Cooperation Between PDGF and FGF Converts Slowly Dividing O-2A<sub>adult</sub> Progenitor Cells to Rapidly Dividing Cells with Characteristics of O-2A<sub>perinatal</sub> Progenitor Cells

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Abstract. We have shown previously that oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells isolated from adult rat optic nerves can be distinguished in vitro from their perinatal counterparts on the basis of their much slower rates of division, differentiation, and migration when grown in the presence of cortical astrocytes or PDGF. This behavior is consistent with in vivo observations that there is only a modest production of oligodendrocytes in the adult CNS. As such a behavior is inconsistent with the likely need for a rapid generation of oligodendrocytes following demyelinating damage to the mature CNS, we have been concerned with identifying in vitro conditions that allow O-2A<sub>adult</sub> progenitor cells to generate rapidly large numbers of progeny cells. We now provide evidence that many slowly dividing O-2A<sub>adult</sub> progenitor cells can be converted to rapidly dividing cells by exposing adult optic nerve cultures to both PDGF and bFGF. In addition, these O-2A<sub>adult</sub> progenitor cells appear to acquire other properties of O-2A<sub>perinatal</sub> progenitor cells, such as bipolar morphology and high rate of migration. Although many O-2A<sub>adult</sub> progenitor cells in cultures exposed to bFGF alone also divide rapidly, these cells are multipolar and migrate little in vitro. Oligodendrocytic differentiation of O-2A<sub>adult</sub> progenitor cells, which express receptors for bFGF in vitro, is almost completely inhibited in cultures exposed to bFGF or bFGF plus PDGF. As bFGF and PDGF appear to be upregulated and/or released after injury to the adult brain, this particular in vitro response of O-2A<sub>adult</sub> progenitor cells to PDGF and bFGF may be of importance in the generation of large numbers of new oligodendrocytes in vivo following demyelination.

Regeneration in some adult tissues is associated with rapid division of otherwise slowly dividing or quiescent populations of appropriate precursor cells. For example, in response to damage to skeletal muscle, a pool of quiescent muscle precursor cells (called satellite cells) become mitotically active and generate a population of myoblasts which subsequently fuse to form new muscle fibers (Allen and Rankin, 1990). To date, very little is known about the cellular and molecular mechanisms that promote regenerative events in adult tissues.

The ability of the adult central nervous system (CNS) to recover from damage to oligodendrocytes, the myelin-forming cells of the CNS, has been well documented. Remyelination appears to occur in many experimental models of CNS demyelination (Ludwin, 1981) and there is also evidence of limited myelin repair in patients suffering from the human demyelinating disease, multiple sclerosis (MS) (Prineas and Connell, 1979; Raine et al., 1981; Prineas et al., 1989).

To obtain insights into the mechanisms that may underlie myelin repair, we have been examining the biology of adult oligodendrocyte progenitors, cells that may provide a source for the oligodendrocytes that are needed to repair demyelinated lesion. Two lines of evidence suggest that oligodendrocyte progenitor cells, possibly in addition to mature oligodendrocytes (Aranella and Herndon, 1984; Ludwin, 1984; Ludwin and Bakker, 1988; Wood and Bunge, 1991), may be responsible for the generation of new oligodendrocytes following demyelinating damage. Firstly, Godfraind et al. (1989) found that after virally induced demyelination of mouse spinal cord, a population of cells with the antigenic profile of oligodendrocyte progenitor cells grown in vitro incorporated <sup>3</sup>H-thymidine and increased in number during the recovery phase. Secondly, many of the oligodendrocyte lineage cells that proliferate in response to experimentally induced demyelination (Ludwin, 1979, 1984; Areenla and Herndon, 1984) and those that are present at the edges of MS lesions (Raine et al., 1981) have an ultrastructure that resem-
bles that of oligodendrocyte progenitor cells isolated from adult CNS tissue and grown in vitro (Wolswijk et al., 1991a).

The identification of factors that may play a role in controlling the proliferation and differentiation of oligodendrocyte progenitor cells in regenerative responses in the adult brain is hampered by the relative complexity and inaccessibility of this tissue. To circumvent this, we have been using tissue culture approaches to study the biology of oligodendrocyte progenitor cells isolated from adult rat optic nerves, the simplest myelinated tracts of the CNS. Like their perinatal counterparts (Raff et al., 1983b), adult optic nerve-derived oligodendrocyte progenitor cells have the ability to differentiate into type-2 astrocytes in vitro (French-Constant and Raff, 1986; Wolswijk and Noble, 1989). However, oligodendrocyte-type-2 astrocyte (O-2A) progenitor cells derived from the optic nerves of mature and developing rats differ in many properties when such cells are grown in the presence of cortical astrocytes or PDGF (Wolswijk and Noble, 1989; Wren and Noble, 1989; Wolswijk et al., 1991b; Wren et al., 1992). The O-2A progenitor mitogen that is produced by cortical astrocytes in vitro (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988). In these conditions, O-2A 

