodendrocytes developed from known numbers of spinal cord cells in culture and could be accurately quantified (Warf et al., 1991). Statistical comparison of cell numbers in the presence of the different culture supplements were made using an unpaired *t*-test.

**RESULTS**

Spinal cord oligodendrocyte precursors go through a series of maturational stages, which can be identified by the sequential expression of cell surface epitopes. Discrete developmental stages can be recognized by the antibodies A2B5 (detectable as early as E12), O4 (first detectable at E16) and anti-GC (first detectable at E17/18) in thoracolumbar rat spinal cord (Warf et al., 1991). This temporal sequence of oligodendrocyte maturation occurs on the same schedule both in vivo and in vitro, and, thus, it has been possible to examine the influence of a variety of putative cell regulatory factors such as PDGF, bFGF and retinoic acid on the differentiation of spinal cord oligodendrocytes in vitro.

**Inhibition of oligodendrocyte differentiation by RA**

To assess the influence of PDGF and retinoic acid on the differentiative program of spinal cord oligodendrocytes in vitro, cultures derived from E14 thoracic and lumbar spinal cord were grown either without supplements (control), in the presence of PDGF or in the presence of PDGF and RA. After 9 days in culture (the equivalent of P1), cultures were labeled with either A2B5, O4 or anti-GC antibodies and the number of immunoreactive cells in the culture determined.

In both control and PDGF-supplemented cultures, similar numbers of A2B5-, O4- and GC-immunoreactive cells were present (Fig. 1A), suggesting that addition of PDGF did not substantially alter the timing or extent of differentiation of spinal cord oligodendrocytes. By contrast, cultures supplemented with PDGF and RA contained virtually no O4- or GC-immunoreactive cells (Fig. 2), and the number of A2B5-immunoreactive cells was over two-fold that seen in control cultures (Fig. 1A). A number of explanations may account for the absence of O4+ and Gal-C+ cells and the increase in A2B5+ cells in PDGF- and RA-supplemented cultures. For example, supplement addition may (1) inhibit oligodendrocyte differentiation at an early A2B5*/O4*+/GC− stage, (2) permanently block the capacity of spinal cord cells to give rise to oligodendrocytes, (3) selectively kill oligodendrocyte precursors that have matured beyond an O4 stage, or, (4) induce dedifferentiation of O4+ or GC+ oligodendrocytes back to an A2B5+ stage.

To determine if PDGF- and RA-treated E14 spinal cord cultures retained the capacity to give rise to O4- and GC-immunoreactive cells, a recovery experiment was performed. Following labeling of the initial set of cultures at E14+9, all supplements were removed from a parallel set of experimental and control cultures by rinsing thoroughly and adding fresh non-supplemented medium. The cultures were then grown for four additional days (until E14+13) and labeled with A2B5, O4 and anti-GC antibodies. In control and previously PDGF-supplemented cultures, the number of A2B5-, O4- and GC-immunoreactive cells was approximately twice that seen at E14+9 (Fig. 3A), indicating both the normal differentiation and the continued generation of oligodendrocyte precursors. In cultures previously supplemented with both PDGF and RA, however, there was a substantial increase in the number of O4- and GC-immunoreactive cells (Fig. 4) and a concomitant decrease in the number of A2B5-immunoreactive cells (Fig. 3A compare with Fig. 1A). These observations demonstrate that E14 spinal cord cultures grown in the presence of PDGF and RA for 9 days retain the capacity to give rise to oligodendrocytes, even though such cells do not develop in the continuous presence of the supplements.

Two observations indicate that retinoic acid is the active factor in the inhibition of oligodendrocyte development. No O4- or CG-immunoreactive cells develop in E14 spinal cord cultures supplemented with only RA even when maintained in culture for 14 days (data not shown). In addition, spinal cord oligodendrocytes develop on schedule in the presence of PDGF alone (Warf et al., 1991 and see above). To eliminate the possibility that the effects of RA, were mediated indirectly through inhibiting the release of PDGF (a major mitogen for oligodendrocyte precursors) from other cell types in the culture.
(Richardson et al., 1988; Yeh et al., 1991), exogenous PDGF was added to all but control experiments to ensure a saturating level of mitogen.

