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Fibroblast Growth Factor 2 (FGF2) and FGF Receptor Expression in an Experimental Demyelinating Disease With Extensive Remyelination

Donna J. Messersmith,[1,2] Joshua C. Murtie,[3] Tuan Q. Le,[1] Emma E. Frost,[1] and Regina C. Armstrong[1–3]★

[1]Department of Anatomy and Cell Biology, Uniformed Services University of the Health Sciences, Bethesda, Maryland
[2]Program in Neuroscience, Uniformed Services University of the Health Sciences, Bethesda, Maryland
[3]Program in Molecular and Cell Biology, Uniformed Services University of the Health Sciences, Bethesda, Maryland

Fibroblast growth factor 2 (FGF2) is an excellent candidate to regulate remyelination based on its proposed actions in oligodendrocyte lineage cell development in conjunction with its involvement in CNS regeneration. To assess the potential for FGF2 to play a role in remyelination, we examined the expression pattern of FGF2 and FGF receptors (FGFRs) in an experimental demyelinating disease with extensive remyelination. Adult mice were intracranially injected with murine hepatitis virus strain A-59 (MHV-A59) to induce focally demyelinated spinal cord lesions that spontaneously remyelinate, with corresponding recovery of motor function. Using kinetic RT-PCR analysis of spinal cord RNA, we found significantly increased levels of FGF2 mRNA transcripts, which peaked during the initial stage of remyelination. Analysis of tissue sections demonstrated that increased levels of FGF2 mRNA and protein were localized within demyelinated regions of white matter, including high FGF2 expression associated with astrocytes. The expression of corresponding FGF receptors was significantly increased in lesion areas during the initial stage of remyelination. In normal and lesioned white matter, oligodendrocyte lineage cells, including progenitors and mature cells, were found to express multiple FGFR types (FGFR1, FGFR2, and/or FGFR3). In addition, in lesion areas, astrocytes expressed FGFR1, FGFR2, and FGFR3. These findings indicate that, during remyelination, FGF2 may play a role in directly regulating oligodendrocyte lineage cell responses and may also act through paracrine or autocrine effects on astrocytes, which are known to synthesize other growth factors and immunoregulatory molecules that influence oligodendrocyte lineage cells. J. Neurosci. Res. 62:241–256, 2000. **Published 2000 Wiley-Liss, Inc.†**

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Remyelination involves a recapitulation of developmental events occurring in a pathological environment. Fibroblast growth factor 2 (FGF2) is likely to be an important regulator of remyelination based on its proposed actions in oligodendrocyte lineage cell development in conjunction with its association with CNS injury. FGF2 is expressed in the developing and adult CNS, and its role may be accentuated in pathological states by enhanced expression and activation. In normal CNS tissues FGF2 is synthesized by neurons and astrocytes and in some areas microglia (Hatten et al., 1988; Woodward et al., 1992; Gonzalez et al., 1995; Redwine et al., 1997). Pathological conditions induce FGF2 synthesis by astrocytes and macrophage/microglia cells (Logan et al., 1992; Mocchetti et al., 1996; Liu et al., 1998). In wound fluids, FGF2 activity may be enhanced by heparanases that degrade the ectodomain of soluble syndecan-1, which then converts from an inhibitor of FGF2 to become a potent activator (Kato et al., 1998). The range of effects of FGF2 in CNS pathology have not been fully elucidated. Improvement in the survival of injured neurons has been demonstrated with FGF2 administration (Grothe and Unsicker, 1992; Teng et al., 1999). In vitro data and developmental studies suggest that FGF2 may also influence oligodendrocyte

Donna J. Messersmith and Joshua C. Murtie contributed equally to this paper.

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★Correspondence to: Dr. Regina Armstrong, Department of Anatomy and Cell Biology, Uniformed Services University of the Health Sciences, 4301 Jones Bridge Rd., Bethesda, MD 20814-4799.
E-mail: rarmstrong@usuhs.mil

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lineage cell responses following CNS demyelination (Wolswijk and Noble, 1995; Armstrong, 2000).