-**Materials and Methods**

**Primary Cultures of Adult Optic Nerve Cells**

Adult optic nerve cells were isolated and cultured as described previously (Wolswijk and Noble, 1989; Wolswijk et al., 1990, 1991b). Optic nerves were dissected from adult Wistar or Sprague-Dawley rats (>21/2 mo old), minced finely with a scalpel blade, and incubated in 1.0 ml Leibovitz L-15 medium (Gibco, Paisley, Scotland) containing 333 i.u./ml collagenase (Worthington Biochemical Corporation, Freehold, NJ). After 60-90 min at 37°C, 1.0 ml 30,000 IU/ml bovine pancreas trypsin type III (Sigma, Dorset, England) in Ca²⁺, Mg²⁺-free DME (DME-CMF) was added, followed by a 20-min incubation at 37°C and a centrifugation at 3,000 g for 2 min. The supernatant was discarded and the tissue was resuspended and incubated further in 2.0 ml of 15,000 IU/ml trypsin and 0.27 mM EDTA (Sigma) in DME-CMF for 20 min at 37°C. The enzymatic digestion was terminated by addition of 4.0 ml SBTI-DNase (5,200 IU/ml soybean trypsin inhibitor Sigma), 74 IU/ml bovine pancreas DNase, and 3.0 mg/ml BSA fraction V (Sigma). The suspension was centrifuged for 2 min at 3,000 g and the tissue was resuspended in 1.0 ml DME + 10% FCS (DME [Gibco], 0.2 μM progesterone [Sigma], 0.1 μM putrescine [Sigma], 0.5 μM α, β-3-iodothyronine [Sigma], modified from Bottenstein and Ewing, 1979) and placed in Falcon 6-well plates (three to four coverslips per well) containing DME-B5 (DME with 4.5 g/liter glucose and supplemented with 25 μg/ml gentamicin, 2 mM glutamine, 0.234 IU/ml bovine pancreas insulin [Sigma], 100 μg/ml human transferrin [Sigma], 0.0286% (vol/vol) BSA, 0.1% bovine pituitary extract [Miles Laboratories, Inc.], 0.2 μM progesterone [Sigma], 0.1 μM putrescine [Sigma], 0.45 μM 3,5-iodothyronine [Sigma], 0.224 μM selenium [Sigma], and 0.5 μM 3,5,3'-triiodothyronine [Sigma], modified from Bottenstein and Sato [1979] as described previously (Wolswijk and Noble, 1989) and, where appropriate, supplemented with bFGF or supplemented with both PDGF-AA and bFGF. In most experiments, growth factors were added at a final concentration of 10 ng/ml. Growth factors were added to the cultures daily, while 90% of the culture medium was changed twice a week. Based on immunolabelings of cultures after 1 d in vitro, each coverslip containe...
120 ± 18 0-2A$^{addd}$ progenitor cells and 17 ± 2 process-bearing oligodendrocytes (mean ± SEM of five separate isolations). Very few astrocytes survived the isolation procedure: each culture contained only 4 ± 1 flat type-1 astrocyte-like cells and an occasional process-bearing type-2 astrocyte-like cell. Previous studies have suggested that type-1 astrocytes and type-2 astrocytes are separate and distinct populations of astrocytes in perinatal nervous tissue and that type-1 astrocytes resemble in their properties cortical astrocytes [Raff et al., 1983a; 1984a]. O-2A lineage cells in cultures of adult optic nerve have characteristic process-bearing cells and can be distinguished both morphologically and antigenically from the contaminating non-O-2A lineage cells. O-2A$^{addd}$ progenitor cells labeled with the A2B5 antibody, but not with antibodies against galactocerebroside (GalC, a marker for oligodendrocytes [Boehringer Mannheim]), and galial acidic protein (GFAP, an astrocyte-specific intermediate filament protein [Bignami et al., 1972]), oligodendrocytes were GalC$^+$ (and initially also A2B5$^+$) and type-2 astrocytes were A2B5$^+$GFAP$^+$. In some conditions described in the present paper, a small number of O-2A$^{addd}$ progenitor cells become A2B5$^+$ (see Table II). However, because such cells had a characteristic process-bearing morphology, they could still be identified unambiguously as O-2A$^{addd}$ progenitor cells. The majority of the non-O-2A lineage cells in the cultures contained vimentin intermediate filaments and had a fibroblast-like morphology. Although a very small proportion of the vimentin$^+$ flat cell bound the A2B5 antibody, they were not included in the population of O-2A lineage cells.

**Growth Factors**

Purified bovine brain bFGF was purchased from R and D Systems, Inc., while the recombinant human bFGF was obtained from either Boehringer Mannheim UK Ltd. (Lewes, England) or was a kind gift of Larry Coussens (Chiron Corporation, Emeryville, CA). C. George Nascimiento (Chiron Corporation) generously supplied us with recombinant human PDGF-AA. Lymphoid growth factors were dissolved in either 0.04 M HCl containing 5% (vol/vol) BSA PATH-O-CYTE$^+$ (PDGF-AA) or in L-15 supplemented with 5% BSA PATH-O-CYTE$^+$ 4 (bFGF). The solution was then filtered and diluted five times in L-15 to a final concentration of 1 μg/ml, aliquoted, and stored at -20°C. After defrosting, aliquots were kept at 4°C for not more than 2 d. As we obtained similar results with the purified bovine bFGF and the recombinant human bFGF, data from experiments with these two different sources of bFGF were pooled. However, the majority of the experiments were carried out with the recombinant human bFGF obtained from Chiron Corporation. Lymphoid human recombinant $^{125}$I-labeled bFGF was obtained from Amersham (starting activity of 900-1,000 Ci/mmol) and was dissolved in L-15 medium containing 5% BSA PATH-O-CYTE$^+$ and stored in aliquots at -20°C.

**Indirect Immunofluorescence**

Before immunolabeling, cultures growing on glass coverslips were fixed in 4.0% paraformaldehyde (TAAB Laboratories Equipment Ltd., Reading, England) in PBS (Gibco) for 15-30 min at room temperature, followed by several rinses in HBSS + 5% NCS (HBSS [Imperial Laboratories] containing 5% heat-inactivated newborn calf serum [NCS] and 0.2 M Hepes [Sigma]). The following antibodies (Abs) were used in the experiments: the mouse IgM mAb A2B5 (hybridoma supernatant, diluted three times; Eisenbarth et al., 1979), the mouse IgM mAb O4 (hybridoma supernatant, diluted three times; Sommer and Schachner, 1981), the mouse IgG anti-GalC mAb (hybridoma supernatant, diluted five times; Ranscht et al., 1982), and the mouse IgG anti-vimentin mAb (Boehringer Mannheim; used at a concentration of 4 μg/ml), the mouse IgG anti-BrdU mAb (Becton Dickinson Immunocytometry Systems, Cowley, England; the Ab solution was diluted 1:100), rabbit anti-cow GFAP polyclonal Abs (Dako Ltd., High Wycombe, England; diluted 1:10000), and rabbit antichicken FGF receptor antisera (UBI, Inc., Lake Placid, NY; diluted 1:30). To visualize the binding of the monoclonal and polyclonal Abs, fluorescent (F) or rhodamine (Rd)-conjugated secondary Abs were used (diluted 1:100; Southern Biotechnology Associates Inc., Birmingham, AL). In some experiments, the binding of the anti-GalC mAbs was visualized with biotinylated goat-anti-mouse IgG (Southern Biotechnology Associates Inc.; diluted 1:50), followed by a streptavidin (Strep) labeled biotinylated (Strep-Tactin) (Molecular Probes Inc., Eugene, OR; diluted 1:50). Abs were diluted in L-15 medium containing 5% heat-inactivated newborn calf serum with HBSS + 5% NCS. In the case of the anti-FGF antibodies, cells were incubated in the antibody solution overnight at 4°C.