The increase in A2B5+ cells observed in PDGF- and RA-supplemented medium at E14+9, along with the recovery of both O4+ and Gal-C+ populations in cultures previously treated with these supplements suggests that RA inhibits oligodendrocyte differentiation at an early stage in the differentiation program, prior to the expression of O4 immunoreactivity. It remained possible however, that either RA was selectively toxic for O4+ and GC+ cells, or that it induced cellular dedifferentiation such that precursors that had matured beyond the A2B5+/O4+ stage were forced to return to an A2B5+/O4+ state.

To assess whether RA was either selectively toxic for maturing oligodendrocyte precursors or had the capacity to induce dedifferentiation in these cells, spinal cord cultures were established from thoracolumbar spinal cord of embryonic rats at E16 and E18. At E16, a small proportion of spinal cord oligodendrocyte precursors have matured to an O4+ stage, but no cells have yet acquired GC immunoreactivity (Warf et al., 1991). Thus, if RA is selectively toxic to, or inducing dedifferentiation in, O4+ cells there should be no O4+ or GC+ cells in RA-supplemented E16 cultures. In E16 cultures after 7 days in vitro (the equivalent of P1), both control and PDGF-supplemented cultures contained large numbers of A2B5- and O4-immunoreactive cells as well as GC+ cells (Fig. 1). Cultures supplemented with PDGF and RA also contained large numbers of A2B5+ cells, however, the numbers of O4- and GC-immunoreactive cells were substantially reduced (Fig. 1B). In contrast to the E14-derived cultures, in which no O4- or GC-immunoreactive cells developed in the presence of RA, E16-derived cultures contained cells of both populations (Fig. 1B). In recovery experiments, following supplement removal, the number of O4+ and GC+ cells in the RA-supplemented cultures recovered to near control levels (Fig. 3B).

Because substantial populations of the O4+ cells and GC+ cells developed in the presence of RA in E16+7 cultures, RA is clearly neither selectively toxic to oligodendrocyte precursors which have matured to an O4-immunoreactive stage, nor is it inducing de-differentiation of O4-immunoreactive cells. Instead, RA appears to be inhibiting the differentiation of oligodendrocyte precursors at a pre-O4 stage.

To determine the effect of RA on GC+ cells, the differentiation of oligodendrocytes in the absence and presence of RA

**Fig. 1.** Quantitation of the initial effects of additives on oligodendrocyte differentiation. Graphs indicate the total numbers of cells at each stage of differentiation. A2B5+ cells represent the most immature oligodendrocytes, O4+ cells represent oligodendrocytes at an intermediate stage of maturation and GC+ cells represent more mature oligodendrocytes. (A) E14 rat spinal cord cultures allowed to develop to the equivalent of P1 (E14+9) in the presence of serum-free medium alone or with PDGF, PDGF and bFGF, PDGF and RA, or PDGF, bFGF and RA added daily. (B) E16 rat spinal cord cultures allowed to develop to the equivalent of P1 (E16+7) either under control conditions or in the presence of the additives as above. (C) E18 cultures allowed to develop to the equivalent of P1 (E18+5). E14+9 cultures exposed to RA, with or without bFGF lack O4+ and GC+ cells, while cultures exposed to bFGF have a large O4+ population, but essentially no GC+ cells (A). The proportion of oligodendrocyte precursors responsive to both RA and bFGF is decreased in the cultures established from animals at either E16 (B) or E18 (C). Numbers represent the mean ± the standard deviation of the total number of immunoreactive cells derived from at least two separate coverslips in three different experiments in each case.