In the CNS, FGF2 can act through three high-affinity receptors (FGFR1, R2, R3 IIIc isoforms) along with multiple low-affinity receptors expressed on a wide variety of neural cell types (Chellaiah et al., 1994; Ornitz et al., 1996). In vitro, FGF2 appears to influence every stage of oligodendrocyte development. FGF2 is an important factor for in vitro treatment of embryonic stem cells to generate oligodendrocytes (Ben-Hur et al., 1998; Brüstle et al., 1999). FGF2 may act independently, or in combination with other factors, to induce oligodendrocyte progenitor (OP) proliferation and migration (McKinnon et al., 1990; Milner et al., 1997; Simpson and Armstrong, 1999). Differentiation of oligodendrocyte lineage cells is arrested and/or reversed in the presence of FGF2 (Grinspan et al., 1996; Bansal and Pfeiffer, 1997). Consistent with these in vitro findings, exogenous FGF2 increases the number of promyelinating oligodendrocytes and retards myelination in vivo (Goddard et al., 1999). These diverse effects of FGF2 on oligodendrocyte lineage cells may reflect the differential expression of FGFRs with cell maturation (Bansal et al., 1996) and the interplay of FGF2 with other regulatory molecules.

The present study examines the expression of FGF2 ligand and receptor types in a murine model of transient focal demyelination that is followed by extensive remyelination and recovery of motor function. We determine the temporal pattern of FGF2 expression relative to the major stages in this disease progression and the spatial distribution of FGF2 relative to focal lesions. In normal and lesioned areas, we characterize expression of FGFR1, FGFR2, and FGFR3 by specific glial cell types, including oligodendrocyte lineage cells as required for predicting a role in the extensive remyelination accomplished in this model.

MATERIALS AND METHODS

MHV-A59 Injection and Preparation of Mouse Spinal Cord Tissue

As was previously described (Redwine and Armstrong, 1998), MHV-A59 stock was injected intracranially into 28-day-old C57Bl/6 female mice (Charles River, Wilmington, MA) to produce focal areas of demyelination throughout the rostrocaudal extent of the spinal cord. Throughout the disease progression, motor impairment and recovery were quantified by recording the period of time when mice were able to hang upside down, supporting their weight by gripping the metal cage-top bars (Redwine and Armstrong, 1998). Additionally, a clinical score was assigned as follows: 0 for no paralysis, 1–5 for paresis/paralysis in one to five appendages, and 6 for morbidity (Redwine and Armstrong, 1998).

Kinetic Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for RNA Quantitation

RNA was isolated from individual spinal cords using Trizol Reagent (Life Technologies, Gaithersburg, MD). The RT reaction was carried out separately from 2 μg total RNA from each mouse using random hexamer primers. According to the methods detailed for the ABI PRISM 7700 "TaqMan" System (PE Applied Biosystems, Foster City, CA), mouse FGF2 primers (forward primer CCCACCAGGCCACTTCAA from nucleotide 60; reverse primer TCTCTCTTCTGCTTGGAGTTGTAGTT from nucleotide 204) were designed to flank an intervening FGF2 probe (CCCAAGCGGCTCTACTGCAAGAACG from nucleotide 82) that was labeled with a fluorochrome (6-FAM) on the 5′ end as well as a fluorescence quencher (TAMRA) on the 3′ end. The total RNA values and RT reaction efficiency was normalized by measuring 18s rRNA for each sample in parallel.

Immunofluorescence Detection of FGF2 and FGFRs

The anti-FGF2 rabbit polyclonal antiserum (Santa Cruz Biotechnology, Santa Cruz, CA) was shown by western blotting and immunohistochemistry to recognize mouse FGF2. The rabbit polyclonal anti-FGFR antisera used were raised against peptide sequences designed to detect FGFR1 differentially (Sigma, St. Louis, MO), FGFR2 (Santa Cruz Biotechnology), and FGFR3 (Santa Cruz Biotechnology) and previously characterized (Johnston et al., 1995; Pirvola et al., 1995; Ballabriga et al., 1997; Del Rio-Tsonis et al., 1997; Rao et al., 1998; Sogos et al., 1998; Cohen and Chandross, 2000).