In addition to the standard double-immunolabeling procedures we have described in detail previously (Wolswijk and Noble, 1989; Wolswijk et al., 1991a, 1991b), four triple-immunolabelings with three different fluorochromes (fluorescein, rhodamine, and coumarin) were carried out according to the following protocols. (Protocol A) Incubation 1, mAb O4; incubation 2, G-anti-MiG-M-F1 plus anti-GalC; incubation 3, mAb A2B5 plus G-anti-MiG-M-Gd plus anti-GalC; incubation 4, G-anti-MiG-M-Rd plus streptavidin-Cou; incubation 5, methanol (10-20 min, -20°C). This immunolabeling procedure allowed us to calculate the proportion of the O-2A$^{addd}$ progenitor cells (i.e., GalC$^+$ O-2A lineage cells) that were A2B5$^+$O4$^+$ (such cells were Rd$^+$Cou$^+$) and those that were A2B5$^+$O4$^+$ (such cells were Rd$^+$Cou$^+$). (Protocol B) Incubation 1, mAb A2B5; incubation 2, G-anti-MiG-M-F1 plus anti-GalC; incubation 3, mAb O4 plus biotinylated G-anti-MiG-M; incubation 4, G-anti-MiG-M-Rd plus streptavidin-Cou; incubation 5, methanol (10-20 min, -20°C). This immunolabeling procedure allowed us to calculate the proportion of the O-2A$^{addd}$ progenitor cells that were A2B5$^+$O4$^+$ (such cells were Rd$^+$Cou$^+$) and those that were A2B5$^+$O4$^+$ (such cells were Rd$^+$Cou$^+$). In addition, this immunolabeling procedure allowed us to determine the proportion of the GalC$^+$ oligodendrocytes that were A2B5$^+$ (such cells were Cou$^+$Rd$^+$). (Protocol C) Incubation 1, mAbs A2B5, O4, and anti-GalC; incubation 2, G-anti-MiG-M-Rd plus biotinylated-G-anti-MiG-M; incubation 3, streptavidin-Cou; incubation 4, methanol (10-20 min, -20°C); incubation 5, anti-tubulin mAbs; incubation 6, G-anti-MiG-M-Cou. This immunolabeling procedure allowed us to determine the proportion of the O-2A$^{addd}$ progenitor cells that contained vimentin intermediate filaments. (Protocol D) Incubations 1-4 were the same as those of Protocol C; incubation 5, anti-GFAP Abs; incubation 6, goat-anti-rabbit IgF. This procedure allowed us to determine the proportion of the GalC$^+$ O-2A lineage cells that were GFAP$^+$ type-2 astrocytes. After the immunolabeling, coverslips were rinsed several times in HBBS + 5% NCS and distilled water, mounted in a drop of anti-fade (glycerol containing 22 mM 1,4-diazobicyclo[2.2.2]octane; Johnson et al., 1982) and sealed with clear nail varnish. Cultures were viewed on a Zeiss Axiopt microscope equipped with phase-contrast and epi-UV illumination and selective filters for rhodamine, fluorescein, and coumarin, using a ×40 Plan NEOFLUAR objective. Immunolabeled cells were photographed using Ilford XP2 400 films.

**bFGF Incorporation Assay**

To determine whether cells were synthesizing DNA, cultures were incubated in the presence of 10 μM 5-bromodeoxyuridine (BrdU; Sigma) for a total period of 24 h. The incorporation of BrdU into the nuclei of those cells that had synthesized DNA was visualized using anti-BrdU mAbs (Gratzner, 1982), as described before (Wolswijk et al., 1991b). Before anti-BrdU m Abs were applied, methanol-fixed (10-20 min, -20°C) cells were exposed first to 0.02% paraformaldehyde in HBBS + 5% NCS for 60 s and then to 0.07 M NaOH for 7-10 min. After each incubation, coverslips were rinsed several times in HBBS + 5% NCS.

**125I-bFGF Labeling of Adult Optic Nerve Cultures and Autoradiography**

Adult optic nerve cells growing on glass coverslips were washed once in L-15 medium containing 5% FCS (binding buffer) and incubated for 1 h at room temperature in 50 μl binding buffer containing various concentrations of $^{125}$I-bFGF with or without a 100-fold excess of unlabeled recombinant human bFGF or unlabeled recombinant human PDGF-AA. After the radiolabeling, cultures were fixed in 4% paraformaldehyde, immunolabeled, washed in distilled water, dehydrated in an ascending series of ethanol, and air-dried. Coverslips were mounted face-up onto glass slides using Gurr fluoromount mountant (BDH Chemicals Ltd.). The following day, slides were dipped twice in Ilford K2 autoradiographic emulsion (diluted in an equal volume of distilled water) and allowed to dry overnight. Slides were exposed at 4°C for varying periods of time (14-32 d) to compensate for differences in the activity of the batch of $^{125}$I-bFGF, the activity of which ranged from 550-920 μCi/mmol. Slides were developed in Ilford Contrast FF (diluted 1:10) and fixed in Ilford Hyperm (diluted 1:5). A second coverslip was mounted in anti-fade on top of the cells and was sealed with nail varnish. Cells were examined using a ×100 Plan NEOFUOR objective and the number of silver grains above the cell body and main processes of O-2A$^{addd}$ progenitor cells in the various conditions was determined using a counting grid.
Results