*Significant difference from control (P<0.05). Note there is no significant difference in the number of O4+ cells between cultures supplemented with PDGF, FGF and RA and cultures supplemented with PDGF and RA at all ages. By contrast, E14 and E18 cultures supplemented with PDGF, FGF and RA contained significantly fewer O4+ cells than those supplemented with PDGF and FGF. (P<0.05).
was assayed in E18-derived spinal cord cultures. At E18 a larger proportion of oligodendrocyte precursors have matured to an O4+ stage, and some have also begun to express GC. If the effect of RA was predominantly on pre-O4+ cells the proportion of cells potentially able to disregard an inhibition of differentiation by RA should be greater in culture established from E18 animals than in cultures established from E16 animals. In both control and PDGF-supplemented E18 cultures, large numbers of A2B5-, O4- and GC-immunoreactive cells were present after 5 days in vitro (Fig. 1C). In the presence of PDGF and RA, however, although a similar number of A2B5-immunoreactive cells were present, the number of O4+ cells were reduced by approximately 50% compared with controls (Fig. 1C), and the number of GC+ cells was also reduced. As anticipated if the effect of RA was mainly on pre-O4+ cells, the number of O4- and GC-immunoreactive cells in the E18 RA-supplemented cultures was significantly larger than that seen in the E16 RA-supplemented cultures (Compare Fig. 1B and C). In recovery experiments, 4 days after removal of the supplements, the number of O4- and GC-immunoreactive cells in E18-derived RA-supplemented cultures was similar to that seen in control and PDGF-supplemented cultures, further demonstrating the reversible nature of the inhibition (Fig. 3C).

Taken together, these results indicate that RA is delaying the differentiation of spinal cord oligodendrocyte precursors. When exposed to RA early enough during their differentiation, oligodendrocyte precursor cells are prevented from maturing to an O4+ or subsequent GC+ stage. Cells that have already progressed beyond the maturational stage characterized by the expression of O4 immunoreactivity are neither selectively eliminated, nor induced to dedifferentiate in the presence of RA.

**FGF promotes proliferation and inhibits differentiation of spinal cord oligodendrocyte precursors**

For brain- and optic nerve-derived oligodendrocyte precursors, basic fibroblast-derived growth factor (bFGF) both promotes cell proliferation and inhibits cellular differentiation (McKinnon et al., 1990; Bogler et al., 1990). To examine the effect of bFGF on spinal cord oligodendrocyte precursor differentiation, E14 spinal cord cultures were supplemented with bFGF (10 ng/day), and PDGF (8 ng/day) for 9 days and then assayed for A2B5-, O4- and GC-immunoreactive cells. Compared to control and PDGF-supplemented cultures, E14 spinal cord cultures grown in the presence of both PDGF and bFGF contained approximately three times as many A2B5+...
cells, twice as many O4+ cells and virtually no GC+ cells (Figs 1A, 5). This substantial increase in the number of A2B5- and O4-immunoreactive cells combined with the reduction in the number of GC+ cells in the presence of bFGF is consistent with an inhibition of spinal cord oligodendrocyte differentiation at the O4+ stage, as previously proposed in other CNS tissue (Bogler et al., 1990; McKinnon et al., 1990). When parallel cultures were allowed to recover from supplement addition for 4 days, all three populations showed an increase in cell number over that seen in control cultures (Fig. 3A) consistent with bFGF acting as a powerful mitogen for spinal cord oligodendrocyte precursors (Fok-Seang and Miller, 1993).

To determine if bFGF was effective at blocking the differentiation of more mature spinal cord oligodendrocyte precursors, E16- and E18-derived cultures were grown for 7 and 5 days respectively in the presence of bFGF and PDGF. In E16-derived cultures supplemented with bFGF, the number of A2B5- and O4-immunoreactive cells increased significantly, while there was no increase in GC+ cells (Fig. 1B). Similarly, in E18-derived cultures while both A2B5 and O4 populations of cells were substantially increased, the number of GC+ cells was decreased as compared to controls (Fig. 1C). Four days after removal of supplements from both sets of cultures, the GC+ cell populations recovered to near normal levels (Fig. 3B,C). Thus, bFGF appears to be a powerful mitogenic for spinal cord oligodendrocyte precursors, as well as inhibiting their differentiation beyond an O4+ stage.

