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Cooperation between two growth factors promotes extended self-renewal and inhibits differentiation of oligodendrocyte–type-2 astrocyte (O-2A) progenitor cells

(platelet-derived growth factor/fibroblast growth factor/maturation arrest/glia/precursor)

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ABSTRACT **Bipotential oligodendrocyte–type-2 astrocyte (O-2A) progenitor cells, which give rise to oligodendrocytes and type-2 astrocytes in cultures of rat optic nerve, are one of the few cell types in which most aspects of proliferation and differentiation can be manipulated in a defined *in vitro* environment. Previous studies have shown that O-2A progenitors exposed to platelet-derived growth factor (PDGF) divide as migratory bipolar cells a limited number of times, with a cell cycle time of 18 hr, before clonally related progenitors differentiate into nondividing oligodendrocytes with a timing similar to that seen *in vivo*. In contrast, O-2A progenitors grown in the absence of mitogen do not divide but instead differentiate prematurely into oligodendrocytes, and progenitors exposed to appropriate inducing factors differentiate into type-2 astrocytes. We now have found that O-2A progenitors can be induced to undergo continuous self-renewal in the absence of oligodendrocytic differentiation by exposure to a combination of PDGF and basic fibroblast growth factor (bFGF). With the exception of the inhibition of differentiation, the O-2A progenitors exposed to PDGF and bFGF behaved similarly to those exposed to PDGF alone. In contrast, progenitors exposed to basic bFGF alone were multipolar, had a cell-cycle length of 45 hr, showed little migratory behavior, underwent premature oligodendrocytic differentiation, and did not cease division upon expression of oligodendrocyte marker antigens. Thus, inhibition of differentiation required the presence of both mitogens. Our results demonstrate that PDGF and bFGF act on O-2A progenitors as both inducers of division and as regulators of differentiation that modulate multiple aspects of O-2A progenitor development and, additionally, reveal a previously unrecognized means of regulating self-renewal processes, wherein cooperation between growth factors promotes continuous division in the absence of differentiation.**

To better understand the cellular and molecular interactions that regulate the development of multipotential precursor cells, we have been studying the control of division and differentiation in oligodendrocyte–type-2 astrocyte (O–2A) progenitor cells of the rat optic nerve (1). These glial precursors represent one of the few cell types in which most aspects of differentiation and proliferation can be manipulated in a defined *in vitro* environment. For example, O-2A progenitors grown in chemically defined medium in the absence of mitogen do not divide but instead differentiate rapidly into oligodendrocytes (1–3), while progenitors exposed to appropriate inducing factors differentiate into type-2 astrocytes (1, 4, 5).

Several studies have demonstrated that the induction of cell division in O-2A progenitors is associated with the expression of several characteristics that are quite distinct from the events of mitosis (see Fig. 1 for a summary). O-2A progenitors induced to divide by platelet-derived growth factor (PDGF) or by growth in the presence of type-1 astrocytes (which secrete PDGF; refs. 6–8) are extensively migratory cells that display a characteristic bipolar morphology and have a cell-cycle time of 18 hr (6, 9). Dividing O-2A progenitors also display a particular program of differentiation wherein clonally related cells generally divide a limited and equal number of times before synchronously differentiating into oligodendrocytes with a timing similar to that seen *in vivo* (3, 7). In the presence of PDGF, oligodendrocytic differentiation is associated with expression of a multipolar morphology, a loss of motility, and a cessation of cell division (6).

We now show that O-2A progenitors can display a multitude of distinct behaviors *in vitro*, the expression of which is regulated by the mitogens to which the cells are exposed. Progenitors induced to divide by exposure to basic fibroblast growth factor (bFGF) developed a multipolar phenotype, had an average cell-cycle time of 45 hr, differentiated prematurely into oligodendrocytes, and continued to divide after oligodendrocytic differentiation. More importantly, O-2A progenitors exposed simultaneously to PDGF and bFGF did not undergo any oligodendrocytic differentiation but instead divided continuously as bipolar and motile progenitors. The ability of cooperation between growth factors to elicit continuous division in the absence of differentiation represents a previously unrecognized means of regulating self-renewal processes in a defined population of precursor cells.