Mice were perfused with 4% paraformaldehyde, and the spinal cords were processed as in Redwine and Armstrong (1998). Fifteen-micrometer-thick transverse cryostat sections were permeabilized with 10% Triton X-100 in PBS for 10 min, and nonspecific binding sites were blocked by a 30 min incubation in a solution of 25% normal goat serum, 0.4% Triton X-100, 1% bovine serum albumin, and 100 mM L-lysine. Sections were incubated overnight at 4°C with anti-FGFR or anti-FGF2 rabbit polyclonal antibodies (100 μg/ml stock diluted 1:500), which was subsequently detected with a donkey anti-rabbit IgG antibody conjugated with Cy3. The specificity of the primary antisera (anti-FGF2, -FGFR2, -FGFR3) immunodetection was demonstrated by preincubation with a 100-fold excess of the corresponding peptide antigen overnight at 4°C, which abolished subsequent immunoreactivity.

Immunofluorescence With Glial Cell Type-Specific Markers

Two-color indirect immunofluorescence was used to detect FGFR immunoreactivity associated with distinct glial cell types, identified by immunoreactivity for cell type-specific marker antigens. Platelet-derived growth factor α receptor (PDGFαR) or NG2 chondroitin sulfate proteoglycan (see below) was used to identify OPs in adult rodent CNS (Nishiyama et al., 1996a, 1997, 1999; Reynolds and Hardy, 1997; Trapp et al., 1997; Redwine and Armstrong, 1998). To double immunolabel with the rabbit polyclonal anti-FGFR, PDGFαR was recognized with a rat monoclonal (APA5; Pharmingen, San Diego, CA) and detected with biotinylated anti-rat IgG, followed by tyramide amplification (Renaissance indirect blue kit; New England Nuclear; Boston, MA) to deposit AMCA (blue fluorescence). Mature oligodendrocytes expressing FGFRs were identified by immunolabeling for CC1 or by in situ hybridization for proteolipid protein (PLP; see below). CC1 monoclonal antibody immunostains the cell body of mature oligodendrocytes without labeling the myelin, which facilitates cell identi-

fication (Fuss et al., 2000). The CC1 antibody (100 μg/ml; Oncogene Research Products, Cambridge, MA) was used at 1:20 dilution, which under our conditions did not double immunolabel with an astrocyte marker, glial fibrillary acidic protein (GFAP), or with NG2 (unpublished observations). Double immunolabeling to detect FGFRs in astrocytes utilized a mouse monoclonal IgG1 anti-GFAP antibody (Boehringer Mannheim, Indianapolis, IN). Microglia were identified with anti-Mac-1 rat monoclonal IgG2b (Boehringer Mannheim) detected using tyramide deposition of AMCA (see above).

All secondary antibodies were affinity-purified $F(ab')_2$ fragments with minimal cross-reactivity to serum proteins from the other species used in each protocol (Jackson Immunoresearch; West Grove, PA). For single label and multilabel immunostaining protocols, the sets of primary and secondary antibodies were tested for potential cross-reactivity by omitting each primary antibody from the protocol.

In Situ Hybridization

In situ hybridization and preparation of digoxigenin-labeled riboprobes was performed as previously detailed (Redwine and Armstrong, 1998), with minor modifications to adjust the hybridization temperature and washes for each probe sequence. Digoxigenin was detected with an alkaline phosphatase-conjugated sheep antidigoxigenin (Fab fragment; Boehringer Mannheim) antibody, followed by reaction with NBT/BCIP substrate (Dako, Carpinteria, CA), dehydration in progressive ethanol solutions, and mounting with Permount (Fisher, Fair Lawn, NJ).