While both bFGF and RA inhibit the differentiation of spinal cord oligodendrocyte precursors, the maturation stage at which each molecule exerts its effect is different. For example, in the presence of either RA or bFGF, large numbers of A2B5-immunoreactive cells develop in E14-derived cultures. In the presence of RA, however, no O4+ cells develop, while in the presence of bFGF, the O4+ population is approximately five times that of controls. GC+ cells fail to develop in the presence of either supplement. These observations suggest that in high density cultures, RA is inhibiting the differentiation of spinal cord oligodendrocyte precursors at a stage before they express O4+, while bFGF is inhibiting the differentiation of oligodendrocyte precursors after the expression of O4, but prior to the expression of GC.

RA acts earlier than bFGF and overrides its effects during differentiation of spinal cord oligodendrocyte precursors

Although there are distinct differences in the effects of bFGF and RA on spinal cord oligodendrocyte precursors, the inhibition of differentiation prior to expression of GC+ is common to both. Since both bFGF and RA are likely to be present in the developing spinal cord at the same time (Vaessen et al., 1990; Kalcheim and Neufeld, 1990), the response of spinal cord oligodendrocyte precursors to simultaneous exposure to PDGF, bFGF and RA was determined. In E14 cultures supplemented with PDGF, bFGF and RA for 9 days there was a dramatic increase in the number of A2B5+ cells and an absence of both O4- and GC-immunoreactive cells compared to control cultures (Figs 1A, 5). This response was indistinguishable from that seen in cultures supplemented with PDGF and RA, but differed from that seen in cultures supplemented with PDGF and bFGF, which contained large numbers of O4+ cells. These observations suggest that during maturation of A2B5+/O4-
spinal cord oligodendrocyte precursors, the effect of RA overrides any influence of bFGF.

In cultures derived from E16 and E18 spinal cords supplemented with PDGF, bFGF and RA, the resulting populations of O4- and GC-immunoreactive cells more closely reflected those seen in the presence of PDGF and RA than in the presence of PDGF and bFGF (Fig. 1B,C). For example, in the presence of all three supplements the number of A2B5+ cells increased while the number of O4+ and GC+ cells decreased compared with controls (Fig. 1B,C). In the presence of all three supplements, the decrease in O4+ cells in E16-derived cultures was not as marked as it was in the presence of PDGF and RA alone, probably because of the mitogenic effect of bFGF on O4+ cells, which are particularly prevalent at E16 (Fok-Seang and Miller, 1993). These results demonstrate that the effect of RA on oligodendrocyte precursor differentiation occurs prior to that of bFGF and suggest that the presence of RA during the early stages of oligodendrocyte development abolishes the mitogenic effect of bFGF.

The mitogenic effect of bFGF on spinal cord oligodendrocyte precursors persists after supplement removal. When PDGF-, bFGF- and RA-treated cultures were allowed to recover for 4 days after supplement removal, a persistent mitogenic effect of bFGF was seen in E14-derived cultures and to a lesser extent in E16 cultures. For example, at E14+13 and E16+11 increased numbers of A2B5- and O4-immunoreactive cells were present in supplemented cultures than in control or PDGF- and RA-supplemented cultures (Fig. 3A,B), while the number of GC+ cells was approximately similar. This persistent mitogenic effect of bFGF was less apparent in E18-supplemented cultures (Fig. 3C). A number of explanations may account for the persistent bFGF mitogenic effect on spinal cord oligodendrocyte precursors. First, although RA abolishes both the mitogenic effect and differentiative effect of bFGF on oligodendrocyte precursors, bFGF or bFGF in combination with RA may ‘prime’ precursor cells for a burst of proliferation once RA is removed. Alternatively, bFGF is a heparin-binding molecule (Burgess and Macaig, 1989), and the persistent mitogenic effect following its removal may result from the binding of bFGF to extracellular matrices in the culture. Consistent with this idea, the persistent bFGF mitogenic effect appears to be correlated with the length of time bFGF was initially present in the culture. Thus, E14 cultures, which received bFGF for 9 days, showed the greatest residual mitogenic response, while E18 cultures, which received bFGF for only 5 days, showed little residual effect.

