The O-2A Progenitor during Development and Remyelination

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We are investigating the factors controlling growth and differentiation of oligodendrocytes precursor cells during CNS myelination and remyelination. These studies have stemmed from the description of O-2A progenitor cells in the newborn rat optic nerve (reviewed in Raff, 1989). These progenitor cells are bipotential since they can differentiate either into oligodendrocytes, which form myelin in the CNS, or type 2 astrocytes, which extend processes to nodes of Ranvier. Therefore, the lineage derived from this progenitor is called the oligodendrocyte-type 2 astrocyte (O-2A) lineage. O-2A progenitor cells are identified by immunolabeling with an antibody to cell surface gangliosides (A2B5) or glycoproteins (NSP4). O-2A progenitor cells purified and cultured from newborn rat brain (Behar et al., 1988) are shown in Figure 1.

Figure 1. Purified brain O-2A progenitors in culture stained with NSP4 antibody. Cells are bipolar or multipolar with short processes.

In this short report of our ongoing studies, we describe how O-2A progenitor cells from the rodent CNS move along the oligodendrocyte differentiation pathway during development *in vitro*, and the role of polypeptide growth factors including platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) in the growth and rate of differentiation of this progenitor cell. We will also review the evidence for the persistence of O-2A progenitors in the rodent adult nervous system and how these cells react to a demyelinating episode and may play a role in remyelination.

1. DEVELOPMENT

Earlier studies on newborn rat optic nerve have shown that O-2A progenitor cells cultured in defined medium quickly develop into oligodendrocytes expressing galactocerebroside (GC), a major myelin glycolipid (Raff et al., 1983). In contrast, addition of 10% fetal bovine serum (FBS) drives the cell along the type 2 astrocyte pathway and induces glial fibrillary acidic protein (GFAP) expression. This situation is different *in vivo*: O-2A progenitors give rise to the first oligodendrocytes around birth while they keep proliferating for at least the next 2 weeks (Skoff et al., 1976). Conditioned medium (CM) derived from type 1 astrocytes, the first type of astrocyte emerging in the brain early before birth, restores this normal sequence of events *in vitro*: the progenitor will go through a number of divisions *in vitro* as they do *in vivo* before they differentiate (Raff et al., 1985). One question raised by these studies (reviewed in Raff, 1989) is whether the O-2A progenitor is committed to become an oligodendrocyte after a set number of divisions or whether it is a true stem cell, capable of self renewal in addition to generating oligodendrocytes. The best way to address this question is to perform cultures of single O-2A progenitors and analyse the progeny derived from clonal expansion of single cells.

A. Clonal Analysis of the O-2A progenitor

Initial studies by Temple and Raff (1986) on O-2A progenitors seeded singly on a layer of type 1 astrocytes indicated that the O-2A progenitor divides a set number of times during 8-10 days before its progeny become multipolar differentiated oligodendrocytes. In similar experiments, we seeded single progenitor cells from either optic nerve or cerebrum onto monolayers of type 1 astrocytes (Dubois-Dalcq, 1987; McMorris and Dubois-Dalcq, 1988). We selected the larger clones at 8 days and followed them for up to

3 weeks in culture. Clonal growth progressed steadily during the first week (~1 division per day) then slowed considerably. The majority of the clones were still expanding at 3 weeks and contained a variable ratio of multipolar cells reacting with A2B5 antibody but not expressing GC. In addition these GC negative cells stained with the O4 antibody, which recognized sulfatide and another glycolipid that are present on the surface of the O-2A progenitor at a later stage along the pathway. These O4+ cells had a more complex morphology and did not express vimentin. Gard and Pfeiffer (1989) have also described a population of O4+ multipolar cells which did not acquire GC 10 days after sorting and culture. Our clonal analysis demonstrated that the relative abundance of multipolar O4+GC- cells was increased when insulin was deleted from defined medium, while many more oligodendrocytes formed in the presence of insulin (5µg/ml) or IGF1 (100ng/ml) (McMorris and Dubois-Dalcq, 1988). In the presence of these factors, some clones contained only GC+ cells as described by Temple and Raff (1986). These studies indicated that progenitor cells can be expanded for a prolonged period of time *in vitro*, as *in vivo* studies had suggested. Furthermore IGF1 can promote proliferation of this O4+ progenitor and induces these cells to develop into oligodendrocytes (McMorris and Dubois-Dalcq, 1988).