MATERIALS AND METHODS

Optic Nerve Cultures. Cultures of optic nerve cells from 7-day-old rats or 19-day rat embryos were prepared as described (1–3, 7). For most experiments dissociated optic nerve cells were plated in 25-μl drops onto poly(L-lysine)-coated cover slips (Chance Propper no. 1, 13-mm diameter) in a mixture of 2 mM glutamine in Dulbecco's minimal essential medium (DMEM) containing per ml 5.6 mg of glucose, 0.5 μg of bovine transferrin, 100 μg of human transferrin, 100 μg of bovine serum albumin, 0.6 ng of progesterone, 16 μg of putrescine, 0.4 ng of selenium, 0.4 ng of thyroxine, 0.3 ng of triiodothyronine, 2 mM glutamine, and 25 μg of gentamicin [DMEM-BS; a modification of the medium described by Bottenstein and Sato (10)]. Cells were allowed to adhere for 30–45 min in a humidified 37°C incu-

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Abbreviations: bFGF, basic fibroblast growth factor; GalC, galactocerebroside; O-2A, oligodendrocyte–type-2 astrocyte; PDGF, platelet-derived growth factor; GFAP, glial fibrillary acidic protein.
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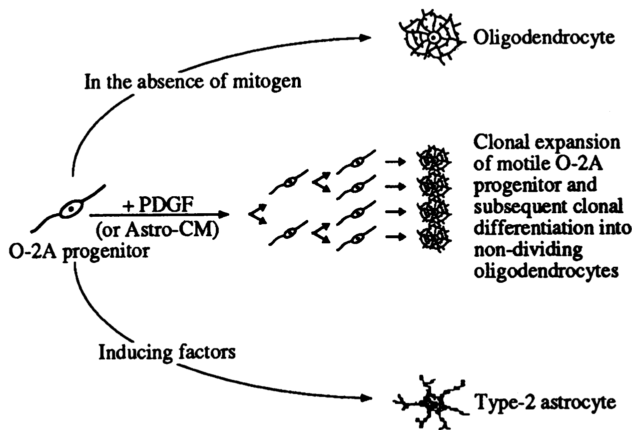

FIG. 1. Diagrammatic summary of division and differentiation in the O-2A lineage. See text for details.

bator, and cultures were then fed to a final volume of 0.5 ml with DMEM-BS.

Cultures prepared from optic nerves of 7-day-old rats were grown for indicated lengths of time in DMEM-BS in the presence of bFGF, PDGF, or both. Cultures prepared from optic nerves of 19-day rat embryos were plated on poly(L-lysine)-coated coverslips (10,000 cells per coverslip) in DMEM-BS. Cultures received per ml 2 or 10 ng of PDGF, 2 ng of PDGF with 5 ng of bFGF, or 10 ng of PDGF with 10 ng of bFGF daily, and approximately half of the DMEM-BS was changed every 2 days.

PDGF-AA was obtained from Chiron Corporation (a gift of C. George-Nascimento), PDGF-BB was from British Biotechnology, and recombinant human bFGF was from Boehringer Mannheim. Sequence grade native bovine brain bFGF was a gift of D. Gospodarowicz. Fresh addition of PDGF, bFGF, or both was carried out on a daily basis, with factors being added in 50-μl aliquots of DMEM-BS. After various periods of time, cultures were labeled on coverslips with antibodies for indirect immunofluorescence analysis.

