PDGF and FGF2 Regulate Oligodendrocyte Progenitor Responses to Demyelination

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ABSTRACT: Acute demyelination of adult CNS, resulting from trauma or disease, is initially followed by remyelination. However, chronic lesions with subsequent functional impairment result from eventual failure of the remyelination process, as seen in multiple sclerosis. Studies using animal models of successful remyelination delineate a progression of events facilitating remyelination. A universal feature of this repair process is extensive proliferation of oligodendrocyte progenitor cells (OPs) in response to demyelination. To investigate signals that regulate OP proliferation in response to demyelination we used murine hepatitis virus-A59 (MHV-A59) infection of adult mice to induce focal demyelination throughout the spinal cord followed by spontaneous remyelination. We cultured glial cells directly from demyelinating and remyelinating spinal cords using conditions that maintain the dramatically enhanced OP proliferative response prior to CNS remyelination. We identify PDGF and FGF2 as significant mitogens regulating this proliferative response. Furthermore, we demonstrate endogenous PDGF and FGF2 activity in these glial cultures isolated from demyelinated CNS tissue. These findings correlate well with our previous demonstration of increased *in vivo* expression of PDGF and FGF2 ligand and corresponding receptors in MHV-A59 lesions. Together these studies support the potential of these pathways to function *in vivo* as critical factors in regulating remyelination. © 2003 Wiley Periodicals, Inc.* J Neurobiol 54: 457–472, 2003

***Keywords:* PDGF; FGF2; remyelination; oligodendrocyte progenitor; demyelinating disease**

INTRODUCTION

Oligodendrocyte myelination of CNS axons facilitates conduction of electrical signals between neurons. In demyelinating diseases, such as multiple sclerosis (MS), myelin damage results in impaired conduction and progressive axonal degeneration. Limited spontaneous remyelination occurs in acute MS lesions, yet recurring myelin damage is not sufficiently repaired (Raine and Wu, 1993; Prineas et al., 1993). The cellular and molecular mechanisms of remyelination are not clear, however, a key feature is the generation of remyelinating cells from local precursor cells. Several studies have identified immature oligodendrocyte lineage cells (OLCs) in the normal adult human CNS (Wolswijk and Noble, 1989; Armstrong et al., 1992; Scolding et al., 1995; Roy et al., 1999), and the density of these immature OLCs is increased in acute MS lesions (Chang et al., 2000; Maeda et al., 2001). These precursor cells may serve as targets for developing therapeutic strategies to improve OLC regeneration and the extent of remyelination in human demyelinating diseases.

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Regulatory mechanisms involved in remyelination can be readily analyzed in adult rodent CNS models that achieve successful remyelination in response to acute demyelination. Oligodendrocyte progenitor cells (OPs) persist in the adult rodent CNS (ffrench-Constant and Raff, 1986; Armstrong et al., 1990; Gensert and Goldman, 1997; Horner et al., 2000). In several models of transient demyelination, OP proliferation occurs prior to remyelination (e.g., Carroll et al., 1998; Keirstead et al., 1998; Redwine and Armstrong, 1998). Several developmentally important trophins are expressed in normal adult rodent CNS, and may be more abundant in remyelinating rodent CNS (reviewed in Armstrong, 2000). As candidate molecules regulating OP responses in remyelinating tissue, we focus on PDGF and FGF2, two relatively well-characterized trophins for developing OPs.

We use murine hepatitis virus A-59 (MHV-A59) to induce transient focal demyelination throughout the spinal cord, which is followed by spontaneous remyelination and functional recovery (Jordan et al., 1989; Redwine and Armstrong, 1998). Our previous studies have shown an up-regulation of platelet-derived growth factor receptor-α (PDGFαR) expressing cells associated with MHV-A59 lesions, and corresponding increases in PDGF-A ligand (Redwine and Armstrong, 1998). We have also shown significant increases in FGF2 ligand and receptor expression in these remyelinating lesions (Messersmith et al., 2000). Thus, PDGF and FGF2 ligands and receptors are expressed during remyelination with a temporal, spatial, and cellular distribution consistent with a potential role in generating remyelinating OLCs.

In this study we use glial cultures isolated at specific stages of MHV-A59 disease progression to directly examine the role of PDGF and FGF2 in regulating OLC proliferation and differentiation associated with remyelination. We investigate the activity of PDGF-AA and FGF2, each tested singly and in combination. In addition, we examine the significance of endogenously produced PDGF and FGF2 in the cultures using inhibitors specific to each pathway. Importantly, at two distinct phases of disease progression, demyelination and early remyelination, we compare OLC responses elicited by exogenous and endogenous PDGF and FGF2. We show that adult OP responses are clearly regulated by PDGF and FGF2, especially acting in combination, and that the OP responses differ with disease state.

MATERIALS AND METHODS

MHV-A59 Injection

As previously described (Redwine and Armstrong, 1998), MHV-A59 virus stock (1000 PFU in 10 μL) was injected intracranially into 28-day-old C57Bl/6 female mice (Charles River, Wilmington, MA) to produce focal areas of transient demyelination throughout the rostrocaudal extent of the spinal cord white matter. Motor impairment was quantified by recording the period of time mice were able to hang upside down supporting their weight by gripping the bars of a metal cage top, with a maximum period set at 60 s (Redwine and Armstrong, 1998). Measurements were taken on days 7, 10, 14, 17, 21, 25, 28, and 32 days postinjection (dpi). The extent of demyelination was classified as "severely" affected if the mice were unable to hang on to the cage top for at least 30 s on 2 or more test days during the first 14 dpi. All animals were handled in accordance with procedures approved by the USUHS Institutional Animal Care and Use Committee.

Tissue Preparation

As previously described (Redwine and Armstrong, 1998), mice were perfused with 4% paraformaldehyde and the spinal cords removed. For histological observation, tissue was osmicated, embedded in plastic, cut into 1 μm semithin sections, and stained with toluidine blue. For immunostaining, spinal cord segments were postfixed overnight, cryoprotected with 30% sucrose, embedded in Tissue Tek OCT compound, and stored at −80°C.

Immunostaining of Tissue Sections

Fifteen-micrometer cryosections were thaw mounted onto Superfrost slides and immunostained for NG2 and PDGFαR to identify OPs *in situ* (Redwine and Armstrong, 1998; Messersmith et al., 2000). Primary antibodies used were rabbit polyclonal anti-NG2 antibody (1:500; a generous gift from Dr. William Stallcup) and rat monoclonal anti-PDGFαR antibody (1:200; Pharmingen, San Diego, CA). Donkey antirabbit Cy3 conjugate (Jackson Immunoresearch, West Grove, PA) was used to detect NG2 and the PDGFαR was detected with biotinylated donkey antirat F(ab′)$_2$ (Jackson Immunoresearch) with FITC-tyramide amplification (New England Nuclear, Boston, MA). Sections were stained with DAPI (Sigma) prior to mounting.

In Situ Hybridization

In situ hybridization and preparation of digoxigenin-labeled riboprobes were performed as previously published for analysis of MHV-A59 spinal cord sections (Redwine and Armstrong, 1998; Messersmith et al., 2000). The digoxigenin-labeled riboprobe used to detect FGF2 was prepared from a cDNA template complementary to nucleotides 1–475 of mouse FGF2 (gift from Dr. Gail Martin, University of California at San Francisco; Hebert et al., 1990). The cDNA template used for PDGF-A (kindly provided by Dr. Mark Mercola, Harvard Medical School) was complementary to an approximately 0.9 kb mRNA fragment of mouse PDGF-A mRNA (Mercola et al., 1990). The cDNA template for the proteolipid protein (PLP) ribonucleotide probe

(gift from Dr. Lynn Hudson, National Institutes of Health) was complementary to a 980 bp fragment of the mouse PLP mRNA sequence (Hudson et al., 1987). An *in vitro* transcription kit (Ambion, Austin, TX) was used to incorporate digoxigenin-UTP (Boehringer Mannheim, Indianapolis, IN). The digoxigenin-labeled riboprobes were hybridized to mixed glial cultures prepared from spinal cords of 4 weeks postinjection (wpi) MHV-A59 severely affected mice. Digoxigenin was detected with an alkaline phosphatase-conjugated sheep antidigoxigenin antibody (Boehringer Mannheim), followed by reaction with NBT/BCIP substrate (DAKO, Carpinteria, CA).