B. PDGF and the O-2A Lineage

The recent observations that type 1 astrocytes synthesize PDGF transcripts and protein and that anti-PDGF antibodies can neutralize the mitogenic effect of type 1 astrocyte CM have suggested a key role of PDGF in the growth of O-2A progenitors (Richardson et al., 1988). PDGF is active as a dimer and composed of 2 chains, designated A and B. The gene coding for PDGF-A chain in man is on chromosome 7, and the B chain gene is on chromosome 22 (reviewed in Ross et al., 1986). All three dimeric forms of PDGF have been identified and are biologically active, such as PDGF AB from human platelets, BB from porcine platelets and AA from osteosarcoma cells. Recent studies have identified both the PDGF ligands and PDGF receptors that are important for O-2A progenitor development in the rat CNS. These results are summarized in Table 1. As mentioned above, type 1 astrocytes are the major source of PDGF (A chain) in the rat, although it is not excluded that O-2A progenitor cells and or oligodendrocytes can also synthesize small amounts of PDGF. In the developing rat CNS, the A chain transcripts start to be expressed at E17 and

Table 1. PDGF ligands and receptors in O-2A lineage cells*

	Type-1 Astrocyte	O-2A Progenitor	Oligodendrocyte
PDGF Ligands:			
A-chain mRNA	++		
A-chain protein	+		
B (sis) mRNA	(+)		
B (sis) protein			
PDGF Receptors:			
α -R mRNA	0	++	+
α -R protein	0	++	+
β -R mRNA			
β -R protein		0	0
Binding assays:	0	AA,AB,BB	AA,AB,BB
Mitogenicity:		AA,AB>BB	0

+ indicates the presence and relative abundance of PDGF ligands and receptors.

0 indicates that levels are non-detectable.

* see text for references.

increase in abundance thereafter, while low and constant levels of PDGF B transcripts are detected throughout rat brain development (Richardson et al., 1988). In addition, A chain transcripts are easily detected by *in situ* hybridization in the newborn rat optic nerve while the B chain transcripts are not detectable in the optic nerve parenchyma (Pringle et al., 1989).

The O-2A progenitor was examined recently for the expression of PDGF receptors, members of the tyrosine-kinase family of growth factors receptors. Two types of PDGF receptors (type-A or α ; type-B or β) have been characterized (Hart et al., 1988; Heldin et al., 1988) and cDNA copies cloned (Yarden et al., 1986; Matsui et al., 1989; Claesson-Welsh et al., 1989). The α receptor binds all 3 dimeric forms of PDGF (AA, AB, BB) whereas the β receptor binds the BB dimers with high affinity and AB dimers with low affinity. Ligand binding studies have indicated that O-2A progenitor cells from optic nerve express the PDGF- α receptor (Hart et al., 1989), and PDGF AA dimers are a more potent mitogen than BB dimers in this system (Pringle et al., 1989) as discussed by Richardson et al. in this volume. The identity of the PDGF receptor on rat O-2A progenitor cells as the α -receptor has been confirmed by Northern blot analysis of the receptor mRNA transcripts in purified brain O-2A progenitor cells. Western blot analysis further

determined that O-2A cells express abundant levels of the α -receptor but non-detectable levels of the PDGF- β receptor (McKinnon et al., 1989). The O-2A progenitor appears to be the first example of a mammalian cell expressing only the PDGF- α receptor.

PDGF drives the proliferation of O-2A progenitor cells from optic nerve and regulates the timing of their differentiation (Raff et al., 1988). We have examined the induction of myelin basic protein (MBP), a gene expressed in differentiated oligodendrocytes (Zeller et al., 1985), in cultures of purified brain progenitor cells treated with PDGF. PDGF delayed MBP transcript accumulation but did not prevent the expression of this differentiation gene (McKinnon et al., 1989).