Taken together, these results suggest that in high density embryonic spinal cord cultures RA inhibits the differentiation of oligodendrocyte precursors early in maturation and apparently reduces the effects of bFGF treatment. Removal of RA,
however, allows precursors to proceed through the normal differentiative program and respond to any residual bFGF present in the environment.

RA inhibits spinal cord oligodendrocyte differentiation in low density cultures

To determine if retinoic acid inhibited oligodendrocyte differentiation in the absence of other spinal cord cells, the experiments were repeated on low density cultures of embryonic spinal cord, enriched in oligodendrocyte precursors. In most cases, the results obtained in such low density cultures were similar to those from high density cultures. For example, E14-derived cultures grown in the presence of PDGF and RA, or PDGF, bFGF and RA for 9 days contained many bipolar A2B5+ cells but no O4- or GC-immunoreactive cells (Fig. 6), while parallel cultures grown in the presence of PDGF alone contained many O4+ and GC+ cells. Four days after supplement removal many O4+ and some GC+ cells were found in previously RA-treated cultures (Fig. 6), demonstrating the reversible nature of the inhibition of differentiation. Similarly, the presence of RA reduced the numbers of O4+ or GC+ cells in E16- and E18-derived spinal cord cultures by approximately 50% compared to PDGF-treated cultures after 7 and 5 days in culture. This reduction in O4 and GC cell number was reversible and recovered to near PDGF levels 4 days after RA removal.

There were two significant differences between the results obtained from low density, oligodendrocyte-enriched cultures and high density spinal cord cultures, however. Control low density cultures from E14 embryos failed to survive in the absence of any supplement, and viability was reduced in E16-derived cultures. More importantly, E14-derived low density cultures supplemented with bFGF and PDGF for 9 days, contained large numbers of A2B5+ cells but no O4+ or GC+ cells. By contrast, high density cultures supplemented with bFGF and PDGF contained large numbers of A2B5+ and O4+, but no GC+ cells (see Fig. 1). Similarly, in both E16- and E18-derived cultures, the number of O4+ and GC+ cells was reduced in the presence of bFGF. The inhibition of oligodendrocyte differentiation by PDGF and bFGF was reversible At all ages 4 days after bFGF removal, O4+ and GC+ cell numbers recovered to close to PDGF values. Thus, in low density cultures enriched in oligodendrocyte precursors the effects of bFGF and RA on inhibition of oligodendrocyte differentiation are similar. These results are consistent with earlier observations on the inhibition of oligodendrocyte differentiation by PDGF and bFGF in purified cell cultures (Bogler et al., 1990; McKinnon et al., 1990).

Fig. 5. In the presence of both RA and bFGF, oligodendrocyte precursors behave as if in the presence of RA alone. Phase contrast micrographs (A'-F') and corresponding immunofluorescence micrographs of cultures labeled with A2B5 antibodies (A,D), O4 antibodies (B,E), or anti-GC antibodies (C,F). (A-C) Cultures grown in the presence of PDGF and bFGF and labeled after 9 day. Note the substantial A2B5+ and O4+ cell populations, but the absence of GC+ cells. (D-F) Parallel cultures grown in the presence of PDGF, bFGF and RA, and assayed at the same stage. These cultures contain large numbers of A2B5+ cells but no O4+ or GC+ cells. Such cultures are indistinguishable from cultures grown in the presence of PDGF and RA (compare Fig. 2 D-F) but lack the O4+ cells seen in cultures grown in the presence of PDGF and bFGF (A-C). Bar, 100 µm.
DISCUSSION