Neonatal Brain Oligodendrocyte Precursor Cell Isolation

Neonatal OPs (nOPs) were prepared from postnatal day 2 rat brains, by a method detailed in Armstrong (1998). Approximately 10–14 days after plating, the nOPs and microglia were dislodged by shaking the flasks, followed by plating on noncoated tissue culture plastic to allow the differential adhesion of microglial cells. The nOP cell suspension was collected, concentrated, and resuspended in Sato's defined medium with high insulin (Bottenstein and Sato, 1979), which is Dulbecco's modified Eagle's medium (DME; Life Technologies, Rockville, MD) supplemented with the following reagents (from Sigma, St. Louis, MO unless otherwise stated): 25 μg/mL gentamicin (Life Technologies), 5 μg/mL bovine insulin, 50 μg/mL human transferrin, 100 μg/mL bovine serum albumin (BSA) fraction V, 66 ng/mL progesterone, 16 μg/mL putrescine, 5 ng/mL sodium selenite, 400 ng/mL thyroxine (T4), and 400 ng/mL tri-iodothyroxine (T3) (Biofluids Inc., Rockville, MD).

Inhibitors of PDGF and FGF2

Rat brain nOP cells proliferate extensively and reproducibly in response to either PDGF-AA or FGF2 (McKinnon et al., 1990; Baron et al., 2000). Therefore, nOPs were used to characterize the specificity of inhibitors of each growth factor pathway.

OPs express only one of the PDGF receptor isoforms, the alpha receptor (PDGFαR) (Hart et al., 1989; Pringle et al., 1992). The PDGFαR is a protein tyrosine kinase receptor with numerous sites of phosphorylation that interact with several different signal transduction molecules (Heldin and Westermark, 1999). Therefore, to block activity of the PDGF pathway, we selected a specific inhibitor of the kinase activity of the PDGFαR. The AG1295 compound (Calbiochem, La Jolla, CA) is 6,7-dimethyl-2-phenylquinoxaline, a tyrphostin that selectively inhibits phosphorylation by the PDGF receptor tyrosine kinase. AG1295 has previously been shown to have no effect on autophosphorylation of the epidermal growth factor receptor even at concentrations up to 100 μM (Kovalenko et al., 1994). Kovalenko et al. (1994) further showed that the IC_{50} for AG1295 inhibition of DNA synthesis in Swiss 3T3 cells was 2.5 μM. Other studies have used 10 μM AG1295 to specifically inhibit PDGF receptor activation (Banai et al., 1998; Iwamoto et al., 2000). A dose response curve (1.0, 2.5, 10, and 25 μM) of AG1295 was used to test the inhibition of proliferation of nOPs in response to 10 ng/mL PDGF-AA. Based upon the results with nOPs a single concentration of 10 μM AG1295 was used for the adult OLC proliferation studies.

In contrast to the single receptor types of the PDGF pathway, OLCs express at least three different FGF receptor types (Bansal et al., 1996; Messersmith et al., 2000). In addition, the FGF receptors are activated by multiple ligands of the FGF family (Ornitz et al., 1996; Ornitz and Itoh, 2001). Therefore, to specifically investigate the role of FGF2 ligand in the regulation of OLC proliferation, we used an anti-FGF2 neutralizing antibody (FGF2nAb) that has been used to block activity of FGF2 *in vivo* (Nilsson et al., 2001). The FGF2nAb (AF-233-NA; R&D Systems, Minneapolis, MN) was used at a concentration of 1 μg/mL, which is the manufacturer recommended $ND_{50,}$ and is 10X the concentration required to block maximal proliferation of NIH3T3 cells in response to rhFGF2.

Proliferation Assay

The purified nOPs were gently triturated to produce a single cell suspension. Cells were then plated at 10,000 cells per 12 mm PDL-coated glass coverslip in Sato's defined medium supplemented with 0.5% fetal bovine serum (FBS) (Life Technologies), 10 ng/mL PDGF-AA (R&D Systems), and 10 ng/mL FGF2 (R&D Systems) overnight. The following day the medium was replaced with serum/growth factor-free Sato's medium for 6 h. The medium was then replaced with serum-free Sato's medium containing 10 ng/mL PDGF-AA or FGF2 with or without inhibitors, and 10 μM BrdU (Boehringer Mannheim) for 19 h.

To prepare cells for immunodetection of incorporated BrdU, the cells were washed with cold HEPES buffered Minimal Essential Medium (Life Technologies), fixed in methanol at −20°C for 10 min, then treated for 1 min with 0.2% paraformaldehyde, washed, treated with 0.07 M NaOH for 7 min, washed, then treated with 2% paraformaldehyde for 3 min. Monoclonal anti-BrdU antibody (0.25 μg/mL in PBS; Beckton Dickinson, San Jose, CA) was added to the cells for 30–60 min at room temperature (RT) and detected with donkey antimouse IgG (H+L) $F(ab')_2$ FITC conjugate (Jackson Immunoresearch). Cells were stained with the nuclear stain DAPI (Sigma) prior to mounting. Cell proliferation was assessed as the percentage of $DAPI^+$ nOPs that were also $BrdU^+$. At least 100 cells per coverslip were counted with three to four coverslips per experiment and three experiments per inhibitor.

Cell Viability Assay

Purified nOPs were prepared as for the proliferation assay. Propidium iodide (PI) (20 μg/mL; Sigma), which is excluded from live cells, was added to each coverslip for 10 min. Cells were then fixed with 4% paraformaldehyde for 10 min at RT and stained with DAPI (see above). Cell viability was assessed as the percentage of $DAPI^+$ PI^- OPs

per coverslip. At least 100 cells per coverslip were counted, with three to four coverslips per experiment and three experiments per growth factor pathway inhibitor.

Mixed Glial Cell Preparation from Spinal Cord

Only mice meeting the criteria of severely affected were used for tissue culture. Mice were sacrificed at either of two stages of the disease progression: at 14–17 dpi ("2 wpi"), or at 28–32 dpi ("4 wpi"). Spinal cords were removed from six mice for each mixed glial cell preparation. Whole spinal cords were used in this study because of the uncertainties of the anatomical locations of various potential pools of precursor cells that may contribute to remyelination, and because prolonged microdissection time to isolate the multiple focal lesion areas throughout each cord would significantly decrease cell yield in cultures. The spinal cords were minced and dissociated according to a protocol modified from Armstrong et al. (1990). Briefly, tissue was incubated at 37°C in 0.25% trypsin (Sigma) with 50 U/mL DNase I (Sigma) in cold isolation medium [MEM-HEPES supplemented with 25 μg/mL gentamicin, 5000 U/mL penicillin, and 5 mg/mL streptomycin (Sigma)] twice for 14 min each. After each incubation period, the supernatant was collected into DME plus 10% FBS (DFM) and replaced with fresh enzyme solution. The tissue was then passed through a flame polished glass Pasteur pipette, then through a 26 G needle prior to being pooled with the supernatants. Glial cells were separated from myelin using a 30% Percoll (Pharmacia, Piscataway, NJ) gradient at 30,000$\times g$ for 30 min at 4°C in 50 mL Oak Ridge centrifuge tubes (Fisher Scientific, Pittsburgh, PA). The glial cell layer, between the myelin cap and the red blood cell pellet, was removed carefully and resuspended in cold isolation medium. The cells were centrifuged for 10 min at 230$\times g$. The supernatant was removed and the cell pellet was resuspended in DFM. The cells were plated onto extracellular matrix coated 35 mm tissue culture dishes (Accurate Chemical, Westbury, NY) at a density of one spinal cord per two to three dishes, in a volume of 200 μL/dish, and incubated at 37°C. After 2 h a further 1.3 mL DFM was added to each dish. Two days later the medium was removed and replaced with Sato's medium plus growth factors and/or inhibitors. After a further 48 h *in vitro*, BrdU was added (10 μM), and cells were fixed 19 h later for analysis.

Immunocytochemistry

Cultured OLCs from MHV-A59 were identified using three-color immunofluorescence with differentiation stage-specific markers. Anti-NG2 antibody recognizes a chondroitin sulphate proteoglycan that is expressed by OPs (Nishiyama et al., 1996a,b). O1 monoclonal antibody recognizes a cell surface galactocerebroside that is a marker of mature oligodendrocytes (Bansal and Pfeiffer, 1992).

BrdU was added to the cultures for 19 h, with O1 antibody (1:10 supernatant from hybridoma cultures containing 10% FBS in DME) being added for the final hour at 37°C. The cells were then fixed, permeabilized, and incubated with anti-BrdU as detailed above. Donkey antimouse IgG (H+L) F(ab′)2 FITC conjugate was used to detect the BrdU, and goat antimouse IgM TRITC conjugate (Jackson Immunoresearch) was used to detect the O1, for 30 min at RT. Cells were then incubated overnight at 4°C with rabbit polyclonal anti-NG2 antibody (1:250–500 dilutions; generous gifts from Dr. Joel Levine and Dr. William Stallcup). NG2 was detected with AMCA conjugated, donkey antirabbit secondary antibody (Jackson Immunoresearch) at RT for 30 min. Cultures were also analyzed for O4 and GFAP immunoreactivity to identify a broad range of OLC stages and the astrocytic cell population. In these experiments, cultures were treated as detailed above, substituting O4 antibody (1:10 dilution of supernatant from hybridoma cultures and containing 10% FBS in DME) for the O1 antibody, and GFAP (0.41 μg/mL; Dako, Carpinteria, CA) for the NG2 antibody. Cells were then counted using a Zeiss Photoscope III. Thirty different fields of view using a 25X objective with a 1.25-optivar were counted per dish, which covered approximately 12.5% of the total measurable area of the dish. Prior to analyzing the dishes, we verified that 30 fields of view were a representative sample and complied with assumptions necessary for this analysis (i.e., normality of distributions and homogeneity of variances).