Immunofluorescence. All antibodies used have been described previously. These antibodies were monoclonal anti-galactocerebroside (GalC) antibody (11), monoclonal antibody A2B5 (12), and rabbit anti-glial fibrillary acidic protein (GFAP) antiserum (13). The A2B5 monoclonal antibody (12) specifically labels O-2A lineage cells in these cultures (1), anti-GalC specifically labels oligodendrocytes (14), and anti-GFAP antiserum specifically labels astrocytes (1, 13). All fluorescein- and rhodamine-conjugated second layer antibodies were purchased from Southern Biotechnology and were used at a dilution of 1:100. Anti-GalC and A2B5 antibodies were applied to living cultures, while anti-GFAP antiserum was applied to cultures following fixation in methanol at −20°C for 10 min. These antibodies were used in various combinations for two fluorochrome immunofluorescence analyses as described previously (1, 2). After labeling for immunofluorescence, any unfixed cultures were fixed in methanol as before, placed cells-down in a drop of Citifluor to retard fading of fluorescein (15, 16), and sealed with nail varnish. Cultures were viewed on a Zeiss Axiphot microscope equipped with phase-contrast and epi-UV illumination and selective filters for fluorescein and rhodamine.

RESULTS

O-2A Progenitors Induced to Divide by bFGF Undergo Premature Oligodendrocytic Differentiation. We first examined the effects of bFGF on the differentiation of O-2A progenitors because this mitogen has been shown to induce DNA synthesis in these progenitors (6) as well as in oligodendrocytes (17, 18) and is abundant in the central nervous system (19, 20).

When grown in the presence of bFGF, >90% of the O-2A progenitors derived from optic nerves of 7-day-old rats differentiated into oligodendrocytes within 72 hr, as compared with differentiation of only 41% in cultures exposed to PDGF (Table 1). In addition, almost all (≥96%) cells with the antigenic phenotype of O-2A progenitors found in bFGF-treated cultures after 72 hr were multipolar cells and did not express the typical bipolar morphology previously observed for dividing O-2A progenitors (6, 9). Similar results were obtained with concentrations of bFGF ranging from 1 to 10 ng/ml (data not shown) and with both sequence-grade and recombinant material (see the legend to Table 1).

Direct time-lapse microcinematographic observations (as in refs. 6 and 9) confirmed that, when grown in the presence of bFGF (5 ng/ml, added daily), O-2A progenitors developed a multipolar morphology within 24 hr (i.e., prior to oligodendrocytic differentiation, determined as described) and divided as multipolar cells. O-2A lineage cells induced to divide by bFGF had an average cell cycle time of 45 ± 12 hr (n = 21 cells) and exhibited little migratory behavior. Moreover, bFGF stimulated division of multipolar cells for several generations (i.e., both prior to and subsequent to oligodendrocytic differentiation of virtually all O-2A lineage cells in parallel cultures), in agreement with previous reports (17, 18) that bFGF promotes division of oligodendrocytes. In agreement with these previous reports and our own time-lapse data, we also have observed stimulation by bFGF of DNA synthesis in GalC$^+$ oligodendrocytes (D.W. and M.N., unpublished observations).

Induction of Progenitor Self-Renewal Through Cooperation Between PDGF and bFGF. In striking contrast to the effects of exposure to bFGF or PDGF alone, oligodendrocytic differentiation of O-2A progenitors was strongly inhibited by simultaneous exposure to both mitogens. While oligodendrocytic differentiation proceeded normally in cultures of 7-day-old rat optic nerve grown in PDGF so that >55% of the O-2A lineage cells in these cultures were oligodendrocytes after 7 days *in vitro*, progenitors grown in the presence of PDGF and bFGF proliferated extensively in the relative absence of differentiation (Fig. 2). Even after 7 days of *in vitro* growth

Table 1. Premature oligodendrocytic differentiation of O-2A progenitors grown in bFGF