bFGF Is a Potent Mitogen for O-2A und Progenitor Cells

bFGF induced DNA synthesis in O-2A und progenitor cells in a dose-dependent manner. Cells derived from the optic nerves of adult rats (>2 1/2 mo old) were cultured for 3 d in DME-BS supplemented with various concentrations of bFGF. Stimulation of DNA synthesis in O-2A und progenitor cells (identified as A2B5+GalC - cells) in response to bFGF was monitored by the incorporation of BrdU during a 24-h terminal pulse. Maximal levels of BrdU incorporation in O-2A und progenitor cells occurred at a concentration of ~10 ng/ml bFGF, when 460 ± 2.5% of O-2A und progenitor cells were BrdU+ (60 ± 4 cells per culture; Fig. 1). Half-maximal effects occurred with a dose of ~1 ng/ml bFGF (Fig. 1). Even with a dose as low as 0.05 ng/ml bFGF, 10.1 ± 2.0% of the 103 ± 4 O-2A und progenitor cells in the cultures had incorporated BrdU. In contrast, no O-2A und progenitor cells were stimulated to synthesize DNA when adult optic nerve cells were grown in DME-BS lacking growth factors, as shown previously (Wolswijk et al., 1988), in preventing the oligodendrocytic differentiation of O-2A und progenitor cells that is seen in DME-BS lacking growth factors. Only 30 ± 1.5% of the O-2A lineage cells in the cultures expressed the oligodendrocyte marker galactocerebroside (GalC; Raff et al., 1978) when adult optic nerve cells were grown for 3 d in the presence of 10 ng/ml bFGF. In contrast, 19.5 ± 2.3% of the O-2A lineage cells were GalC+ in adult optic nerve cultures treated for 3 d with 10 ng/ml PDGF (see also Wolswijk et al., 1991b). When adult optic nerve cells were grown in the absence of growth factors, O-2A und progenitor cells differentiated prematurely into oligodendrocytes (as described previously [Wolswijk and Noble, 1989]), such that after 3 d of growth in DME-BS, 47.5 ± 3.2% of the O-2A lineage cells expressed GalC.

Very few cells in the cultures expressed the antigenic phenotype of type-2 astrocytes after 5 and 8 d of exposure to bFGF (data not shown), suggesting that bFGF did not induce astrocytic differentiation of O-2A und progenitor cells.

O-2A und Progenitor Cells Bind Both 125I-bFGF and FGF Receptor Antibodies

O-2A und progenitor cells bound 125I-bFGF in a dose-dependent manner and the binding of radiolabeled bFGF was reduced greatly when cells were incubated additionally in an
Figure 2. Direct binding of $^{125}$I-bFGF to O-2A$^{\text{adult}}$ progenitor cells. Adult optic nerve cells were grown for 4 d in 10 ng/ml PDGF-AA and then incubated in 0.1, 0.33, 1.0, or 3.3 ng/ml $^{125}$I-bFGF with or without a 100-fold excess of unlabeled bFGF or PDGF-AA, immunolabeled, and processed for autoradiography as described in Materials and Methods. The total number of silver grains above the cell body and main process of ten A2B5$^+$GalC$^-$ O-2A$^{\text{adult}}$ progenitor cells in each condition was determined. The graph shows that the number of silver grains above O-2A$^{\text{adult}}$ progenitor cells increased with increasing concentrations of $^{125}$I-bFGF. Addition of a 100-fold excess of unlabeled bFGF at the indicated concentrations of $^{125}$I-bFGF reduced significantly the number of grains/cell ($P$ value <0.001, $t$ test), while addition of a 100-fold excess unlabeled PDGF-AA at each concentration of $^{125}$I-bFGF did not have a significant effect on the binding of radiolabeled bFGF (0.11 < $P$ < 0.73). Each symbol represents the mean ± SD of one experiment.

excess of unlabeled bFGF. In these experiments, adult optic nerve cultures that had been maintained for 4 d in 10 ng/ml PDGF-AA were incubated in increasing concentrations of $^{125}$I-bFGF, immunolabeled and processed for autoradiography. Adult optic nerve cells were grown in PDGF-AA instead of bFGF to prevent possible down regulation of FGF receptors as a result of exposure to bFGF. The specificity of the binding of radiolabeled bFGF was demonstrated by incubating adult optic nerve cultures in the presence of $^{125}$I-bFGF and a 100-fold excess of unlabeled bFGF or PDGF-AA.

The number of silver grains over O-2A$^{\text{adult}}$ progenitor cells increased significantly from 16 ± 8 grains/cell at a concentration of 0.1 ng/ml $^{125}$I-bFGF to 153 ± 59 grains/cell at a concentration of 3.3 ng/ml $^{125}$I-bFGF (Fig. 2). Only 1 ± 1 silver grains were found above O-2A$^{\text{adult}}$ progenitor cells that had been incubated with the binding buffer only. The addition of a 100-fold excess unlabeled bFGF at each concentration reduced the binding of $^{125}$I-bFGF to the cells by >70% (Fig. 2). For example, the number of silver grains per O-2A$^{\text{adult}}$ progenitor cell was reduced by 94.7 ± 2.9% when adult optic nerve cells were exposed to 1.0 ng/ml $^{125}$I-bFGF plus a 100-fold excess of unlabeled bFGF (mean ± SEM of three separate experiments). The number of grains per O-2A$^{\text{adult}}$ progenitor cell was reduced by only 9.5 ± 7.2% when cells were exposed to 1 ng/ml $^{125}$I-bFGF plus a 100-fold excess of unlabeled PDGF-AA; this reduction was not significant. More silver grains were found generally over the non-O-2A lineage cells in the cultures (data not shown).

The possibility that O-2A$^{\text{adult}}$ progenitor cells express FGF receptors was substantiated further by the observation that such cells bound antibodies against the chicken FGF receptor. We found that all O-2A$^{\text{adult}}$ progenitor cells that had been grown for 3-5 d in 10 ng/ml PDGF-AA or bFGF bound the anti-FGF receptor antibodies (although most cells were only weakly positive; Fig. 3). Oligodendrocytes and the non-O-2A lineage cells in the adult optic nerve cultures also bound the antibody. However, as was the case in the $^{125}$I-bFGF binding studies, the non-O-2A lineage cells in the adult optic nerve cultures were generally more strongly labeled with the anti-FGF receptor antibodies than O-2A$^{\text{adult}}$ progenitor cells and oligodendrocytes (see Fig. 3). Thus, the $^{125}$I-bFGF and immunolabeling studies suggest that O-2A$^{\text{adult}}$ progenitor cells express FGF receptors and that bFGF acts directly on these cells.