Clonal Analysis

Mixed glial spinal cord cultures were prepared as detailed above. A combination of PDGF-AA and FGF2, or a combination of the inhibitors AG1295 and FGF2nAb, was added to the dishes at day 2. After a further 24 h, the BAG replication-deficient retrovirus [80 plaque forming units (PFU); Price et al., 1987; Levison et al., 1999; generously provided by Dr. Steve Levison, Penn State University, Hershey, PA] was added to the cultures, which were returned to the incubator for a further 48 h. O1 or O4 monoclonal antibody (as detailed above) was added to the medium for the final 60 min. The dishes were then fixed in 2% paraformaldehyde and the O1 or O4 monoclonal antibody was detected as detailed above. The cultures were then incubated for 24 h at 4°C with anti-NG2 (as above) and β-galactosidase (β-gal) using the 40-1A monoclonal antibody (1:2 dilution of supernatant from hybridoma cultures containing 15% FBS in DME). The hybridoma cells were obtained from the Developmental Studies Hybridoma Bank, developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences (Iowa City, IA, 52242). Monoclonal 40-1A was detected with donkey antimouse IgG FITC (Jackson Immunoresearch). All labeled clones were counted within each dish.

Imaging

All images were collected using a Spot II Camera (Diagnostic Instruments, Inc.) attached to an Olympus IX70 epifluorescence microscope, with associated software and Photoshop 5.0.

RESULTS

The role of PDGF and FGF2 in the regulation of OLC proliferation and differentiation during demyelination and remyelination was investigated using glial cell cultures isolated from spinal cords of MHV-A59 infected adult mice. To ensure that reproducibly affected mice were used in each culture preparation, motor impairment was monitored by measuring the length of time the MHV-A59 injected mice were able to hang upside down from the wires of a cage top and support their weight (Fig. 1). Mice unable to hang for a minimum of 30 s or less, for at least two out of the first three test dates (days 7, 10, 14), were classified as severely affected (Redwine and Armstrong, 1998). Previous studies have shown demyelinated lesions beginning at 1 wpi with more extensive areas of myelin degeneration by 2 wpi (Jordan et al., 1989; Armstrong et al., 1990). In subsequent weeks, myelin debris is cleared and remyelination is initiated (Jordan et al., 1989; Redwine and Armstrong, 1998).

In the current study, the overall area of demyelination throughout the spinal cord was assessed in a representative severely affected mouse by measuring the area of lesion in toluidine blue stained sections [Fig. 2(A); $n = 152$ sections throughout the spinal cord]. Characteristic areas of focal demyelination were observed in each funiculus. The proportion of white matter area with myelin loss was 18.3 ± 3.6%, 12.9 ± 2.2%, and 24.0 ± 4.2% for cervical, thoracic, and lumbar levels, respectively. In addition to myelin loss, lesions typically exhibited vacuolation and in-

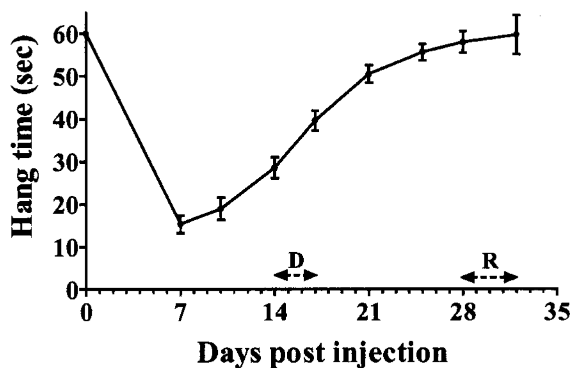

Figure 1 Time course of murine hepatitis virus induced disease progression. Intracranial injection of MHV-A59 results in motor function impairment. Mice were tested for limb motor impairment (hang time) prior to being sacrificed for mixed glial cultures between 14–17 days postinjection (dpi) (during demyelination "D"), and between 28–32 dpi (remyelination "R"). Hang time was scored as the period that each mouse could hang upside down from a metal cage top. Prior to infection and upon recovery, mice could hold on for a maximum allowed period of 60 s. Hang time scores shown are combined for all the mice used in this study, so that values given include the mice sacrificed later on that day. Error bars indicate S.E.M. Days 0–14, $n = 83$; day 17, $n = 71$; days 21–28, $n = 52$; day 32, $n = 34$.

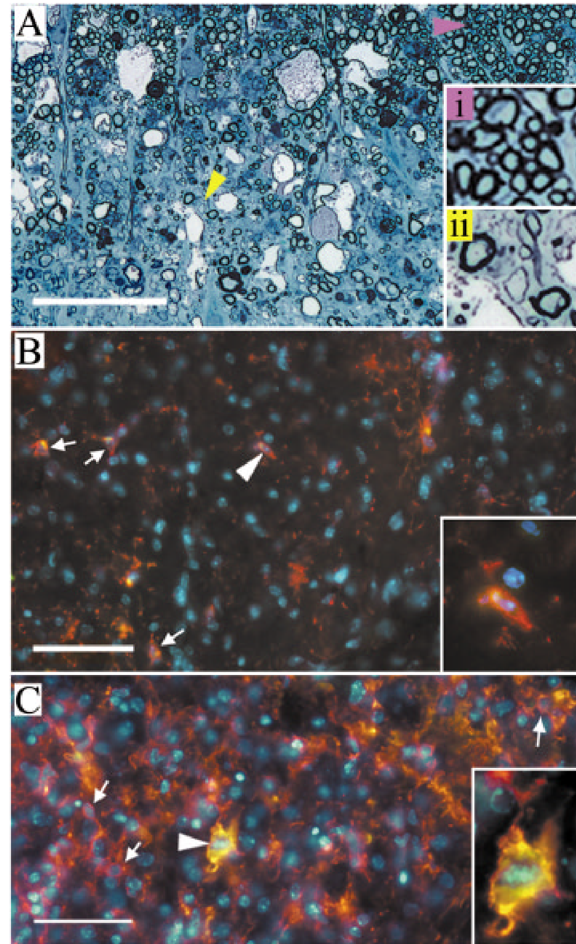

Figure 2 MHV induced spinal cord lesions. Transverse sections of lesioned spinal cord segments from MHV-A59 injected mice sacrificed during remyelination (4 wpi). (A) Toluidine blue staining reveals the characteristic histopathology of lesioned tissue, with reduced dark myelin staining, increased cell density, and vacuolation. Adjacent tissue demonstrates normal appearing myelin [pink arrowhead indicates example, shown enlarged as inset (i)]. In addition, thin myelin sheaths are evident in the lesion [yellow arrowhead indicates example, shown enlarged as inset (ii)], which is consistent with remyelination. (B, C) Immunohistochemistry for NG2 (red) and PDGFαR (green), with DAPI staining of DNA in nuclei (blue) to identify OPs in normal appearing white matter (B) and in an MHV-A59 induced lesion (C). Arrowheads indicate cells shown enlarged as insets. During remyelination (4 wpi), lesions show increased density of OPs and increased intensity of NG2 immunoreactivity. Mitotic cells in lesioned white matter [(C), inset] show extremely strong immunoreactivity for NG2 and PDGFαR (dual immunoreactivity appears yellow). Scales bars = 50 μm.

creased cell density [Fig. 2(A)]. Lesion areas showed accumulation of OPs, which were identified by immunostaining for NG2 and PDGFαR [Fig. 2(B,C)].

To analyze OP responses, cultures were prepared from spinal cords of mice sacrificed at two distinct phases of disease progression. The time of sacrifice was restricted to within a 5 day window of each phase to minimize variation within disease progression. Within the demyelination phase, 14–17 days after MHV-A59 injection was used as the earliest point at which the mice could be classified as "severe" to be used for preparations of cultures. These cultures are referred to as 2 wpi. This period corresponds with severe loss of motor function and the viral infection of the white matter (Jordan et al., 1989). Cultures were also prepared from tissue 28–32 days postinjection (referred to as 4 wpi). This period corresponds with recovery of motor function (Fig. 1) and viral clearance (Jordan et al., 1989; Redwine and Armstrong, 1998). For each cell preparation, spinal cords from six severely affected mice were combined in order to minimize the variability between mice.