Condition	O-2A lineage cells that are A2B5$^+$ GalC$^-$, %	A2B5$^+$ GalC$^-$ cells that are bipolar, %
DMEM-BS	1.4 ± 1.0	0
bFGF (10 ng/ml)	9.4 ± 3.0	3.5 ± 2.5
PDGF (10 ng/ml)	58.9 ± 3.0	46.4 ± 3.1
bFGF + PDGF	76.3 ± 5.0	60.0 ± 7.0

bFGF does not prevent the premature oligodendrocytic differentiation of O-2A progenitors. Most (>90%) O-2A progenitors (i.e., A2B5$^+$ GalC$^-$ cells; see below) grown in the presence of 10 ng of bFGF per ml for 72 hr differentiated into oligodendrocytes (i.e., GalC$^+$ cells) and most (>96%) of the remaining GalC$^-$ cells were multipolar. In contrast, cultures grown in the presence of PDGF (porcine BB homodimer; British Biotechnology) or in the presence of PDGF and sequence grade bFGF (a gift of D. Gospodarowicz) contained many O-2A progenitors that expressed the bipolar morphology previously seen in cultures of dividing O-2A progenitors (6, 9). The A2B5$^+$ GalC$^-$ cells seen in cultures exposed to PDGF or to PDGF and bFGF were also negative for expression of glial fibrillary acidic protein and thus had not simply differentiated into type-2 astrocytes (1). Similar results were obtained with samples of recombinant AA and BB homodimers of PDGF (Chiron Corporation) and with recombinant bFGF (Boehringer Mannheim). Figures are means ± SEM for triplicate coverslips averaged from three separate experiments, with a minimum of 400 cells counted per coverslip.

in the presence of PDGF and bFGF, >90% of the O-2A lineage cells were still O-2A progenitors.

A more dramatic demonstration of the ability of PDGF and bFGF to inhibit oligodendrocytic differentiation was obtained by adding these mitogens to cultures of optic nerve cells from embryonic day 19 rats (Fig. 3). Cells from embryonic day 19 rats exposed to PDGF or grown in the presence of PDGF-secreting type 1 astrocytes normally begin to generate oligodendrocytes after 2 days *in vitro* (3, 7), a timing that mimics that seen *in vivo* (21). In contrast, cells cultured in the presence of PDGF and bFGF did not differentiate into oligodendrocytes even after 10 days *in vitro* (Fig. 3). Similar results (M.N. and D.W., unpublished observations) were seen when cells from embryonic rats were exposed from the time of plating to bFGF and medium conditioned by type-1 astrocytes (which contains PDGF; refs. 6–8). During the time of coapplication of PDGF and bFGF, the O-2A progenitors expressed the bipolar morphology and extensive motility previously seen in cells induced to divide by PDGF (6).

Inhibition of Differentiation Requires the Continued Presence of PDGF and bFGF. The inhibition of differentiation seen in embryonic cultures exposed to both PDGF and bFGF could be maintained for prolonged periods but was dependent on continued application of the mitogens. For example, cells derived from embryonic day 19 rat optic nerves were grown on glass coverslips (as in Fig. 3) in the presence of PDGF and bFGF (10 ng/ml of each) for 14 days and then switched to medium containing PDGF but not bFGF by washing cultures and refeeding with PDGF alone (10 ng/ml), replacing cultures on new coverslips in the presence of PDGF, or replating cultures on monolayers of purified type-1 astrocytes (which secrete PDGF). As the amount of PDGF secreted by astrocyte monolayers is variable (unpublished observations), some cultures replated on astrocytes also received an additional 10 ng of PDGF per ml. Under all conditions, oligodendrocytes appeared within 24 hr. The most satisfactorily quantifiable experiments were those carried out on astrocyte monolayers, where small numbers of progenitors (≤500 optic nerve-derived cells plated per coverslip) could be replated with high plating efficiency. In these cultures 6.2 ± 1.6% (mean ± SD) of O-2A progenitors differentiated into oligodendrocytes within 24 hr, and 71.4 ± 7.1% differentiated within 6 days (analyzed as in Fig. 2). Similar differentiation occurred with cells grown on astrocyte monolayers in the presence of PDGF (unpublished observations). The oligodendrocytic differentiation seen in all of these cases was not due to effects of washing or trypsinizing cells, as addition of PDGF and bFGF to cultures in all conditions continued to inhibit differentiation and to promote continued self-renewal, thus indicating that the inhibition of differentiation induced by exposure to PDGF and bFGF was maintained after passaging of O-2A progenitors. The presence of type-1 astrocytes in all optic nerve-derived cultures that have been maintained for prolonged periods *in vitro* meant that PDGF could not be completely removed from the medium, and thus we were unable to satisfactorily examine effects of exposure to bFGF alone.