C. A Possible role for FGF in the O-2A Lineage

Both acidic and basic FGFs are abundant in the adult CNS (reviewed by Barde, 1989) and have been shown to have mitogenic effects on oligodendrocyte precursors and possibly oligodendrocytes (Eccleston and Silberberg, 1985; Saneto and DeVellis, 1985; Besnard et al., 1989). The basic form of FGF (bFGF) has been purified from cell lysates of cultured astrocytes (Hatten et al., 1988; Ferrara et al., 1988). As shown in Fig 2, bFGF is a more potent mitogen than PDGF for purified brain O-2A progenitors as assayed by BUdR incorporation, using double immunofluorescence with A2B5 to identify progenitor cells with BUdR labeled nuclei.

Figure 2. Mitogenic response of O-2A progenitor cells to porcine PDGF-BB (pPDGF), human PDGF-AB (hPDGF), and bFGF. Purified progenitors were cultured for 24 hrs with growth factors, pulsed 2 hrs with 10 μ M BUdR, then stained with anti-A2B5 and anti-BUdR. Values represent the % A2B5 positive cells that incorporated BUdR.

We have also used Northern blot analysis of myelin-specific gene transcripts to examine the effects of bFGF on O-2A differentiation (McKinnon et al. 1989). Expression of MBP was completely inhibited by treatment of O-2A progenitors by 10 ng/ml bFGF, a situation different from that seen with PDGF (see above). When both FGF and PDGF were present for 2 days, the same inhibition of MBP expression was seen. The inhibition of myelin expression was reversible when FGF was removed from the cells. FGF also induced a rapid and dramatic increase in the levels of the PDGF- α receptor expressed by brain O-2A progenitors. We are presently examining whether this FGF induced increase in receptor levels results in an enhanced sensitivity of the progenitor cells to lower doses of the different dimeric forms of PDGF.

Our present results suggest that FGF can regulate the O-2A progenitor interactions with PDGF. Thus FGF and PDGF can influence the timing of oligodendrocyte differentiation *in vitro*. This indicates that a subtle interplay between these factors may set the timing of myelination in specific tracts *in vivo*. Since FGF appears to be increased locally in brain wounds (Finkelstein et al., 1988), this factor may also play a role in glial cell regeneration and remyelination (see below). bFGF may in fact be produced by neurons (Pettmann et al., 1986; Finkelstein et al., 1988; review by Barde, 1989) and FGFs have been shown to promote survival and neurite outgrowth of a variety of neurons (reviewed in Barde, 1989). Thus this polypeptide may successively have an effect on neuronal development and the timing of myelination of axons. Interestingly purified cultures of cerebellar interneurons secrete in their medium a factor which has mitogenic activity for O-2A progenitor cells and appears to arrest their differentiation (Levine, 1989). Whether cerebellar interneuron CM contains FGF-like factors is presently not known.

If FGF allows O-2A progenitor cells to divide for prolonged periods of time, one would postulate that other factors made in the CNS might inhibit PDGF induced mitosis. One possible candidate for this function would be transforming growth factor β , TGF- β (Van Obberghen-Schilling et al., 1987). TGF β is synthesized by type 1 astrocytes and can decrease the number of O-2A progenitors dividing in the presence of PDGF. The possibility of upregulation of the O-2A progenitor response to PDGF by one factor versus down regulation by another needs to be further explored. It would constitute an attractive mechanism by which the number of oligodendrocytes could be controlled with flexibility during development and regeneration. In addition the factors mentioned above may have a direct effect on the remyelination program (see below).

2. Remyelination

A. The O-2A progenitor in adult CNS

Evidence that rare O-2A progenitor cells exist in the normal adult rodent CNS has arisen from recent studies on optic nerve, rat brain and mouse spinal cord (French-Constant and Raff, 1986; Armstrong et al., 1988; Hunter et al., 1988; Wolswijk and Noble, 1989). These investigators have successfully isolated progenitor cells by enzymatic and mechanical dissociation of the nervous tissue, sometimes followed by a Percoll gradient to separate the glial cells from the myelin. The *in vitro* properties of the progenitors isolated from adult CNS are compared to those of the newborn progenitors in table 2. The latter differ from the adult O-2A progenitor by their antigenic phenotype, cell shape and growth potential. Adult O-2A progenitors may extend branched processes which contrast with the simple bipolar morphology of the neonatal progenitor (Wolswijk and Noble, 1989). These adult O-2A progenitors simultaneously express the glycolipids recognized by the A2B5 and O4 antibodies whereas the neonatal O-2A progenitor stains only for A2B5 at birth and then moves on to express O4 one week later (Sommer and Noble, 1986). Vimentin is clearly present in the A2B5+ neonatal progenitor (Raff et al., 1984). However, vimentin is not detectable in adult progenitors of optic nerve. Similarly, O4+, A2B5+, vimentin- progenitors are present in 3 week old clones derived from single O-2A progenitors of newborn optic nerve and brain (Dubois-Dalcq, 1987; McMorris and Dubois-Dalcq, 1988).