Identification of Glial Cell Types in Cultures from Spinal Cords of MHV-A59 Infected Mice

The cell types in the cultures were characterized using three-color immunofluorescence. Cell type-specific antibodies facilitated the identification of specific stages of differentiation of the OLCs within the cultures (see Materials and Methods). In addition, BrdU incorporation was used to identify proliferating cells with each antigenic phenotype. OPs were identified as $NG2^+$, $O1^-$. Transitional oligodendrocytes were identified as $NG2^+$, $O1^+$. Differentiated oligodendrocytes were identified as $NG2^-$, $O1^+$ (Fig. 3).

An extracellular matrix culture substrate was used to promote the adhesion of OLCs of all differentiation stages as well as astrocytes and microglia, as previously shown (Armstrong et al., 1990). Experiments using glial fibrillary acidic protein (GFAP) as a marker for astrocytes confirmed that these cultures contained astrocytes, upon which the OLCs were often observed (not shown). Cells with astrocytic morphology and those with microglial morphology were also prevalent within the cultures (Fig. 4).

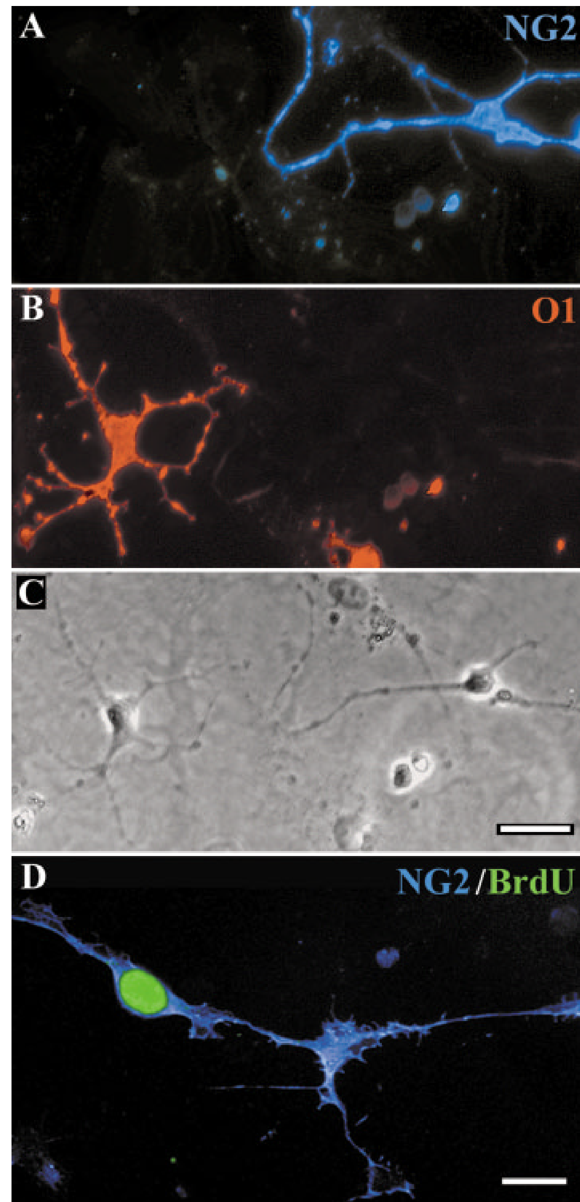

Figure 3 Characterization of oligodendrocyte lineage cells within glial cultures isolated from remyelinating spinal cord. Three-color immunofluorescence was used to identify two different antigen-specific stages of the oligodendrocyte lineage within the cultures, in addition to BrdU incorporation, which was used to identify proliferating cells. (A) An oligodendrocyte progenitor cell (OP) was identified as an $NG2^+$, $O1^-$ cell. (B) A differentiated oligodendrocyte was identified as being $NG2^-$, $O1^+$. (C) Phase contrast shows the glial cells present in the culture. (A), (B), and (C) show the same field. (D) A proliferating OP was identified as $NG2^+$ $BrdU^+$ (also $O1^-$, not shown). Scale bar in (C) = 50 μm; in (D) = 25 μm.

In Situ Hybridization Analysis of Growth Factor mRNA Expression in Cultures from Spinal Cords of MHV-A59 Infected Mice

Previous studies have shown that activated astrocytes and microglial cells synthesize and secrete both PDGF and FGF2 within demyelinated lesions (Redwine and Armstrong, 1998; Messersmith et al., 2000; Liu et al., 1998). Therefore, *de novo* synthesis of

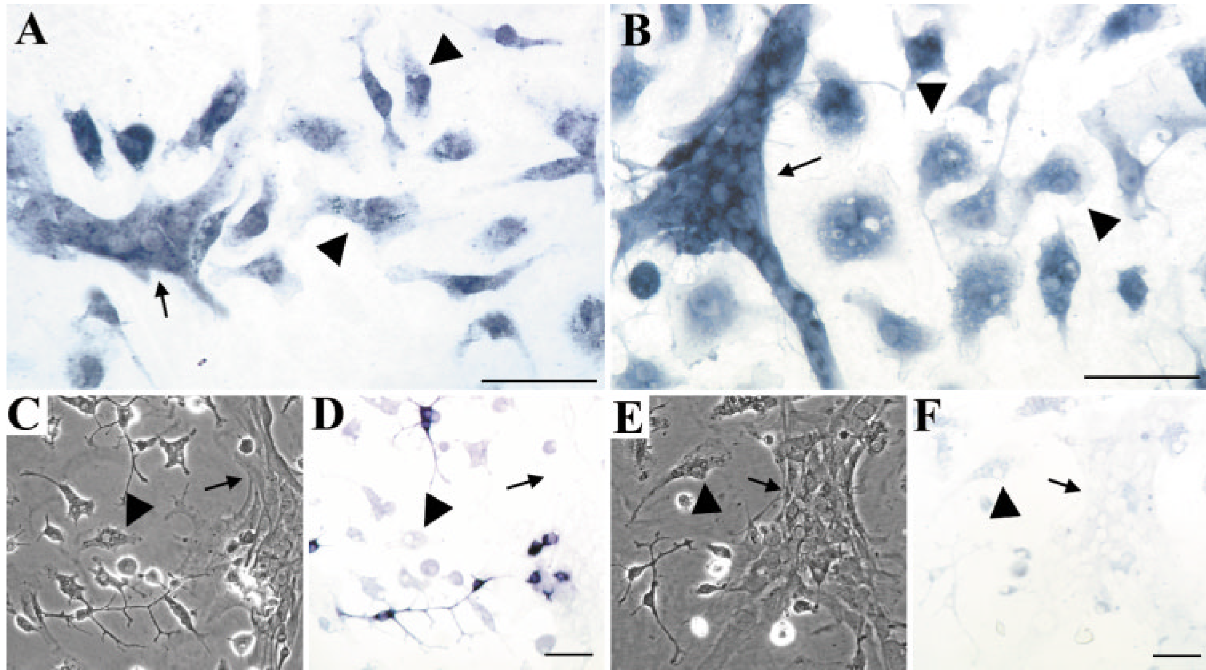

Figure 4 Characterization of growth factor expression by *in situ* hybridization. *In situ* hybridization was performed on cultures derived from spinal cords isolated during remyelination, at 4 wpi. Signal was detected in astrocytes (examples at arrows) and microglial cells (examples at arrowheads) within the cultures. (A) Bright field photomicrograph showing the PDGF antisense riboprobe signal. (B) Bright field photomicrograph showing the FGF2 antisense riboprobe signal. (C) Phase contrast photomicrograph showing the presence of astrocyte clusters and microglial cells that are PLP negative, compared to (D) bright field photomicrograph showing PLP antisense riboprobe signal in oligodendrocytes. (E) and (F) FGF2 sense probe was used to demonstrate hybridization specificity and background. (E) Phase contrast photomicrograph showing an astrocyte cluster, microglial cells, and oligodendrocytes present in the cultures. (F) Bright field photomicrograph of panel (E) showing lack of hybridization with the FGF2 sense probe. Scale bars = 50 μm.

PDGF-A and FGF2 by cells within the mixed glial cultures was demonstrated using *in situ* hybridization. Mixed glial cultures were prepared as described above. In cultures isolated at 4 wpi, both astrocytes and microglial cells exhibited PDGF-A and FGF2 mRNA signal [Fig. 4(A,B)]. Signal intensity in astrocytes appeared to be greater for FGF2 than for PDGF-A, which is consistent with results seen in this model *in vivo* (Redwine and Armstrong, 1998; Messersmith et al., 2000). Cultures were also analyzed for PLP mRNA [Fig. 4(C,D)], a marker of mature oligodendrocytes, and FGF2 sense mRNA [Fig. 4(E,F)], to demonstrate specificity of the *in situ* hybridization detection.

Characterization of Growth Factor Inhibitors

To investigate the role of endogenously produced PDGF and FGF2 in the regulation of proliferation in these cultures we used reagents that inhibited each growth factor pathway. The tyrphostin compound AG1295 was used to inhibit PDGFαR tyrosine kinase phosphorylation. The activity of FGF2 was inhibited with a neutralizing antibody (FGF2nAb).