The digoxigenin-labeled ribonucleotide probes used to detect FGF2 transcripts were prepared from cDNA templates complementary to a 477 bp fragment of the rat FGF2 mRNA (Shimasaki et al., 1988; Dr. Andrew Baird, Ciblex Corporation, San Diego, CA) or a 475 bp ribonucleotide probe to the mouse FGF2 mRNA (nucleotides 1–475; Dr. Gail Martin, University of California at San Francisco; Hebert et al., 1990). The FGFR1 ribonucleotide probe (Dr. Alka Mansukhani, New York University School of Medicine) contained 1.2 kb of the 5′ end of the murine *flg* receptor (nucleotides 1–1,177). Similar results were also observed using a smaller probe that hybridized to nucleotides 843–1,170 of the mouse FGFR1 sequence (Dr. David Ornitz; Washington University Medical School, St. Louis, MO). The FGFR3 ribonucleotide probe (Dr. David Ornitz) hybridizes to nucleotides 1,233–1,663 of the mouse sequence, which should detect each of the differentially spliced FGFR3 isoforms (Chellaiah et al., 1994; Johnston et al., 1995; Ornitz et al., 1996). These cDNA probes have been shown to be specific for each of the FGFR types based on detection of appropriately sized bands and differential expression on Northern blots of neonatal rat oligodendrocyte lineage cells (Bansal et al., 1996). The cDNA template for the proteolipid protein

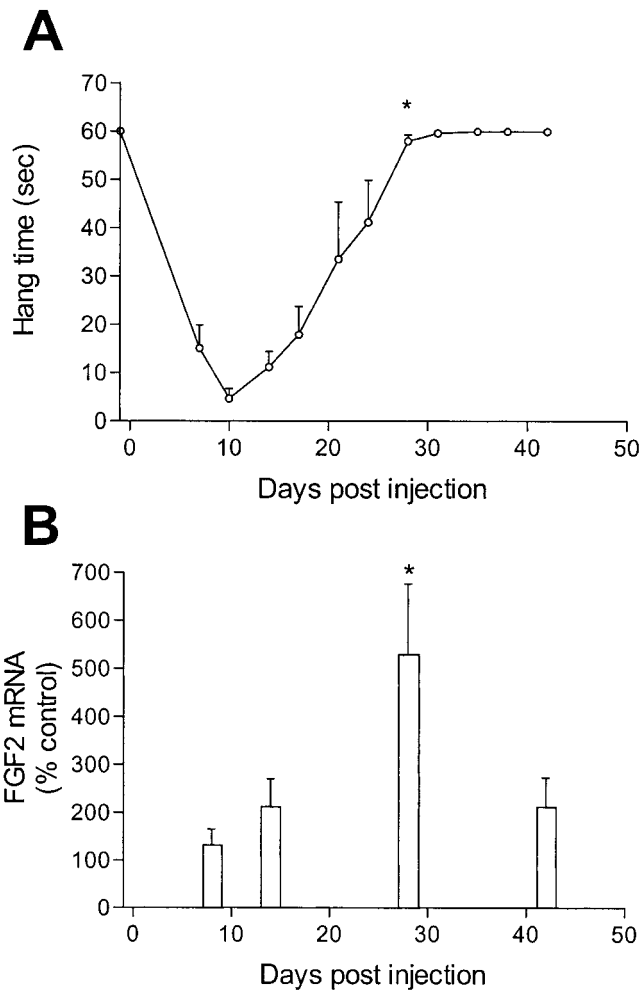

Fig. 1. FGF2 mRNA peak expression corresponds with recovery of motor function. **A:** Mice intracranially injected with MHV-A59 or PBS were tested for limb motor impairment (hang time) and sacrificed for RNA isolation from spinal cords. Analysis was carried out in parallel for MHV-injected and age-matched PBS-injected (control) mice at various days postinjection (dpi) throughout the progression of demyelination and remyelination. Limb motor impairment was scored by the time that mice could support their weight by grasping the bars of a cage top. Hang time scores indicate significant motor function impairment at 7–21 dpi, with maximal impairment at 10 dpi ($P < 0.001$ for 7, 10, 14, 21 dpi each; compared to preinjection baseline values in which each mouse recorded a maximal time of 60 sec for this assay; ANOVA with Tukey's post hoc analysis), with almost complete recovery at 28 dpi ($^{\star}P < 0.001$ for 28 dpi vs. 10 dpi; ANOVA with Tukey's post hoc analysis). **B:** FGF2 mRNA transcript abundance was determined in these behaviorally tested mice by kinetic RT-PCR analysis using the ABI Prism 7700 "TaqMan" system. FGF2 mRNA values were normalized to 18s rRNA values for the cDNA sample generated from the same RT reaction. FGF2 mRNA transcript levels for MHV affected mice were 533% higher than PBS control values at the 28 dpi stage, when motor function recovery was almost complete. FGF2 mRNA values from MHV-affected mice were significantly elevated at this 28 dpi stage of the disease progression ($P < 0.05$ for 28 dpi vs. 8 dpi; ANOVA with Tukey's post hoc comparison). The same mice were used in the behavioral tests and the RNA isolation (8 dpi, N = 4 MHV and 3 PBS; 14, 28, and 42 dpi, N = 3 MHV and 3 PBS for each dpi). Hang time scores shown are combined for all mice so that values for a given dpi include the mice sacrificed on that dpi as well as mice that were sacrificed for RNA isolation at any later dpi. Error bars indicate SEM.