Figure 3. O-2A$^{\text{adult}}$ progenitor cells bind anti-FGF receptor antibodies. Adult optic nerve cultures that had been grown for 4 d in 10 ng/ml PDGF-AA were immunolabeled with the A2B5 antibody (a) and antichick FGF receptor polyclonal antibodies (b) followed by fluorochrome-conjugated second antibodies. Like all other O-2A$^{\text{adult}}$ progenitor cells in the culture, the A2B5$^+$ cell shown in the figure was FGF receptor positive. A strongly FGF receptor positive A2B5$^+$ non-O-2A lineage cell is indicated with an arrow. (a) Rhodamine optics; (b) fluorescein optics. Bar, 10 μm.
Table I. Increases in the Number of O-2A Lineage Cells Following Growth in bFGF or PF

| Number of days in vitro | Condition | Total number of O-2A lineage cells | Number of O-2A<sup>ablat</sup> progenitors | Number of oligodendrocytes |
|------------------------|-----------|-----------------------------------|------------------------------------------|---------------------------|
| 1                      | bFGF      | 145 ± 38                          | 129 ± 36                                  | 17 ± 3                    |
|                        | PF        | 147 ± 35                          | 128 ± 33                                  | 20 ± 3                    |
| 5                      | bFGF      | 547 ± 63                          | 520 ± 64                                  | 27 ± 10                   |
|                        | PF        | 693 ± 114                         | 671 ± 108                                 | 22 ± 7                    |
| 8                      | bFGF      | 1,006 ± 18                        | 913 ± 54                                  | 93 ± 36                   |
|                        | PF        | 1,525 ± 111                       | 1,482 ± 107                               | 44 ± 5                    |

Adult optic nerve cells were cultured in DME-BS supplemented with either bFGF or PF. After 1, 5, and 8 d of in vitro growth, cultures were immunolabeled and the total number of O-2A<sup>ablat</sup> progenitor cells and oligodendrocytes present on each coverslip in each condition was determined. A small proportion of the O-2A<sup>ablat</sup> progenitor cells was not labeled with the A2B5 or O4 antibody (see Table II), but such cells were identified as being O-2A<sup>ablat</sup> progenitor cells on the basis of their morphology, which was indistinguishable from O-2A<sup>ablat</sup> progenitor cells that had bound these antibodies. The O-2A lineage population expanded with a doubling time of 60 h between day 1 and day 8 when adult optic nerve cells were cultured in bFGF alone, while the O-2A lineage population in cultures exposed to PF expanded with a 50-h doubling time during the same period. However, the most rapid increase in the number of O-2A lineage cells in both conditions occurred during the first 5 d in culture. The data shown are the mean ± SEM of a minimum of nine cultures from three separate experiments.

The O-2A Lineage Population Expands Rapidly When Cultured in bFGF

Prolonged growth of adult optic nerve cells in the presence of bFGF was associated with more rapid increases in the number of O-2A lineage cells than when such cultures were treated with PDGF-AA or cortical astrocyte-derived PDGF. For example, the average number of O-2A lineage cells per coverslip increased 6.9-fold, from 145 ± 38 cells on day 1 to 1,006 ± 18 cells on day 8, when adult optic nerve cultures were maintained in DME-BS supplemented with 10 ng/ml bFGF (Table I). The increase in the number of O-2A lineage cells over this 7-d period was almost twofold higher than when adult optic nerve cultures were exposed to 10 ng/ml PDGF-AA (3.5-fold increase in the number of O-2A lineage cells during the same period of culture; see also Wolswijk et al., 1991b).

The most rapid increase in the number of O-2A lineage cells occurred between day 1 and day 5 (Table I), when the O-2A lineage population increased by 3.8-fold, corresponding to a doubling time for the total O-2A lineage population of 50 h. In contrast, there was only a 1.8-fold increase in the number of O-2A lineage cells between day 5 and day 8 of in vitro growth (Table I), corresponding to a population doubling time of 82 h.

Although the largest expansion occurred in the O-2A<sup>ablat</sup> progenitor population, the number of oligodendrocytes increased 5.5-fold between day 1 and day 8 of in vitro growth (Table I). This suggests that bFGF was not able to inhibit completely oligodendrocytic differentiation of O-2A<sup>ablat</sup> progenitor cells. This was substantiated by the observation that 91 ± 68% of the oligodendrocytes were labeled with the A2B5 antibody after 8 d in vitro, an antigenic phenotype that appears to characterize newly generated oligodendrocytes (Raff et al., 1983b). As following a 24-h pulse with BrdU some oligodendrocytes (<5%) were BrdU<sup>+</sup> on day 5 and day 8, the increase in the number of oligodendrocytes could have been due in part to division of these cells. This observation is in agreement with previous suggestions that bFGF is a mitogen for oligodendrocytes (Eccleston and Silberberg, 1985; Saneto and de Vellis, 1985; Bögl er et al., 1990).

Many O-2A<sup>ablat</sup> Progenitor Cells Cultured in bFGF Divide Rapidly, Migrate Slowly, and Have a Multipolar Morphology

To determine directly whether O-2A<sup>ablat</sup> progenitors exposed to bFGF had a cell cycle time that was shorter than the 59 ± 5 h cell cycle of O-2A<sup>ablat</sup> progenitor cells treated with PDGF-AA (Wolswijk et al., 1991b), adult optic nerve cultures were grown in DME-BS supplemented with 10 ng/ml bFGF and followed between day 1 and day 9 of in vitro growth using time-lapse cinemicroscopy (see Materials and Methods). Cell cycle times of individual O-2A<sup>ablat</sup> progenitor cells (identified on the basis of their characteristic process-bearing morphology) could only be measured if such cells remained in the field of photography and divided at least twice. Thus, O-2A<sup>ablat</sup> progenitor cells that had very long cell cycles (>100 h) were not included in the analysis.

We found that O-2A<sup>ablat</sup> progenitor cells cultured in bFGF had an average cell cycle time of 38.2 ± 3.9 h (n = 45; Fig. 4) and that these cells underwent their first division 58 ± 3 h after the cultures had been prepared. A large variation in the cell cycle lengths of individual O-2A<sup>ablat</sup> progenitor cells was observed (Fig. 4). For example, 23 cells (51%) had a cell cycle time of less than 30 h, while 13 cells (29%) had a cell cycle time of over 50 h. Some O-2A<sup>ablat</sup> progenitor cells did not divide during the filming period (10% of the O-2A<sup>ablat</sup> progenitor cells that were in the field of photography when the filming started), while others only divided once (12.5% of the starting cells).