During their differentiation, oligodendrocyte precursors go through a series of distinct maturational stages. These stages are associated with the expression of particular cell surface antigens, mitogenic responses (Gard and Pfeiffer, 1990) and migratory behavior (Small et al., 1987; Temple and Raff, 1986; Pfeiffer et al., 1993). What regulates the progression of oligodendrocyte precursors through this differentiative program is currently unclear, although spinal cord oligodendrocyte differentiation occurs on the same schedule both in vivo and in vitro (Warf et al., 1991). Here we show that when E14 rat spinal cord cultures are grown in the presence of retinoic acid, oligodendrocyte precursors are inhibited from maturing beyond an early differentiative stage characterized by expression of A2B5 immunoreactivity. The absence of more mature oligodendrocyte precursors in E14 RA-supplemented cultures is not due to selective toxicity or RA-induced dedifferentiation of O4+ or GC+ cells as demonstrated by cultures derived from older animals. These cultures initially contained O4+ and GC+ cells and both populations were retained during RA exposure. The RA inhibition of oligodendrocyte differentiation is reversible and removal of RA from cultures of all ages allowed immature oligodendrocyte precursors to progress through the normal differentiative program.

It is unclear if RA is acting directly on oligodendrocyte precursors to inhibit their differentiation, or whether its effects are mediated through other cell types such as neurons or astrocytes which are present in spinal cord cultures (Warf et al., 1991; Fok-Seang and Miller, 1993). In low density cultures, RA inhibits the differentiation of spinal cord oligodendrocyte precursors suggesting that contact of the precursors with other cells types is not required. However, RA enhances spinal cord neuronal survival (Wuarin et al., 1990) and promotes differentiation of spinal cord astrocytes in vitro (Wuarin et al., 1990); therefore, its influence on oligodendrocyte precursors could be mediated through soluble factors released by other cell types in spinal cord cultures. Since in the intact spinal cord, oligodendrocyte precursors are surrounded by other neural cells, the effects of RA may be mediated through both direct and indirect mechanisms.

Retinoic acid may play an important role in regulating spinal cord oligodendrocyte development in vivo. Spinal cord oligodendrocytes originate from a distinct cluster of cells located in the ventral ventricular zone (Warf et al., 1991; Pringle and Richardson, 1993; Noll and Miller, 1993). During subsequent development, oligodendrocyte precursors must migrate dorsally and laterally to populate the peripheral developing white matter (Warf et al., 1991; Noll and Miller, 1993). The majority of oligodendrocyte precursor migration is accomplished by immature precursors prior to the expression of O4 and GC (Noble et al., 1988; Temple and Raff, 1985; Small et al., 1987; Warrington et al., 1993). Ventrally derived RA (Wagner et al., 1990) would facilitate precursor cell migration by inhibiting their differentiation until sufficient precursors populate dorsal and lateral regions of the spinal cord where further differentiation may be regulated by levels of locally synthesized retinoids (Wagner et al., 1993).

The pattern of expression of RA-binding proteins in spinal cord is also consistent with a role for RA in regulation of oligodendrocyte precursor development. For example, Cellular Retinoid Binding Protein (CRBP), proposed to store and
release RA where high levels need to be maintained (Ruberte et al., 1991) appears concentrated in ventral (Maden et al., 1990; Ruberte et al., 1993) and central regions of the spinal cord, along the dorsoventral axis (Dolle et al., 1990). This distribution is coincident with one of the observed pathways of oligodendrocyte precursor migration (Noll and Miller, 1993). Conversely, most spinal cord oligodendrocyte precursors differentiate into mature oligodendrocytes in the peripheral white matter (Gilmore, 1971; Hirano and Goldman, 1988). In developing white matter, RA concentrations would have to be maintained at low levels in order to allow oligodendrocyte differentiation to occur. A second RA binding protein, cellular retinoic acid binding protein (CRABP) proposed to sequester RA where low levels are required (Ruberte et al., 1991) appears concentrated in the developing white matter of the embryonic rat spinal cord (Vaessen et al., 1990) as well as in mouse developing white matter (Dencer et al., 1990; Maden et al., 1991). Thus, local mechanisms exist to spatially regulate the levels of retinoids within the spinal cord, and the distribution of RA binding proteins is consistent with a role for RA in regulation of oligodendrocyte differentiation.