The specificity of each inhibitor was demonstrated using BrdU incorporation in neonatal rat cortical OP cultures (nOP), in which PDGF and FGF2 have been characterized as potent mitogens (McKinnon et al., 1990; Baron et al., 2000). AG1295 is a specific inhibitor of PDGF receptor activated DNA synthesis, with an IC_{50} of 2.5 μM in Swiss 3T3 cells (Kovalenko et al., 1994) that has previously been shown to inhibit PDGF induced proliferation of several different cell types (for example Banai et al., 1998; Iwamoto et al., 2000). In this study a dose response analysis of AG1295 showed that 2.5 μM AG1295 only inhibited PDGF induced proliferation by 9.1%, whereas 10 μM AG1295 inhibited PDGF induced proliferation by 35.8%, back to levels seen in cultures without PDGF (Fig. 5). Previous studies have demonstrated that at 10 μM, AG1295 inhibited the phosphorylation of PDGF

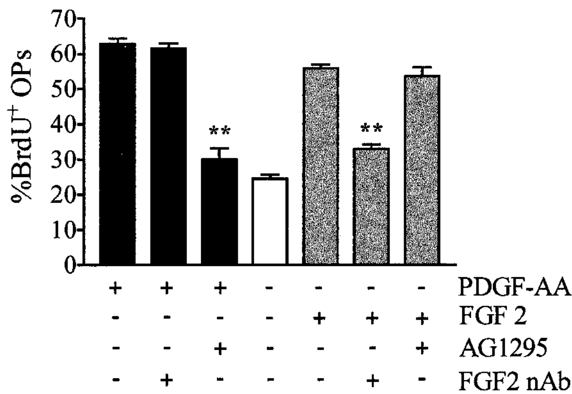

Figure 5 Characterization of growth factor specific inhibitors. The specificity of the PDGFαR inhibitor AG1295 and the anti-FGF2 neutralizing antibody (FGF2nAb) was assessed in neonatal rat brain OPs (nOP) using BrdU incorporation as a marker of proliferation. Black bars denote PDGF-AA treated nOPs and gray bars denote FGF2 (FGF2) treated nOPs. AG1295 (10 μM) maximally inhibited PDGF-AA (10 ng/mL) stimulated proliferation of nOPs, with no marked effect on FGF2 induced proliferation. The FGF2nAb at 1 μg/mL significantly inhibited proliferation induced by FGF2 (10 ng/mL) with no marked effect on PDGF-AA induced proliferation. Each value (±S.E.M.) represents four experiments with three to four replicates and at least 100 cells were counted per coverslip (**$p < .005$ compared to growth factor condition without inhibitor). Statistical analysis was paired two-tailed t test analysis.

receptors and subsequent downstream signaling pathways leading to proliferation, without inhibiting the tyrosine kinase activity of the EGF receptor (Kovalenko et al., 1994; Banai et al., 1998). Therefore, subsequent experiments used 10 μM AG1295 to maximally inhibit PDGFαR signaling in the nOP cultures (Fig. 5) without compromising cell viability (assessed by a propidium iodide exclusion assay; data not shown). Compared to EGF receptors, FGF tyrosine kinase receptors are even more closely related to the PDGFαR. Therefore, we confirmed that AG1295 inhibition was specific to PDGF by showing that AG1295 did not affect proliferation induced by FGF2 (Fig. 5). In parallel experiments, the FGF2nAb (at 1 μg/mL, the recommended ND_{50}) significantly inhibited the nOP proliferation induced by FGF2 (Fig. 5), without compromising cell viability (data not shown), yet had no marked effect on the PDGF-AA response (Fig. 5).

Endogenous PDGF and FGF2 Stimulate Proliferation of OPs in Cultures Isolated during Demyelination (2 wpi)

Within glial cultures derived from demyelinated spinal cord at 2 wpi and then grown for 2 days *in vitro* (div) in DFM, 42% of the OLCs had incorporated BrdU. The majority of these OLCs were $NG2^+$ OPs [Fig. 6(A)]. After a further 3 days in defined medium alone (control), the total percentage of proliferating cells did not change significantly. The addition of exogenous PDGF-AA and FGF2 for these 3 div also did not significantly change the total proportion of proliferating OLCs, even when added in combination [Fig. 6(A)]. One possible explanation for this result is that the OLCs are responding maximally to endogenous growth factors synthesized by cells within the cultures. Indeed, inhibition of endogenously produced PDGF and FGF2 activity using AG1295 and FGF2nAb, either individually or in combination, significantly reduced both the total percentage of proliferating OLCs and the proportion of $NG2^+$ OPs that had incorporated BrdU (Fig. 6).

Endogenous PDGF and FGF2 Prevent OLC Differentiation in Cultures of Demyelinated Spinal Cord (2 wpi)

The OLC population within cultures derived from demyelinated spinal cord (at 2 wpi) consisted primarily of $NG2^+$ OPs [Fig. 7(A), black bars] after 2 div in DFM. After a further 3 div, in defined medium alone (control), there was no change in the proportion of $NG2^+$ OPs [Fig. 7(B)]. The addition of exogenous growth factors did not significantly alter the antigenic phenotype of the OLCs. In contrast, the inhibitors AG1295 and FGF2nAb, both individually and in combination, significantly reduced the proportion of $NG2^+$ OPs among the OLC populations in these cultures (Fig. 7). Consistent with the proliferation analysis (above), these findings indicate that endogenous PDGF and FGF2 signaling prevents OP differentiation during the demyelination stage of the disease progression.

OP Proliferation in Cultures Derived during Remyelination (4 wpi)

Within cultures derived from remyelinating spinal cord (at 4 wpi) and then grown for 2 div in DFM, 23% of the OLCs incorporated BrdU [Fig. 8(A)]. This proportion of proliferating OLCs was less than that seen at 2 wpi [Fig. 6(A)] ($p < .005$). Approximately half of the BrdU labeled OLCs were $NG2^+$ OPs [Fig. 8(A), black bars]. After a further 3 days in defined medium alone (control), the percentage of $NG2^+$ OPs that had incorporated BrdU was significantly reduced by approximately 60%. The addition of exogenous PDGF-AA with FGF2 significantly increased $NG2^+$ OP proliferation [Fig. 8(B)]. The growth factor inhibitors AG1295 and FGF2nAb did not reduce the total

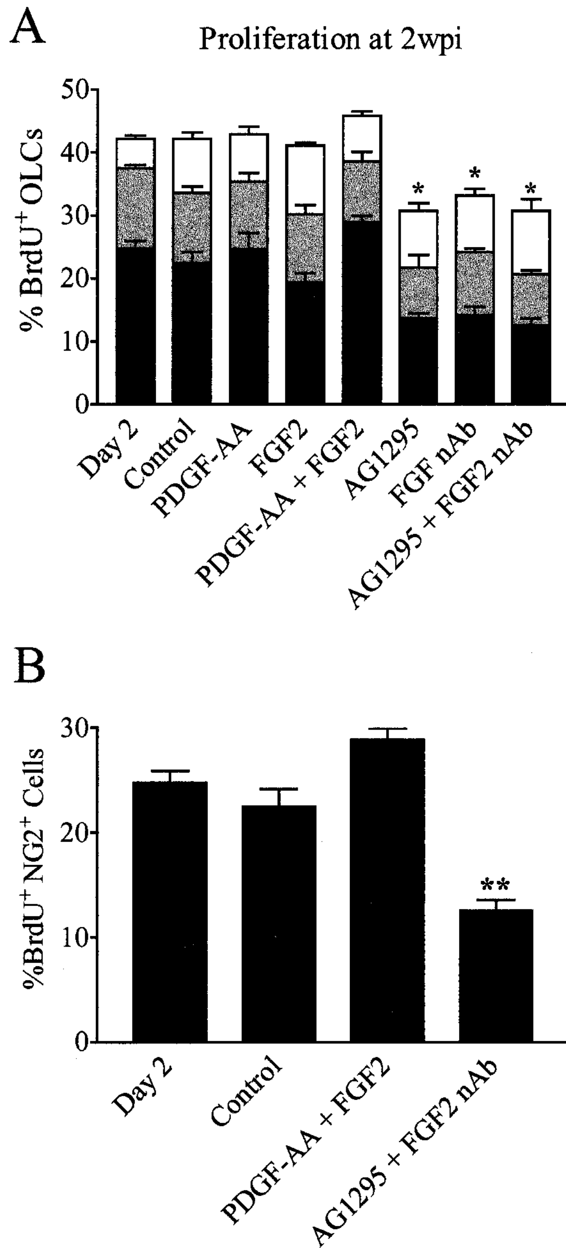

Figure 6 Endogenous PDGF and FGF2 enhance proliferation of oligodendrocyte lineage cells in mixed glial cultures of demyelinated spinal cords. In cultures prepared from 2-week postinfection (2 wpi) tissue, OLC proliferation was assessed as the percentage of total OLCs (labeled with NG2 and/or O1 stage-specific immunomarkers) that incorporated BrdU during a terminal 19 h pulse. Black bars represent $NG2^+O1^-$ cells, gray bars represent $NG2^+O1^+$ cells, and white bars represent $NG2^-O1^+$ cells. (A) OLC proliferation is not altered by the addition of exogenous PDGF AA and/or FGF2. In contrast, inhibition of either or both endogenous growth factors results in significant inhibition of proliferation. (B) $NG2^+O1^-$ cell proliferation is not affected by exogenous PDGF-AA or FGF2, however, it is significantly reduced by the inhibition of endogenous PDGF and FGF2. For each value (±S.E.M.) $n = 4-6$ dishes per treatment across three to four different glial preparations. (p vs. control $*p < .05$, $**p < .01$). Statistical analysis was one-way analysis of variance, followed by paired two-tailed t test analysis.