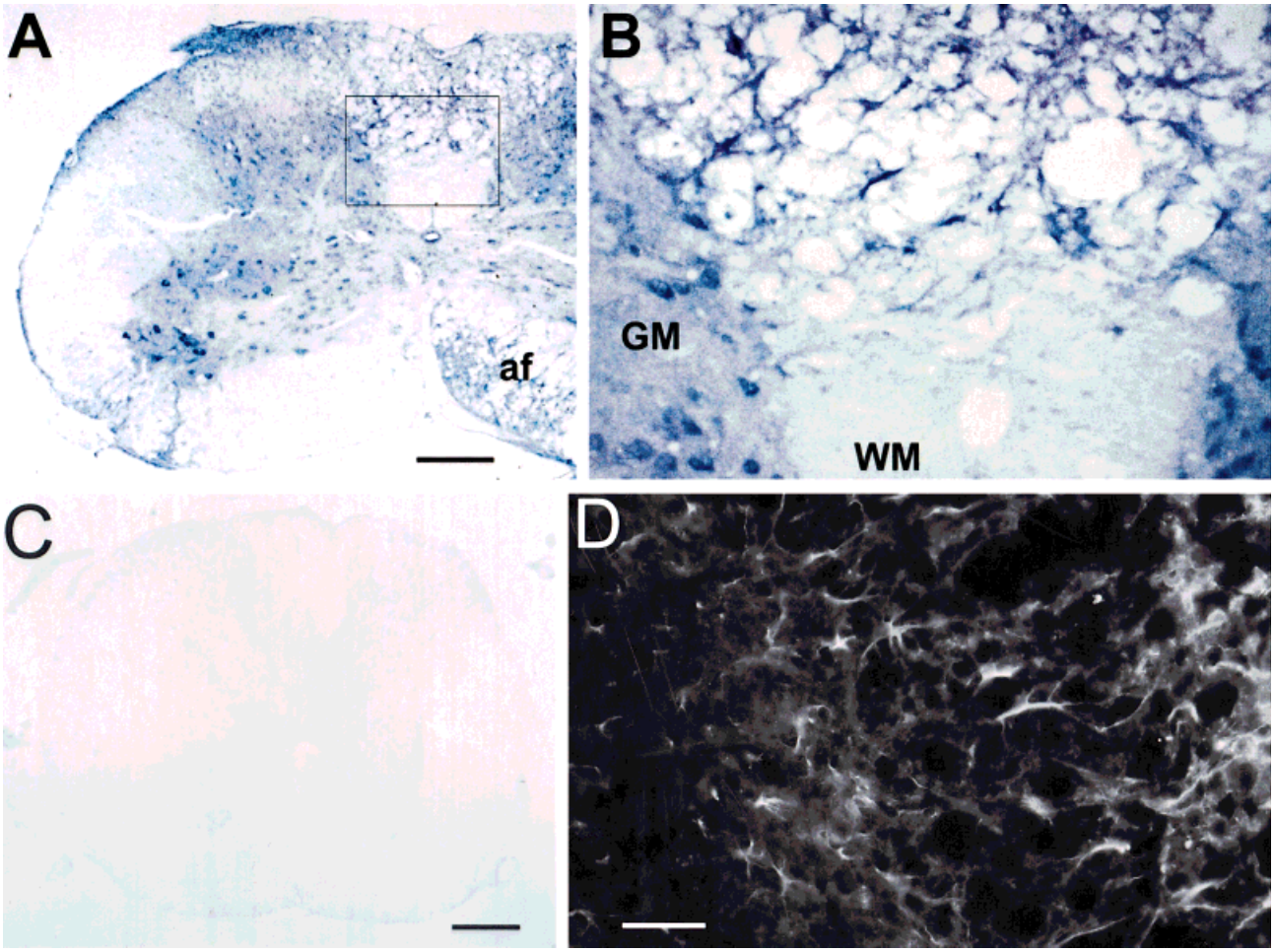

Fig. 2. Increased expression of FGF2 mRNA and protein is localized to white matter lesion areas. In situ hybridization for FGF2 mRNA transcripts (A–C) and immunofluorescence for FGF2 protein (D). **A:** In spinal cord sections from lesioned mice (MHV, 4 wpi), FGF2 mRNA signal is evident in neurons (as in controls, not shown) and is strong in lesioned white matter areas. **B:** Higher magnification (dorsal column region from box in A) shows signal in the cell body and processes of astrocyte-like cells that is clearly increased in the vacuolated lesion area (upper dorsal column area) relative to the normal-appearing white matter (lower dorsal column area). **C:** Similar FGF2 mRNA signal was not found in sections of FGF2 null mice (no injections), demonstating the specificity of the FGF2 mRNA detection. **D:** In sections of lesioned white matter, FGF2 immunoreactivity was most evident in cells with an astrocytic morphology, similar to the findings with FGF2 mRNA detection. Scale bars = 200 μm in A,C; 50 μm in D.

(PLP) ribonucleotide probe (Dr. Lynn Hudson, National Institutes of Health) is complementary to a 980 bp fragment of mouse PLP mRNA (Hudson et al., 1987).

In Situ Hybridization Combined With Immunostaining

The in situ hybridization reaction was carried out, as described above, followed by immunostaining. However, because the NBT/BCIP precipitate can impair distinction of the immunostaining reaction, adjustments to the protocols were chosen based on the optimal combination of detection methods required by the subcellular localization and relative intensity of each signal. The immunoreactivity for GFAP was detected as a brown DAB precipitate, using the ABC Elite kit (Vector, Bulingame, CA), which could be distinguished from the blue/purple NBT/BCIP hybridization reaction. PLP mRNA was detected with a Cy3-conjugated antidigoxin mouse monoclonal (Jackson Immunoresearch) that recognizes digoxigenin, and the anti-FGFR2 was detected with an FITC-conjugated anti-rabbit secondary antibody.

Preparation and Immunostaining of Glial Cell Cultures From Remyelinating Spinal Cord

Because the OPs have small cell bodies with very little cytoplasm, cell cultures were prepared to more readily detect NG2 immunofluorescence along the cell membrane of the soma and processes relative to the NBT/BCIP reaction in the cytoplasm. Mice infected with MHV-A59 were sacrificed at 4 weeks postinjection (wpi), and glial cell cultures were prepared from the spinal cords, as previously detailed (Armstrong et al., 1990). For each glial cell isolation spinal cords were combined from six