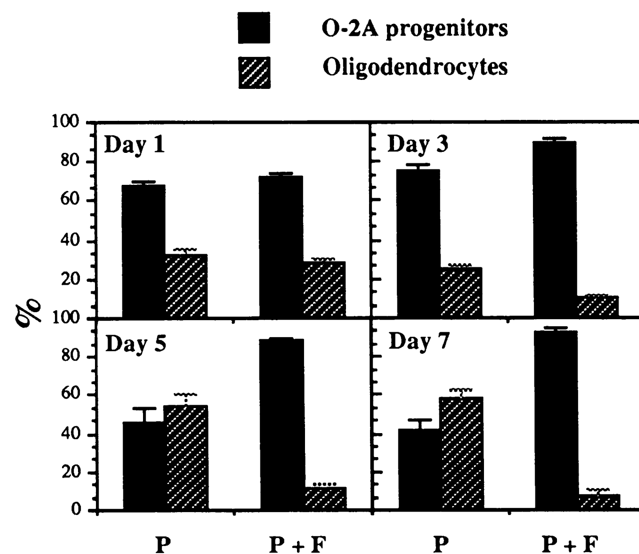

FIG. 2. Simultaneous application of PDGF and bFGF inhibits oligodendrocytic differentiation of O-2A progenitors in cultures prepared from optic nerves of 7-day-old rats. The data show that many O-2A progenitors exposed to PDGF differentiate into oligodendrocytes, as seen previously (6, 7). In contrast, oligodendrocytic differentiation was inhibited in cultures exposed to PDGF and bFGF. Means ± SEM are shown from several experiments.

It appears that the cooperative interaction between PDGF and bFGF directly inhibits oligodendrocytic differentiation and thus overrides the limitation on progenitor proliferation seen in previous experiments. The possibility that the inhibition of differentiation was due to lengthening of the cell cycle (thus necessitating a longer period of time to reach a particular number of cell divisions) seems unlikely, as timelapse microcinematographic analysis indicates similar cell-cycle times for cells grown in PDGF or in PDGF and bFGF (unpublished observations). Support for the view that cell-cycle times were similar in cultures treated with PDGF or PDGF and bFGF also came from direct comparison of the numbers of cells generated in embryonic cultures. In the experiments shown in Fig. 3, similar numbers of O-2A lineage cells per coverslip were found up until 6 days in culture in the