The time-lapse cinemicroscopy studies also revealed that bFGF was able to induce motility in O-2A<sup>ablat</sup> progenitor cells. Proliferating O-2A<sup>ablat</sup> progenitor cells exposed to bFGF migrated with average speeds of 4.9 ± 0.7 μm/h (Fig. 4), which is very similar to the average rate of migration of O-2A<sup>ablat</sup> progenitors cultured in PDGF-AA (4.1 ± 0.6 μm/h; Wolswijk et al., 1991b) or in medium conditioned by purified cortical astrocytes (Astro-CM) (4.3 ± 0.7 μm/h; Wolswijk and Noble, 1989). Although we observed some variation in the migration rates of individual O-2A<sup>ablat</sup> progenitor cells, 84% (38/45 cells) migrated with speeds of <10 μm/h (Fig. 4). The majority of the proliferating and migrating O-2A<sup>ablat</sup> progenitor cells expressed a multipolar morphology (Figs. 4 and 5). However, five cells, all of which were members of one family, had the bipolar morphology characteristic of O-2A<sup>ablat</sup> progenitor cells cultured in PDGF or Astro-CM (Temple and Raff, 1986; Fig. 4). Like such O-2A<sup>ablat</sup> progenitor cells (Small et al., 1987; Noble et al., 1988), these five cells also divided and migrated rapidly (Fig. 4; average cell cycle time: 15.7 ± 2.4 h; average rate of migration: 14.8 ± 3.8 μm/h).

O-2A<sup>ablat</sup> progenitor cells grown for 5 or 8 d in bFGF were still bipotential and differentiated into oligodendrocytes when the medium of the adult optic nerve cultures was changed to DME-BS and differentiated into type-2 astrocytes when the culture medium was changed to DME + 10% FCS (data not shown).
The Number of O-2A Lineage Cells Increases More Rapidly When Exposed Simultaneously to bFGF plus PDGF-AA than When These Cells Are Exposed to bFGF Alone

The O-2A lineage population increased more rapidly when adult optic nerve cells were cultured in bFGF plus PDGF-AA (PF) than when such cells were exposed to either PDGF-AA or bFGF alone. For example, the number of O-2A lineage cells on each coverslip increased 10.4-fold between day 1 and day 8 of in vitro growth, from 147 ± 35 cells to 1,525 ± 111 cells in cultures exposed to PF (Table I), corresponding to a population doubling time of ~50 h. As was the case for adult optic nerve cultures grown in bFGF, >90% of the O-2A lineage cells in the cultures were O-2A<sub>adult</sub> progenitor cells (Table I and II). The most rapid increase in the number of O-2A lineage cells occurred between day 1 and day 5 when the O-2A lineage population expanded with a doubling time of 43 h (Table I).

Two observations suggested that PF was more effective in inhibiting the differentiation of O-2A<sub>adult</sub> progenitor cells into oligodendrocytes than bFGF. (a) The number of oligodendrocytes increased much more slowly when adult optic nerve cells were cultured in PF than when such cells were grown in bFGF alone (Table I). (b) The proportion of O-2A lineage cells that were oligodendrocytes was lower in cul-
Many O-2A<sub>adult</sub> Progenitor Cells Exposed Simultaneously to PF Divide and Migrate Rapidly and Possess a Bipolar Morphology

To examine whether O-2A<sub>adult</sub> progenitor cells exposed to PF divided more rapidly than O-2A<sub>adult</sub> progenitor cells cultured in bFGF alone, cultures of adult optic nerve were followed between day 1 and day 10 of in vitro growth using time-lapse cinemicroscopy. O-2A<sub>adult</sub> progenitor cells exposed to PF divided for the first time 60 ± 5 h after the cultures had been prepared. We found that the 44 O-2A<sub>adult</sub> progenitor cells examined divided with an average cell cycle time of 31.1 ± 2.7 h (Fig. 4). 26 of these O-2A<sub>adult</sub> progenitor cells (59%) had a cell cycle length of <30 h (average: 19.1 ± 0.9 h), like O-2A<sub>primitive</sub> progenitor cells (Noble et al., 1988). Seven cells (16%) divided very slowly, with cell cycle times of over 50 h (average: 63.5 ± 4.3 h) (Fig. 4). Furthermore, 11.4% of the starting cells did not divide during the filming period, while a further 11.4% divided only once.

Many proliferating O-2A<sub>adult</sub> progenitor cells growing in cultures exposed to PF migrated rapidly, with average speeds of 14.9 ± 1.1 μm/h (Fig. 4). 25% (14/44) of the O-2A<sub>adult</sub> progenitor cells, like O-2A<sub>primitive</sub> progenitor cells (Small et al., 1987; Noble et al., 1988), were highly motile and migrated with speeds of over 20 μm/h, with some cells achieving average speeds of >30 μm/h (Fig. 4). Only two cells had a rate of migration of <2 μm/h. O-2A<sub>adult</sub> progenitor cells with a cell cycle time of >50 h tended to migrate at slower rates than those with a cell cycle time of <50 h (86 ± 2.0 μm/h [n = 7] versus 161.1 ± 1.2 μm/h [n = 37]).

80% (35/44) of the O-2A<sub>adult</sub> progenitor cells growing in cultures of adult optic nerve exposed to PF had a bipolar morphology (Figs. 4 and 5) and resembled morphologically O-2A<sub>primitive</sub> progenitor cells cultured in PDGF or Astro-CM (Temple and Raff, 1986). In total, 47% (21 cells) of the O-2A<sub>adult</sub> progenitor cells examined in cultures of adult optic nerve exposed to PF expressed O-2A<sub>primitive</sub>-like characteristics, i.e., were bipolar, divided with a cell cycle of <30 h, and migrated with average speeds of >10 μm/h (Fig. 4).

As was the case for O-2A<sub>adult</sub> progenitor cells exposed to bFGF alone, cells that had been treated for 5 or 8 d with PF were still able to differentiate into oligodendrocytes or type-2 astrocytes depending on the constituents of the culture medium (data not shown).