percentage of proliferating OLCs when used individually [Fig. 8(A)]. In combination, however, the inhibitors did significantly reduce the total percentage of OLCs incorporating BrdU [Fig. 8(A)] and the proportion of $NG2^+$ OPs that were $BrdU^+$ [Fig. 8(B)]. Thus, at 4 wpi, endogenous PDGF and FGF2 still contribute to OP proliferation. However, the significant response to exogenous PDGF-AA and FGF2 during remyelination also indicates that endogenous PDGF and FGF2 are no longer acting at saturation levels, and the appropriate growth factor receptors are still available to respond at 4 wpi.

OP Differentiation in Cultures Derived during Remyelination (4 wpi)

The OLC population within the cultures from 4 wpi tissue consisted of predominantly $NG2^+$ OPs [Fig. 9(A)] after 2 div in DFM, similar to that seen in 2 wpi cultures [Fig. 7(A)]. However, in contrast to cultures from 2 wpi, after a further 3 div in defined medium alone, there were significantly fewer $NG2^+$ OPs, with the majority of cells being in later stages of the lineage [Fig. 9(A)] (p = .0027 vs. control). This pattern indicates that OLCs derived from 4 wpi tissue exhibit an increased tendency to differentiate. Thus, OLC differentiation within this culture system correlates with OLC transitions expected for the onset of remyelination at this stage of the disease progression *in vivo*.

Analysis of OLC differentiation in the cultures from tissue at 4 wpi, with either inhibition or addition of PDGF and FGF2, also indicated differences relative to cultures derived from 2 wpi tissue. In cultures from 4 wpi tissue, AG1295 and FGF2nAb had no effect on OLC differentiation when used either individually or in combination [Fig. 9(A)]. This further supports the suggestion that the OLC response to endogenous PDGF and FGF2 signaling is reduced at 4 wpi relative to 2 wpi. Moreover, addition of PDGF-AA or FGF2, either individually or in combination, significantly increased the proportion of $NG2^+$ OPs compared to defined medium alone (Fig. 9), which is again consistent with maintained expression of growth factor receptors at 4 wpi. This increase may be due to proliferation (see above) that generates more $NG2^+$ OPs. Alternatively, the exogenous PDGF-AA

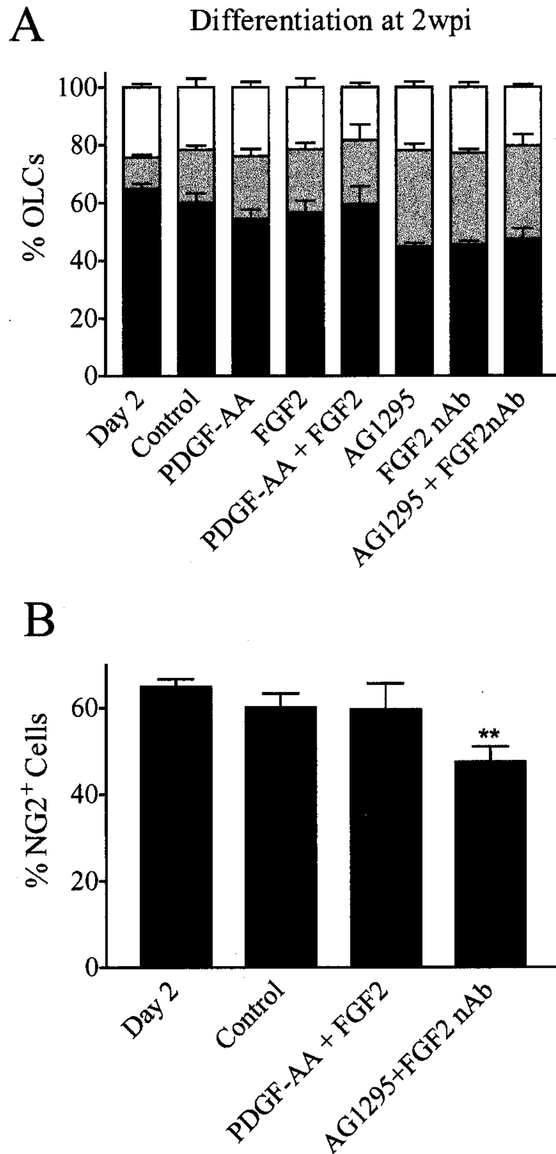

Figure 7 Endogenous PDGF and FGF2 prevent oligodendrocyte lineage cell differentiation in cultures of demyelinated spinal cord. Differentiation of the OLC population in mixed glial cultures of demyelinated spinal cords (at 2 wpi) was assessed using stage-specific markers. Black bars represent early stage $NG2^{+}O1^{-}$ progenitor cells, gray bars represent the transitional stage $NG2^{+}O1^{+}$ cells, and white bars represent differentiated $NG2^{-}O1^{+}$ oligodendrocytes. (A) The addition of exogenous growth factors did not significantly alter the antigenic phenotype of the OLCs. (B) The inhibition of endogenous PDGF-AA and FGF2 with the inhibitors AG1295 and anti-FGF2 neutralizing antibody (FGF2nAb), both individually and in combination, significantly reduced the number of $NG2^{+}$ cells in the cultures. For each value (±S.E.M.) $n = 4$–6 dishes per treatment across three to four different glial preparations. (p vs. control $^{**}p < .01$). Statistical analysis was one-way analysis of variance, followed by paired two-tailed t test analysis.

and FGF2 may be maintaining the $NG2^{+}$ OP population by blocking maturation to the $O1^{+}$ stage.

Clonal Analysis Distinguishes between the Mitogenic and Differentiation Effects of PDGF and FGF2

To more specifically delineate the roles of PDGF and FGF2 in the regulation of OLC proliferation and differentiation during early remyelination, cells isolated from spinal cords at 4 wpi were infected with the BAG replication-deficient retrovirus. Cells infected with the BAG retrovirus expressed β-gal as a heritable marker that allowed identification of clonally derived cells 48 h after infection (Fig. 10). Because the BAG retrovirus is integrated into the chromosome after mitosis (Hajihosseini et al., 1993), the number of β-gal^{+} OLC clones per dish is proportional to the extent of proliferation of the OLC population, whereas the antigenic phenotype indicates the stage of differentiation.

Cultures grown in defined medium alone (control) contained a mean of six OLC clones per dish (Fig. 11), whereas cultures treated with a combination of PDGF-AA and FGF2 contained approximately twice as many OLC clones per dish. Importantly, in both control dishes and in PDGF-AA- and FGF2-containing medium, the antigenic phenotype of β-gal^{+} OLCs was similar (control = 64.5 ± 3.7% $NG2^{+}$ cells; PDGF + FGF2 treatment = 60.1 ± 1.9% $NG2^{+}$ cells). Therefore, exogenous PDGF-AA and FGF2 did not directly alter differentiation of newly generated OPs at 4 wpi. Further, a combination of the PDGF and FGF2 inhibitors significantly reduced the mean number of clones per culture (Fig. 11), but also had no marked effect on the differentiation state within the clones (51.0 ± 11.6% $NG2^{+}$ cells). Taken together, these data indicate that PDGF and FGF2 in combination regulate OLC generation during remyelination by acting directly as mitogens for OPs.

DISCUSSION

Several studies using experimentally induced demyelination have shown increases in OP proliferation associated with remyelination (e.g., Redwine and Armstrong, 1998; Mason et al., 2000; McTigue et al., 2001). However, the mechanisms that induce OP proliferation remain unclear. Using an *in vitro* assay system, this study demonstrates that the proliferation of OPs, during experimentally induced demyelination and remyelination of the adult CNS, is significantly influenced by PDGF and FGF2 acting in combination.