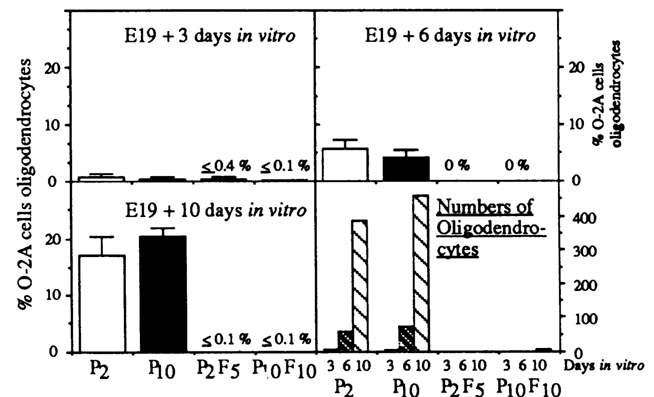

FIG. 3. Simultaneous application of bFGF and PDGF to cultures of embryonic optic nerve inhibits oligodendrocytic differentiation. In cultures derived from embryonic day 19 (E19) rats and grown in the presence of PDGF and bFGF there was, on average, <1 oligodendrocyte per coverslip on days 3 and 10 and no oligodendrocytes on day 6. The lack of increase in oligodendrocyte numbers with time suggests that a small number of O-2A progenitors were already fully committed to oligodendrocyte differentiation at the time of dissection, but that no further oligodendrocytes appeared once these initial cells differentiated. All oligodendrocytes and O-2A progenitors were counted on every coverslip until day 6 in all conditions and were similar in all conditions up until this time (see text for more detail). By day 10 the number of O-2A progenitors in cultures receiving PDGF and bFGF was so high as to make accurate counting impossible, so at this point all cells were counted only in the cultures exposed to PDGF alone, whereas in cultures receiving PDGF and bFGF the total numbers of oligodendrocytes were counted, and a minimum estimate was determined for the number of O-2A progenitors. It was clear, however, that at day 10 cultures grown in the presence of PDGF and bFGF contained many more O-2A lineage cells than did cultures grown in the presence of PDGF alone. The concentration of PDGF was either 2 (P_2) or 10 (P_{10}) ng/ml, and the concentration of bFGF was either 5 (F_5) or 10 (F_{10}) ng/ml, as indicated in the figure. Each time point represents the mean ± SEM of triplicate coverslips averaged over several experiments.

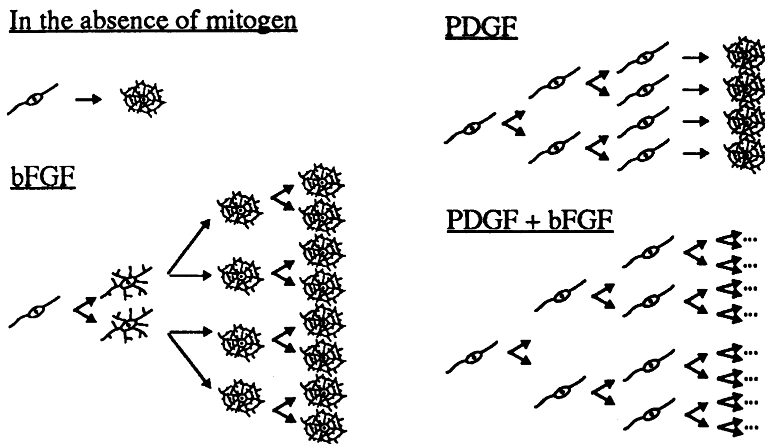

FIG. 4. Summary of the relationship between mitogen stimulation and the control of O-2A progenitor differentiation. In the absence of mitogen, progenitors do not proliferate and instead differentiate rapidly into multipolar oligodendrocytes. Limited proliferation of bipolar progenitor cells occurs in the presence of PDGF, followed by synchonous differentiation of clonally related cells into oligodendrocytes and a withdrawal from further cell division. Stimulation with bFGF, in contrast, is associated with division of progenitors as multipolar cells, premature oligodendrocytic differentiation, and continued proliferation of oligodendrocytes. Cooperation between PDGF and bFGF inhibits oligodendrocytic differentiation and causes O-2A progenitors to undergo continuous self-renewal.

presence of either PDGF or PDGF and bFGF. For example, cultures examined on day 6 contained 1356 ± 275 O-2A lineage cells per coverslip when grown in PDGF and 1336 ± 337 O-2A lineage cells per coverslip when grown in PDGF and bFGF. After this time, the number of O-2A lineage cells found in cultures grown in the presence of PDGF and bFGF was greater than that seen in the presence of PDGF alone because in the latter cultures many clonal families of cells ceased division upon oligodendrocytic differentiation (7).