Growth of O-2A<sub>adult</sub> Progenitor Cells in bFGF or PF Is Associated With the Acquisition of an O4-Vimentin Antigenic Phenotype

After 1 d of growth in DME-BS supplemented with bFGF or PF, the vast majority of the O-2A<sub>adult</sub> progenitor cells
Figure 5. O-2A<sup>adult</sup> progenitor cells exposed to bFGF are multipolar, vimentin<sup>+</sup> cells, while many O-2A<sup>adult</sup> progenitor cells grown in PF have a bipolar morphology and contain vimentin. Cells derived from the optic nerves of adult rats were maintained for 5 d in either bFGF alone (a–c) or PF (d–f), immunolabeled with A2B5 (a and d), anti-GalC (not shown), and antivimentin antibodies (b and e), followed by fluorochrome-conjugated second antibodies. The photographs illustrate that many O-2A<sup>adult</sup> progenitor cells cultured in bFGF had a multipolar morphology and acquired vimentin intermediate filaments, and that many O-2A<sup>adult</sup> progenitor cells maintained in PF were bipolar and vimentin<sup>+</sup>. Note that because of the limited migratory capacities of O-2A<sup>adult</sup> progenitor cells exposed to bFGF, colonies generated by O-2A<sup>adult</sup> progenitor cells were much more compact than those in PF. (a and d) Rhodamine optics; (b and e) fluorescein optics; (c and f) phase-contrast optics. Bar, 50 μm.

were labeled with both A2B5 and O4 mAbs and were devoid of vimentin intermediate filaments (Table II), as shown previously (Wolswijk and Noble, 1989). In contrast, virtually all O-2A<sup>adult</sup> progenitor cells exposed to either bFGF or PF for several days acquired vimentin filaments in vitro (Table II and Fig. 5). For example, >90% of the O-2A<sup>adult</sup> progenitor cells contained vimentin in both conditions after 5 and 8 d of culture (Table II). Furthermore, the proportion of O-2A<sup>adult</sup> progenitor cells that were A2B5<sup>+</sup>O4<sup>-</sup> increased substantially in both conditions from <10% on day 1 to >65% on day 5 (Table II). After 8 d of culture in PF, 69.7 ± 11.0% O-2A<sup>adult</sup> progenitor cells were still A2B5<sup>+</sup>O4<sup>-</sup>. In contrast, only 18.0 ± 3.7% of the O-2A<sup>adult</sup> progenitor cells grown in bFGF alone were A2B5<sup>+</sup>O4<sup>-</sup> after 8 d in vitro, while 75.5 ± 1.1% of these cells were A2B5<sup>+</sup>O4<sup>+</sup> (Table II). In these conditions, a small proportion (<5%) of the O-2A<sup>adult</sup> progenitor cells grown in bFGF or PF were A2B5<sup>+</sup>O4<sup>-</sup> when cultures were examined on day 5 and day 8 (Table II). As such cells expressed the morphology characteristic of O-2A<sup>adult</sup> progenitor cells, they were considered to be O-2A<sup>adult</sup> progenitor cells. Thus, our immunolabeling studies suggest that many O-2A<sup>adult</sup> progenitor cells cultured in bFGF or PF acquired the O4-vimentin<sup>+</sup> antigenic phenotype typical of O-2A<sup>present</sup> progenitor cells (Raff et al., 1984b; Wolswijk et al., 1990; Sommer, I. and M. Noble, unpublished observations) and that this antigenic phenotype
was retained more effectively when O-2A adult progenitor cells were grown in PF than when such cells were cultured in bFGF.

Discussion

We have found that growth of adult optic nerve cells in the presence of bFGF or in the presence of PF was associated with more rapid increases in the number of O-2A lineage cells than when such cells were exposed to PDGF-AA. The O-2A lineage population expanded most rapidly when adult optic nerve cells were cultured in PF. In addition, O-2A adult progenitor cells exposed to either bFGF or PF expressed a distinct pattern of cellular behavior which was unlike that of O-2A adult progenitor cells grown in PDGF-AA. In particular, PF elicited the expression of O-2A progenitor-like properties in many O-2A adult progenitor cells. In addition, bFGF and PF almost completely prevented the differentiation of O-2A adult progenitor cells into oligodendrocytes.

O-2A adult Progenitor Cells Express Phenotypic Plasticity In Vitro

We have shown previously that O-2A adult progenitor cells exposed to PDGF-AA had a unipolar morphology expressed an O4+vimentin- antigenic phenotype, and divided and migrated slowly (Wolswikj et al., 1991b). O-2A adult progenitor cells expressed the same range of properties as cells cultured in the presence of purified cortical astrocytes (Wolswikj and Noble, 1989; Wolswikj et al., 1990), which secrete PDGF in vitro (Noble et al., 1988; Raff et al., 1988; Richardson et al., 1988). However, it appears that the phenotype we have described previously for O-2A adult progenitor cells only applies to cells grown in the presence of PDGF or cortical astrocytes.

In our present study, we have shown that many O-2A adult progenitor cells in cultures of adult optic nerve exposed to bFGF alone were prevented from differentiating into oligodendrocytes, had a multipolar morphology, and divided relatively rapidly, but showed little motility. Many O-2A adult progenitor cells treated with bFGF alone gained vimentin intermediate filaments and became transiently O4-.

Exposure of adult optic nerve cultures to PF elicited the expression in O-2A adult progenitor cells of many of the characteristics we observed previously for O-2A progenitor cells cultured in PDGF or Astro-CM, such as bipolar morphology, short cell cycle time, high rate of migration, and O4+vimentin+ antigenic phenotype. This observation is of particular interest in the light of our recent time-lapse cinemicroscopy studies which have suggested that O-2A adult progenitor cells are derived directly from a subpopulation of O-2A progenitor cells in cultures of developing optic nerve exposed to Astro-CM (Wren et al., 1992). Thus, our present results suggest that the molecular mechanisms responsible for the expression of the O-2A progenitor phenotype are not irreversibly inactivated with the generation of O-2A adult progenitor cells.

The observation that O-2A adult progenitor cells bound higher amounts of bFGF and anti-FGF receptor antibodies suggests that bFGF acts directly on these cells and not indirectly through other non-O-2A lineage cells in the cultures. However, it can not be excluded that growth factors secreted by non-O-2A lineage cells in the cultures cooperated with bFGF or PF to induce the observed change in O-2A adult progenitor phenotype when cultures were exposed to bFGF or PF.