The MHV-A59 coronavirus model used in this

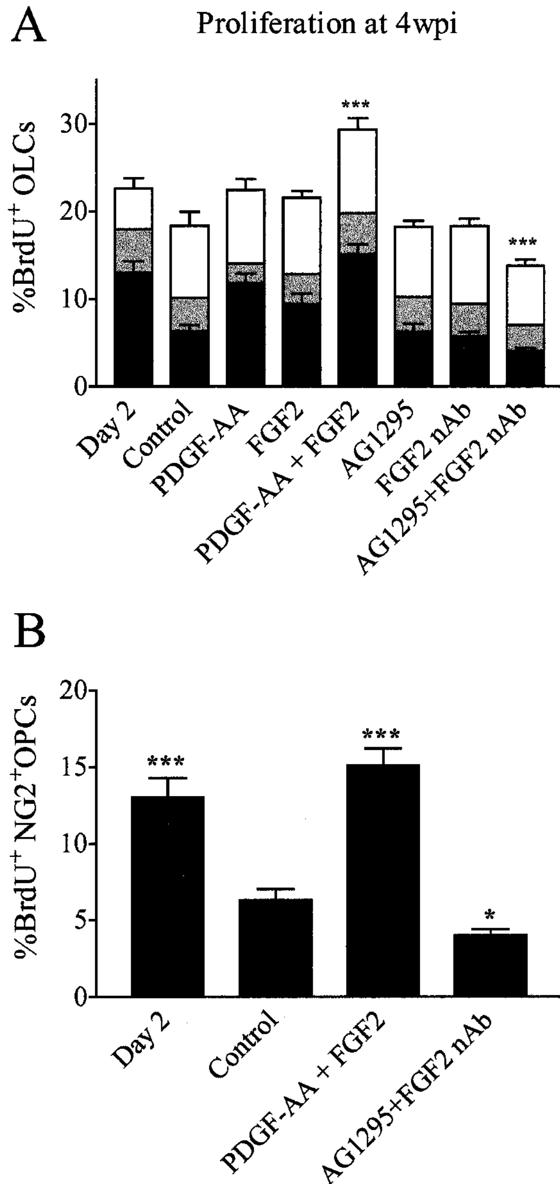

Figure 8 Oligodendrocyte progenitor proliferation in cultures derived during remyelination is regulated by PDGF and FGF2. In mixed glial cultures of remyelinating spinal cords (4 wpi), OLC proliferation was assessed as the percentage of total OLCs immunolabeled with anti-NG2 and/or O1 that had incorporated BrdU. Black bars represent $NG2^+O1^-$ oligodendrocyte progenitor cells, gray bars represent transitional $NG2^+O1^+$ cells, and white bars represent $NG2^-O1^+$ oligodendrocytes. (A) OLC proliferation is significantly increased by a combination of exogenous PDGF-AA and FGF2. Inhibition of both endogenous growth factors with a combination of AG1295 and anti-FGF2 neutralizing antibody (FGF2nAb) results in significant inhibition of proliferation of the OLC population. (B) Specifically, the $NG2^+O1^-$ cell proliferation is significantly increased in the presence of a combination of exogenous PDGF-AA and FGF2, and significantly inhibited in the presence of a combination of AG1295 and FGF2nAb. For each value (±S.E.M.) $n = 3$–6 dishes per treatment across four different glial preparations. (p vs. control $*p < .05$, $***p < .005$). Statistical analysis was one-way analysis of variance, followed by paired two-tailed t test analysis.

study is well suited for examining the events surrounding spontaneous remyelination following acute demyelination of the adult mouse CNS. During the first weeks after intracranial injection of the virus, throughout the spinal cord focal lesions develop and are characterized by significant oligodendrocyte loss with subsequent myelin destruction (Jordan et al., 1989). This demyelination of the spinal cord results in paresis, paralysis, and grasping difficulty (represented in Fig. 1; Godfraind et al., 1989; Jordan et al., 1989; Redwine and Armstrong, 1998). Remyelination can be identified by light microscopy at 3–4 wpi, with several wraps of myelin visible by electron microscopy at 4 wpi, which coincides with recovery of motor function (Jordan et al., 1989; Redwine and Armstrong, 1998). The number of remyelinated axons and thickness of myelin continues to progress in subsequent weeks (Jordan et al., 1989; Armstrong et al., 1990).

This study and our previous work (Armstrong et al., 1990) used cultures prepared from spinal cords of MHV-A59 infected mice to examine cellular responses to demyelination and remyelination. To ensure inclusion of all potential pools of progenitors (discussed in Reynolds et al., 2001), entire spinal cords from severely affected mice were used to prepare mixed glial cultures for this study. Using this *in vitro* system we have previously shown that the total number of OLCs isolated from spinal cords at progressive stages of the MHV-A59 induced disease is significantly increased when compared to age matched controls (Armstrong et al., 1990). The present study shows significantly more OLC proliferation occurring during the demyelination stage of the disease, and more specifically identifies $NG2^+$ OPs as the major proliferative phenotype within the OLC population (Fig. 6). During remyelination (4 wpi) we also found that the total number of OLCs (Armstrong et al., 1990), and more specifically the number of $NG2^+$ OPs (current study, data not shown), was significantly increased compared to age matched control animals (Armstrong et al., 1990). The most proliferative $NG2^+$ cells were negative for O1 immunostaining (Figs. 6 and 8). Thus, this technique appears to distinguish progenitors from remyelinating spinal cord compared with a report of normal adult progenitors from optic nerve expressing NG2 or A2B5 with O1 (Shi et al., 1998).

The present study further demonstrates that OPs

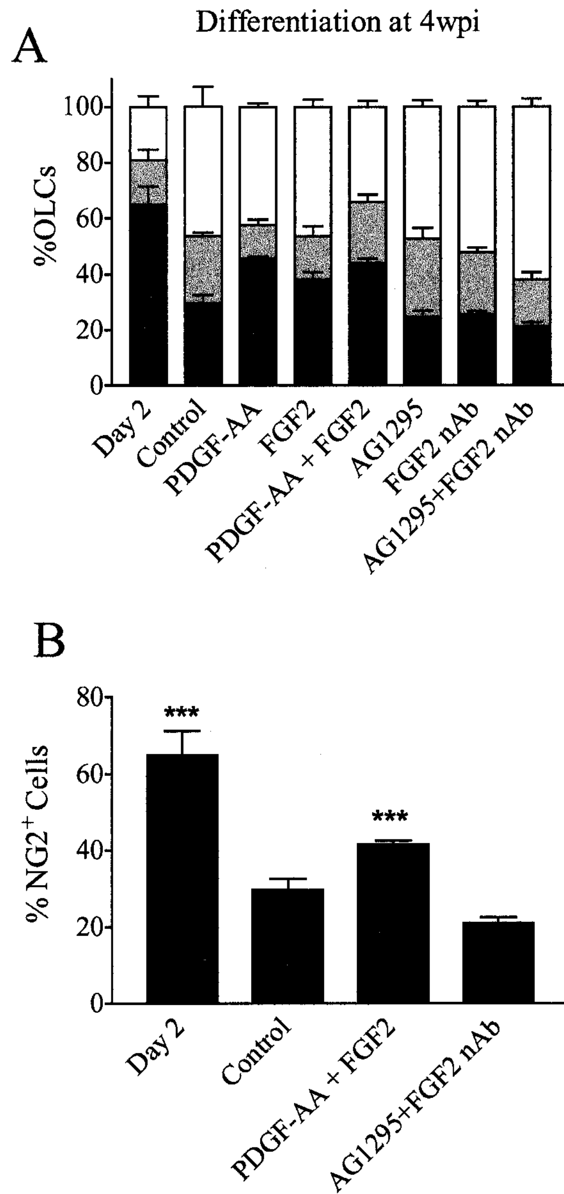

Figure 9 Oligodendrocyte lineage cells are more likely to differentiate in cultures derived during remyelination. Differentiation of the OLC population in mixed glial cultures of remyelinating spinal cords (4 wpi) was assessed using stage-specific markers. Black bars represent $NG2^+O1^-$ OPs, gray bars represent transitional $NG2^+O1^+$ cells, and white bars represent premyelinating $NG2^-O1^+$ oligodendrocytes. (A) After 2 days *in vitro* (div) in DME plus 10% FBS, the OLC population within the cultures consisted of predominantly $NG2^+O1^-$ OPs. However, in contrast to cultures from 2 wpi (Fig. 7), after a further 3 days in Sato's defined medium alone (control), there were significantly fewer $NG2^+$ OPs, with the majority of cells being in later stages of the lineage. (B) The addition of exogenous PDGF-AA and/or FGF2 significantly increases the percentage of $NG2^+O1^-$ cells in the cultures. The growth factor inhibitors, AG1295 and anti-FGF2 neutralizing antibody (FGF2nAb), had no effect on OLC differentiation in the cultures when used either individually or in combination. For each value (±S.E.M.) $n = 3$–6 dishes per treatment across at least four different glial preparations. (p vs. control $^{***}p < .005$). Statistical analysis was one-way analysis of variance, followed by paired two-tailed t test analysis.