DISCUSSION

We have found that O-2A progenitor cells can be induced to express a variety of distinct and multifaceted developmental programs *in vitro* by exposure to different defined growth factors (see Fig. 4 for a summary). Cells exposed to PDGF were bipolar, divided rapidly, were extensively motile, and differentiated into oligodendrocytes *in vitro* with a timing that mimicked that seen *in vivo* (6, 7). On the other hand, cells exposed to bFGF were multipolar, divided slowly, showed little migratory behavior, and underwent premature oligodendrocytic differentiation. In contrast to the generation of oligodendrocytes associated with exposure to either individual mitogen, progenitors exposed to a combination of PDGF and bFGF underwent continuous self-renewal in the absence of any oligodendrocytic differentiation.

Our results reveal a previously unknown means of modulating self-renewal of a precursor cell. Our data indicate that control of this process in O-2A progenitor cells resides in the cooperative interaction between two molecules more commonly known for their ability to modulate cell division than for their modulation of precursor differentiation. In contrast, studies on embryonic stem (ES) cells have demonstrated that inhibition of ES cell differentiation is induced by a factor (known as differentiation-inhibiting activity, or DIA; refs. 22 and 23) that seems to function as a modulator of differentiation in several cell types. Along with its effects on ES cells, DIA can also induce macrophage differentiation of the leukemic myeloid M1 cell line (where it is known as "LIF"; ref. 24) and can induce cholinergic differentiation of sympathetic neurons (where it is known as "CNDF"; ref. 25). We know of no other instances where the control of precursor self-renewal has been reduced to the action of a defined molecule or group of molecules.

The differences between the effects of PDGF and bFGF examined individually suggest that there must be qualitative differences in the signal transduction pathways stimulated by these mitogens. The ability of PDGF and bFGF to induce division of O-2A progenitors and also to differently influence the timing of oligodendrocyte production, the expression of cellular morphology, and the promotion of cell migration *in vitro* suggests that a combinatorial system of intracellular signals controls a complex behavioral repertoire in these cells. Moreover, the dependence of continuous self-renewal of O-2A progenitors upon the cooperative action of two mitogens, each of which is independently capable of promoting O-2A progenitor division, further suggests that induction of self-renewal in these cells requires simultaneous activation of multiple signal-transduction pathways.

Our results indicate that the relationship between the induction of cell division in O-2A progenitors and the promotion of appropriately timed oligodendrocytic differentiation is more complex than was previously envisaged. Results of studies conducted on O-2A progenitors grown in the presence of type-1 astrocytes or PDGF have suggested that the induction of division in these cells is associated with promotion of the activity of a biological clock, which limits clonally related families to a small number of cell divisions before there is a withdrawal from the cell cycle in association with commencement of oligodendrocytic differentiation (3, 7, 26). In contrast, our present results demonstrate that dividing O-2A progenitor cells can differentiate prematurely into oligodendrocytes when exposed to bFGF (and can continue to divide as oligodendrocytes) or can continue to divide repeatedly in the absence of differentiation when exposed to PDGF and bFGF. Thus, there is no necessary relationship between the induction of cell division and the control of oligodendrocytic differentiation.

It is important to note that it is necessary to reconcile our results with claims that bFGF is widely present in the central nervous system during development (19, 20). If bFGF availability were as widespread as has been suggested, then cooperation of this factor with PDGF in the central nervous system (8) would be expected to inhibit the differentiation of oligodendrocytes *in vivo*. Alternatively, if bFGF were produced in an accessible form *in vivo*, then there must exist an as yet undiscovered means of causing dividing progenitors to generate oligodendrocytes with an appropriate timing, adding a further level of complexity to the control of this developmental pathway.

We find it of particular interest that the inhibition of O-2A progenitor differentiation produced by cooperation of PDGF and bFGF resembles, at least superficially, the inhibition of differentiation induced by expression of simian virus 40 (SV40) large tumor (T) antigen in these cells (M.N. and H.L., unpublished observations). Such a similarity of effect raises the possibility that these rather different types of stimuli might overlap in their mechanism of action in ways that may have relevance for attempts to understand the molecular basis for the inhibition of differentiation associated with neoplastic transformation.

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