The Population of O-2A adult Progenitor Cells Is Heterogeneous in Its Response to bFGF and PF

It is important to note that O-2A adult progenitor cells were heterogeneous in their response to either bFGF or PF. For example, although the majority of O-2A adult progenitor cells cultured in bFGF were multipolar, a small number of such cells were bipolar. Interestingly, these O-2A adult progenitor cells all belonged to one family and had short cell cycle times and high rates of migration, i.e., had characteristics of O-2A progenitor cells. Thus, these few O-2A adult progenitor cells were able to express O-2A progenitor-like properties in cultures exposed to bFGF alone. In addition, some O-2A adult progenitor cells did not divide during the filming period or divided only once when grown in the presence of bFGF or PF. This suggests that some O-2A adult progenitor cells had long cell cycles in such conditions. Similar observations were made in cultures of adult optic nerve cells growing on monolayers of purified cortical astrocytes, where we found that some oligodendrocyte-free O-2A adult progenitor colonies contained <16 cells even after 25 d of culture (Wren et al., 1992). Whether such O-2A adult progenitor cells are part of a small population of relatively quiescent cells which only divide occasionally in the presence of mitogen(s) needs to be analyzed further.

The In Vitro Response of O-2A adult Progenitor Cells to PF may be of Importance in the Generation of New Oligodendrocytes in Vivo Following Oligodendrocyte Cell Death

We have suggested previously (Wolswikj and Noble, 1989; Noble et al., 1991) that the pattern of cellular behavior expressed by O-2A adult progenitor cells grown in the presence of cortical astrocytes or PDGF may be appropriate for the generation of the small numbers of new oligodendrocytes that may be needed as part of the slow turnover of glial cells in the adult CNS (McCarthy and Leblond, 1988). However, such a pattern of behavior may not allow the generation of the large numbers of oligodendrocytes that would be needed to repair demyelinated lesions in the adult brain. The simultaneous exposure of O-2A adult progenitor cells to PF would allow rapid increases in the number of O-2A adult progenitor cells (as observed in lesions of mice recovering from virally-induced demyelination [Godfraind et al., 1989]) through an increase in the rate of division of these cells and through active migration of O-2A adult progenitor cells into the lesion site. Subsequent differentiation of these cells into oligodendrocytes, followed by re-ensheathment of the denuded axons, could then restore proper impulse conduction.

Is injury to the mature CNS associated with release of PDGF and FGF into the lesion site? Several studies have suggested that both PDGF and FGF and their mRNAs are upregulated transiently in response to mechanical injury to the adult brain (Finklestein et al., 1988; Logan, 1988, 1990; Nieto-Sampedro et al., 1988; Lotan and Schwartz, 1992). It has been suggested that the increased levels of FGF in lesion sites may be the result of release of FGF from damaged neurones, which express acidic FGF and bFGF and their
provided evidence that neurones express PDGF, as well as cytes (Finkelstein et al., 1988) and invading macrophages (Baird et al., 1986). Furthermore, as recent studies have provided evidence that neurones express PDGF, as well as PDGF transcripts (Yeh et al., 1991; Sasahara et al., 1991), damaged neurones also could be a source of PDGF. A further source of PDGF could be endothelial cells (Hermansson et al., 1988) and the astrocytes that proliferate in response to injury, since studies by Raff et al. (1983a) and Miller et al. (1986) have suggested that glial scars may be formed by cortical astrocyte-like cells, the cells that secrete PDGF in vitro. Even though FGF and PDGF may be available to cortical astrocyte-like cells, the cells that secrete PDGF in vitro, it remains to be determined whether these factors are also present in lesions induced by viruses, chemicals, or by immunological means, as these are the experimental lesions that have been studied most extensively with respect to myelin repair (Ludwin, 1981). Furthermore, as there is evidence that limited remyelination occurs in the brains of patients suffering from the human demyelinating disease multiple sclerosis (Prineas and Connell, 1979; Raine et al., 1981; Prineas et al., 1989), it will be of interest to investigate whether FGF and PDGF are expressed in regions of demyelinating damage in humans.

The generation of large numbers of O-2A lineage cells as a result of exposure of O-2A progenitor cells to both PDGF and bFGF in vivo would be clearly inappropriate if continued after sufficient numbers of new cells have been generated. Thus, mechanisms must exist which limit the proliferative response. As the levels of FGF appear to be increased only transiently after CNS damage (Finkelstein et al., 1988; Logan, 1988; Nieto-Sampedro et al., 1988), the reduction in local concentrations of FGF and PDGF could be associated with a reduction in the rate of proliferation of O-2A progenitor cells and their differentiation into oligodendrocytes. In addition, since the increase in the number of O-2A lineage cells in adult optic nerve cultures exposed to PF was less dramatic between day 5 and day 8 of in vitro growth as compared to the increase between day 1 and day 5, it may be that O-2A progenitor cells are limited intrinsically in their ability to generate large numbers of cells.

In addition to the generation of new oligodendrocytes by O-2A progenitor cells in response to demyelination, proliferation of mature oligodendrocytes may be important in the recovery process. Uptake of radiolabeled thymidine by mature oligodendrocytes has been observed in vivo after lysolecithin-induced demyelination (Aranella and Herndon, 1984) and after trauma to the adult brain (Ludwin, 1984). In addition, Ludwin and Bakker (1988) found that even differentiated oligodendrocytes attached to myelin incorporated radiolabeled thymidine after wounding of the cortex of adult mice. However, only a comparatively small proportion of mature oligodendrocytes were radiolabeled in these studies. Furthermore, in vitro studies have suggested that oligodendrocytes are able to divide in response to bFGF (Saneto and De Vellis, 1985; Eccleston and Silberberg, 1985; Bogler et al., 1990), when grown on an endothelial cell-derived extracellular matrix (Ovadia et al., 1984) or on dorsal root ganglion neurones (Wood and Bunge, 1986, 1991).

Although PDGF and FGF may play key roles in postinjury responses of the adult brain, other factors may also be important in these processes. The identification of such factors and the ability to identify O-2A progenitor cells unambiguously in situ would greatly enhance our knowledge of the process of remyelination. This knowledge may prove useful in attempts to enhance myelin repair in diseases in which remyelination is limited, such as in patients with multiple sclerosis.

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