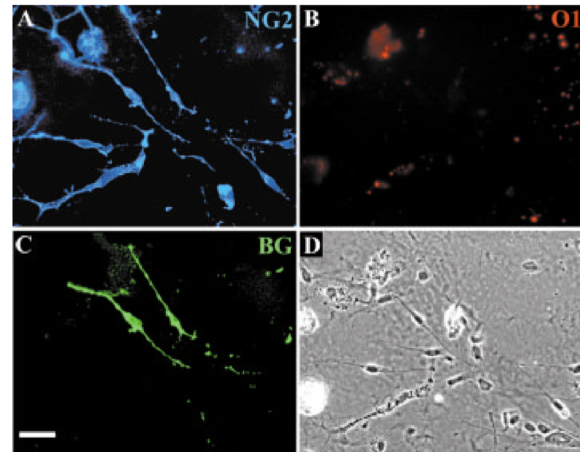

Figure 10 Clonal analysis of mixed glial cultures derived from remyelinating spinal cords. To distinguish between the effects of PDGF and FGF2 on proliferation and differentiation, cultures derived from remyelinating spinal cords (4 wpi) were infected with BAG replication-incompetent retrovirus to introduce β-galactosidase as a heritable marker. OLCs were characterized using antigen-specific markers for the different stages of the oligodendrocyte lineage. (A) OPs were identified by NG2 expression. (B) Differentiated oligodendrocytes were identified with O1 antibody; in this field of view the cells are $O1^-$. (C) β-galactosidase (BG) incorporation was used to identify clonally derived cells within each antigenic phenotype. (D) Phase contrast (PC) shows a variety of glial cell types within the cultures. Scale bar = 25 μm.

show an increased tendency to differentiate in cultures isolated during remyelination (Fig. 9), as would match the transition appropriate for remyelination *in vivo*. Thus, our *in vitro* system maintains the progression of OLC proliferation, accumulation, and differentiation that has been documented to occur in experimental models of demyelination and remyelination *in vivo* (Ludwin, 1979; Godfraind et al., 1989; Mason et al., 2000; McTigue et al., 2001). Therefore, we can take advantage of this *in vitro* model to facilitate a reductionist approach to the investigation of putative growth factor and cytokine interactions that regulate OLC responses during spontaneous remyelination of the adult CNS.

Based upon previous developmental and *in vitro* studies we focused on examining the effects of PDGF and FGF2 signaling to determine the mechanisms

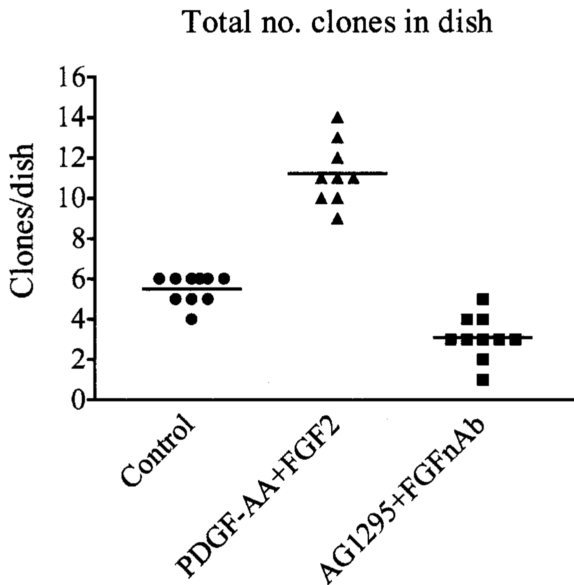

Figure 11 Quantification of clonal analysis. Cultures derived from remyelinating spinal cords (4 wpi) were infected with BAG replication-incompetent retrovirus to introduce β-galactosidase as a heritable marker. OLCs were characterized using antigen-specific markers for the different stages of the oligodendrocyte lineage (as shown in Fig. 10). The number of clones identified within each treatment is an indicator of the level of proliferation within that treatment at the time of retroviral infection. A combination of exogenous PDGF-AA and FGF2 (triangles) increases the total number of OLC clones relative to control conditions (circles). Inhibition of the PDGF and FGF2 (squares) reduces the total number of clones/dish. Each treatment was repeated in four dishes within each of two different glial preparations. Statistical analysis was one-way analysis of variance, followed by paired two-tailed *t* test analysis.

regulating enhanced OP proliferation in response to demyelination. During development PDGF and FGF2, both individually and in combination, are important regulators of nOP proliferation prior to myelination in the developing CNS (Bögler et al., 1990; McKinnon et al., 1990; van Heyningen et al., 2001). In addition, PDGF and FGF2 regulate the proliferation of OP cells isolated from normal adult optic nerve, spinal cord, and corpus callosum (Wolswijk and Noble, 1992; Engel and Wolswijk, 1996; Mason and Goldman, 2002). Studies of ligand and receptor localization in lesions of the MHV-A59 model provide further support for a potential role for PDGF and FGF2 during remyelination *in vivo* (Redwine and Armstrong, 1998; Messersmith et al., 2000).

Proliferation of PDGFαR$^+$ OPs is increased in and around lesions in the MHV-A59 model and is associated with increased numbers of NG2 expressing cells (Redwine and Armstrong, 1998; Armstrong, 2000). Also, the density of cells expressing FGF receptors increases in remyelinating MHV-A59 lesions in comparison to nonlesioned spinal cord tissue (Messersmith et al., 2000). Our previous studies have also identified increases in expression of PDGF and FGF2 ligand in and around lesions in the MHV-A59 model (Redwine and Armstrong, 1998; Messersmith et al., 2000). Specifically, PDGF ligand expression was associated with reactive astrocytes around lesions in the spinal cord (Redwine and Armstrong, 1998). In addition, increased FGF2 expression was reported in activated astrocytes and microglia in lesions resulting from MHV-A59 infection (Messersmith et al., 2000) and from experimental allergic encephalomyelitis (Liu et al., 1999).

These findings support the prediction that PDGF and FGF2 are involved in the regulation of OLC proliferation in response to demyelination. Surprisingly, our earlier study showed no effects of exogenous PDGF and FGF2 on the proliferation of OLCs in the cultures derived from MHV-A59 lesioned spinal cords (Armstrong et al., 1990). The present study demonstrates a logical explanation for these previous results being disparate with the predicted role of PDGF and FGF2, and provides further insights into the potential activity of PDGF and FGF2 in the progression of demyelination and remyelination. The current work clearly shows: the importance of the combination of PDGF and FGF2, as well as endogenously produced PDGF and FGF2, in the regulation of OLC proliferation; the NG2$^+$ OP stage of OLCs is most responsive to PDGF and FGF2; depending upon the disease stage used for preparation of the cultures, the NG2$^+$ OP sensitivity varies, possibly in response to changes in the level of exogenous versus endogenous PDGF and FGF2. The mitogenic activity of endogenous PDGF and FGF2 appears most important during the demyelination phase when overall OP proliferation is most robust. Further studies of the growth factor expression within the cultures and the receptor downstream signaling pathways are required to fully elucidate the regulatory mechanism of these growth factor pathways.

In studies using neonatal CNS, PDGF and FGF2 both affect the differentiation state of nOPs, as well as acting as mitogens for nOPs (Bögler et al., 1990; McKinnon et al., 1993; Grinspan et al., 1996; Goddard et al., 1999). The present study used clonal analysis to distinguish between these two different potential roles of PDGF and FGF2 during remyelination. Our results (Fig. 11) show that the differentiation of β-gal$^+$ OLC clones is not affected by the addition of exogenous PDGF and FGF2, whereas OP proliferation is increased. This implies that other putative factors are present in the cultures, and potentially *in*

vivo, which contribute to the regulation of OLC differentiation.

Increased expression of other growth factors and cytokines has been reported in experimental models of demyelination and may influence OLC responses. Glial Growth Factor 2, TNFα, and TGF-β1 may contribute to a hierarchy of growth factor interaction that, along with PDGF and FGF2, may regulate OP proliferation and differentiation to bring about remyelination (Cannella et al., 1998; Shi et al., 1998; Hinks and Franklin, 1999; Arnett et al., 2001). Other growth factors, such as IGF1, may also promote oligodendrocyte survival in demyelinating diseases (Mason et al., 2000). However, more detailed methods of analysis will be required to clarify the role of other trophins in the regulation of OLCs during remyelination. Further studies will be important to assess the *in vivo* efficacy of a growth-factor-based therapeutic strategy, and to extend this work to the context of human demyelinating diseases such as multiple sclerosis. The present study illustrates how the control of the effects of growth factor treatment for demyelinating disease may require knowledge of the most responsive cell populations, the interactions among combinations of trophins, and how growth factor therapies may need to be modulated throughout a disease course.

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