Table 2. Comparison between neonatal and adult O-2A progenitor cells of rodent CNS*.

	<u>Neonatal Progenitor</u>	<u>Adult Progenitor</u>
A2B5 (GQ Gangliosides)	+	+
O4 (sulfatide other lipid)	(-) → (+)	+
Vimentin	(+) → (-)	-
Galactocerebroside	-	-
Shape	Bipolar or small multipolar	Complex processes
Cell Cycle Time	18 h	65 h
Bipotentiality	+	+

* see text for references.

The adult O-2A progenitor of optic nerve has retained bipotentiality. It can evolve into either an oligodendrocyte or a type 2 astrocyte depending on the media conditions, but it differentiates more slowly (>5 days) than the neonatal progenitor (<3 days) (Wolswijk and Noble, 1989). O-2A progenitor cells in adult rodents can proliferate, although the cell cycle is lengthened relative to the neonatal O-2A progenitor (65 hours versus 18 hours) (Wolswijk, & Noble 1989). The properties of bipotentiality and mitogenic capacity identify the adult cell as a true progenitor of the O-2A lineage which continues to exist in fully myelinated tissue.

B. The O-2A Progenitor in Remyelination

The studies summarized above point to a potential role for the O-2A progenitor of the adult CNS in the regeneration of myelin after a demyelinating episode. The antigenic phenotype and biological properties of these precursors had not previously been described during a demyelinating disease. The fact that new oligodendrocytes are generated in reaction to a demyelinating episode of viral or toxic origin has been established in several electron microscopic studies combined with autoradiography (Herndon et al., 1977; Ludwin et al., 1981; Aranella and Herndon, 1984). Moreover, the emergence of myelin proteins and the expression of myelin genes during remyelination recapitulates the developmental program of myelination (Ludwin and Sternberger, 1984; Jordan et al., 1989a). This suggests that immature oligodendrocytes are the cells responsible for remyelinating denuded axons.

The demyelinating model we have studied is produced in C57Bl/6N mice at four weeks of age by intracerebral inoculation of the A-59 strain of mouse hepatitis virus. One week after intracranial inoculation (1 WPI), virus replication in glial cells is widespread throughout the spinal cord and results in a distinct decrease in all myelin-specific mRNA's prior to demyelination (Jordan et al., 1989b). Areas of demyelination develop by 3 WPI. Within the lesions the number of oligodendrocytes is reduced and total MBP transcripts are decreased by 75% compared to control white matter. Virus is cleared throughout most of the spinal cord by 4 WPI. At the earliest stage of remyelination, we have described an important increase in MBP transcripts containing exon 2 (encoding pre-large and pre-small forms of MBP), which are characteristic of myelination in early development (Jordan et al., 1989a). This increase in MBP transcripts containing exon 2 occurred in and around the lesions. This suggests that oligodendrocyte progenitor cells can repopulate

the demyelinating lesions through of a process of mitosis, migration, and differentiation.

To examine this possibility further, we first attempted to detect directly *in vivo* O-2A lineage cells in the spinal cord of these infected mice at several stages of the demyelinating disease (Godfraind et al., 1989). We used triple-label immunofluorescence for O4, a progenitor marker, GFAP, the astrocyte marker, and 2'-3'-cyclic nucleotide 3'-phosphohydrolase (CNP), an early myelin protein which stains oligodendrocytes more reliably than GC in 1 micron frozen sections. While we found rare O4+ cells in control mice, these same cells were frequent in the spinal cord of infected mice at 1 and 2 WPI. Some of these cells were labeled with tritiated thymidine after a two hour *in vivo* pulse, indicating their ability to divide in response to a stimulus. Between 2 and 4 WPI, both O-2A progenitors (O4+) and type 2 astrocytes (O4+, GFAP+) were found in the lesions, and a proportion of these cells again incorporated thymidine (Godfraind et al., 1989). As remyelination proceeded, tritiated thymidine injected during the demyelinating phase appeared in CNP+ oligodendrocytes, indicating that precursor cells which divided earlier in the disease had generated new oligodendrocytes (Godfraind et al., 1989).