Further support for a role of RA in oligodendrocyte development comes from studies on RA-deficient rats. Developing rats maintained on a vitamin A-deficient diet show a decrease in myelin content in the CNS (Bhat and Rao, 1978). Biochemical analysis of myelin isolated from these animals indicated a relatively normal composition, although histological analysis showed areas of CNS hypomyelination (Bhat and Rao, 1978). Such results may reflect the premature cessation of oligodendrocyte migration and premature differentiation, due to low RA concentrations resulting in decreased amounts of myelin which is normal in composition.

Retinoic acid is not the only regulator of spinal cord oligodendrocyte differentiation. In the presence of bFGF, oligodendrocyte precursor differentiation is inhibited at the stage of O4 expression, prior to expression of GC immunoreactivity in high density cultures. In low density cultures, however, bFGF inhibits oligodendrocyte precursor at an A2B5 stage prior to the expression of O4. It is unclear why bFGF inhibits oligodendrocyte differentiation earlier in low density cultures. One possibility is that positive differentiation signals are provided by other spinal cord cells at high density and these override the earliest effects of bFGF allowing oligodendrocyte precursors to mature at an O4* stage. In addition to inhibiting differentiation, bFGF also appears to have a strong mitogenic effect on spinal cord oligodendrocyte precursors. It seems likely that the effects of bFGF will be common to oligodendrocytes from many regions of the CNS. For example, purified oligodendrocyte precursors from brain and optic nerve are stimulated to proliferate in the presence of bFGF, and their differentiation is inhibited prior to the expression of GC immunoreactivity (Bogler et al., 1990). In such cultures, as in low density spinal cord cultures, combinations of PDGF and bFGF block differentiation prior to O4 expression (Collarini et al., 1992; Pfeiffer et al., 1993) directly paralleling the effect of RA in high density embryonic spinal cord cultures.

Why the differentiation of oligodendrocyte precursors in the spinal cord should be regulated by both RA and bFGF is currently unclear. Since the proliferation of oligodendrocyte precursors is especially protracted during spinal cord development, different regulators of oligodendrocyte differentiation may be required during the early and late stages of development. Labeling of mitotic spinal cord neural precursors with BrdU indicates that oligodendrocyte precursors are rapidly proliferating at E16.5 (Noll and Miller, 1993). Retroviral mediated gene transfer studies suggest they may be proliferating as early as E12 (Zhang and Miller, unpublished observation). Previous studies have documented the continued proliferation of spinal cord oligodendrocyte precursors during the first and second postnatal week (Gilmore, 1971), that is some three weeks after their initial period of proliferation. Since both bFGF (Enfors et al., 1990; Kalcheim and Neufeld, 1990) and FGF receptors (Josef et al., 1990; Wanaka et al., 1991) are present during much of spinal cord development, the differentiation of spinal cord oligodendrocyte precursors in the early embryonic spinal cord could be regulated by a combination of RA and bFGF while during later embryonic and postnatal development the differentiation of oligodendrocyte precursors could be regulated primarily by bFGF levels.

The inhibition of spinal cord oligodendrocyte differentiation by RA is likely to be transient. RA levels appear relatively high in early embryonic spinal cord, but as development proceeds they decrease (Wagner et al., 1993), possibly below the levels required for the inhibition of oligodendrocyte differentiation. Furthermore, the notochord, one of the proposed ventral sources of RA in the early embryonic animal is a transient structure present only during early embryological stages. This concept of multiple regulators of cell proliferation and differentiation is not restricted to development of the CNS. In the hematopoietic system, for example, within a single cell lineage different cytokines influence cell proliferation at different stages of development (Metcalf, 1989).

In conclusion, we propose that retinoic acid plays a primary role in the regulation of differentiation of oligodendrocyte precursors in the early embryonic spinal cord. The RA-induced inhibition of oligodendrocyte differentiation allows immature oligodendrocyte precursors to migrate from their point of origin in the ventral ventricular zone to populate the developing white matter throughout the entire spinal cord.

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