Encouraged by the identification of O-2A lineage cells *in vivo* in these animals, we then isolated these cells from the spinal cord of demyelinating and remyelinating mice (Armstrong et al., 1988). O-2A lineage cells identified in cultures from adult CNS included O-2A adult progenitor cells (figure 3), oligodendrocytes and type 2 astrocytes (figure 4).

Figure 3. An O-2A progenitor cell (O4+ GC- GFAP-) isolated from spinal cords of 10 week old mice during remyelination (6 WPI). Cells were fixed at 3 days *in vitro*. Left: phase contrast. Right: O4 immunostain.

Figure 4. An oligodendrocyte (O4+ GC+ GFAP-) and a type 2 astrocyte (O4+ GC- GFAP+) isolated from spinal cords of 9 week old mice at the onset of remyelination (5 WPI). Cells were fixed at 3 days *in vitro* and processed for 3-color immunofluorescence. Top left: phase contrast. Top right: GFAP. Bottom left: GC. Bottom right: O4.

We found an important increase, relative to controls, in the number of O-2A lineage cells that could be isolated and cultured from demyelinating tissues at three to five WPI. Cultures derived from demyelinating tissues also contained numerous microglial cells which were rarely encountered in controls. Within the O-2A lineage population isolated from demyelinating tissues, there was a higher proportion of type 2 astrocytes and cells with a mixed oligodendrocyte-astrocyte phenotype, as compared to controls. The mixed phenotype cell, almost unique to remyelinating animals, was also identified *in vivo* in demyelinating animals, although rarely (Godfraind et al., 1989). This indicates a greater phenotypic plasticity in O-2A lineage cells from these diseased animals. Such

Figure 5. O-2A lineage cells were isolated from spinal cords of 9 week old mice (5 WPI). Growth factors were added to defined media after 1 day *in vitro*. Cells were fixed at 3 days *in vitro* and identified by 3-color immunofluorescence.

behavior could be related to the release of a number of polypeptides in the lesions; for instance ciliary neurotrophic factor is increased in CNS tissues during regeneration (Nieto-Sampedro et al., 1983) and may transiently induce GFAP in O-2A progenitors (Lillien et al., 1988). The increased number of O-2A lineage cells isolated during demyelination is at least partially due to proliferation. Cultures derived from spinal cords of infected mice contained an increased proportion of tritiated thymidine-labeled O-2A lineage cells after a short *in vivo* or *in vitro* pulse. Dividing cells were rarely seen in control cultures. Thymidine incorporation was not markedly enhanced by addition of human PDGF, basic FGF or IGF1 (Armstrong et al., 1989). In these cultures the ratio of oligodendrocytes relative to type 2 astrocytes was higher in the presence of IGF-1, and was lower in the presence of basic FGF (figure 5) as was seen with cultured O-2A progenitors isolated from early development (see above). Thus, in this *in vitro* system, we can now study the conditions for O-2A lineage cells to proliferate and express phenotypic plasticity as they do *in vivo* in the course of demyelination and remyelination.

3. Conclusions:

Studies on glial O-2A progenitor cells *in vitro* suggest that an interaction between several different polypeptide growth factors may affect the process of O-2A lineage differentiation *in vivo*. FGF and PDGF are mitogenic for O-2A progenitor cells, and PDGF appears to control the timing of O-2A differentiation while FGF may block differentiation into oligodendrocytes. In contrast, IGF-1 promotes oligodendrocyte development. An O4+ O-2A progenitor cell persists in the adult and may represent a self-renewing "stem" cell that proliferates in response to a demyelinating episode. The phenotype of adult O-2A lineage cells can also be modulated by some of these growth factors. Thus polypeptide growth factors that are important for normal glial cell development may also have a role in the process of remyelination. Understanding the mechanisms regulating expression of these factors during development and disease will further our ability to intervene in demyelinating diseases in man.

Acknowledgements:

We thank Ray Rustin for excellent technical assistance. R.McK was supported by a senior fellowship from the US National Multiple Sclerosis Society.

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