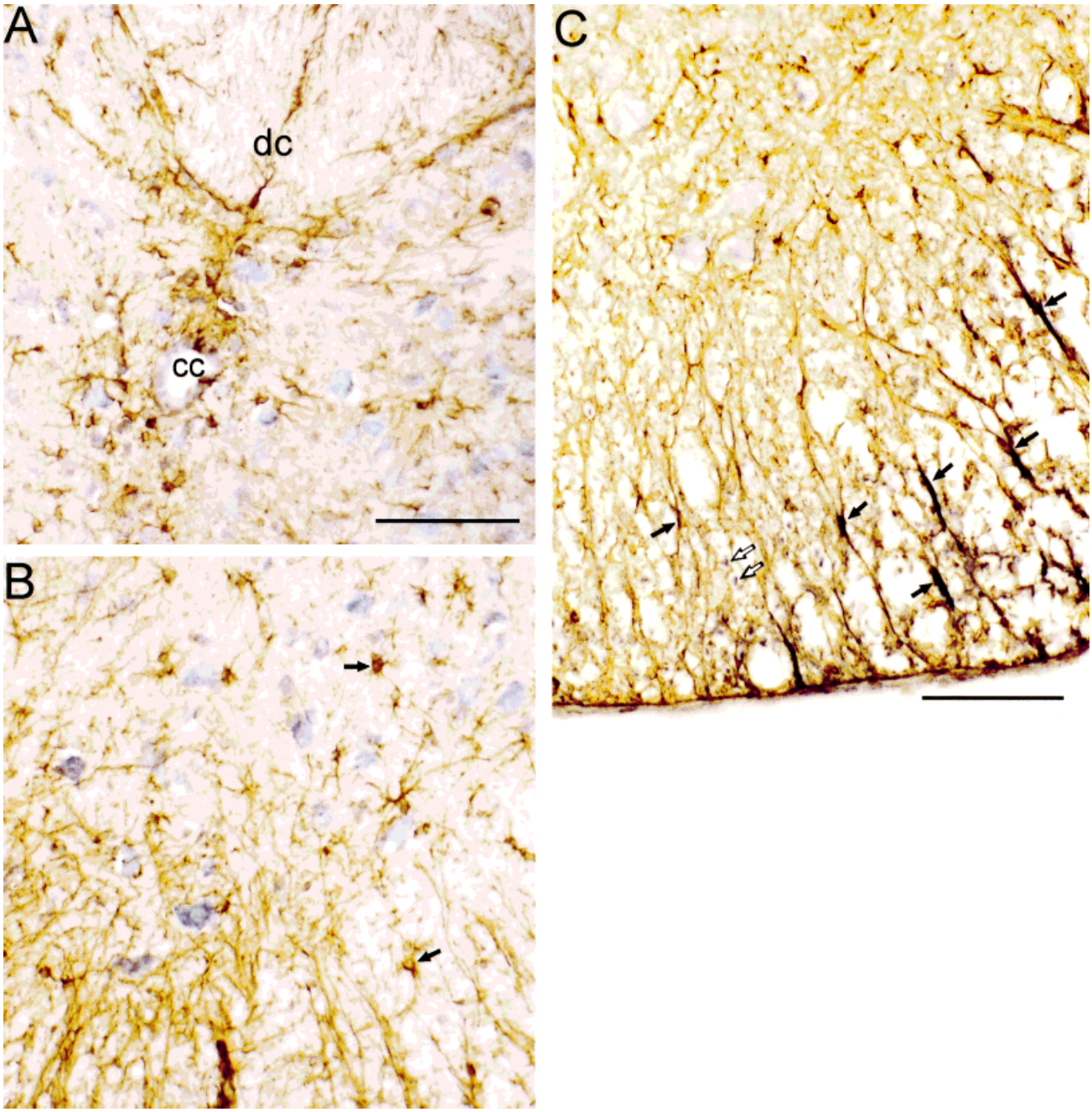

Fig. 3. Reactive astrocytes exhibit high levels of FGF2 mRNA transcripts. In situ hybridization for FGF2 (blue, NBT/BCIP reaction) combined with immunostaining for GFAP (brown, DAB reaction) to identify astrocytes. **A,B:** In control sections of spinal cord (PBS, 4 wpi), the FGF2 mRNA transcripts are most abundant in neurons throughout the gray matter [A shows area adjacent to central canal (cc) and dorsal column (dc); B shows ventral horn]. FGF2 mRNA is also detected in cells around the central canal and in astrocytes of the gray matter and white matter (examples at arrows in B). **C:** In sections of lesioned spinal cord (MHV, 4 wpi), the overall immunoreactivity for GFAP (brown) is increased and is very intense within the vacuolated lesion area, consistent with gliosis. The FGF2 mRNA signal is extremely intense within astrocytes in the lesions (solid arrows) and appears as almost black, because of the NBT/BCIP dark blue combining with the brown anti-GFAP reaction. In the lesioned tissue, the NBT/BCIP reaction had to be weaker in the neurons (large cells in C compared to B) to allow detection of GFAP in astrocytes with intense NBT/BCIP. Scale bars = 100 μm and apply to all panels.

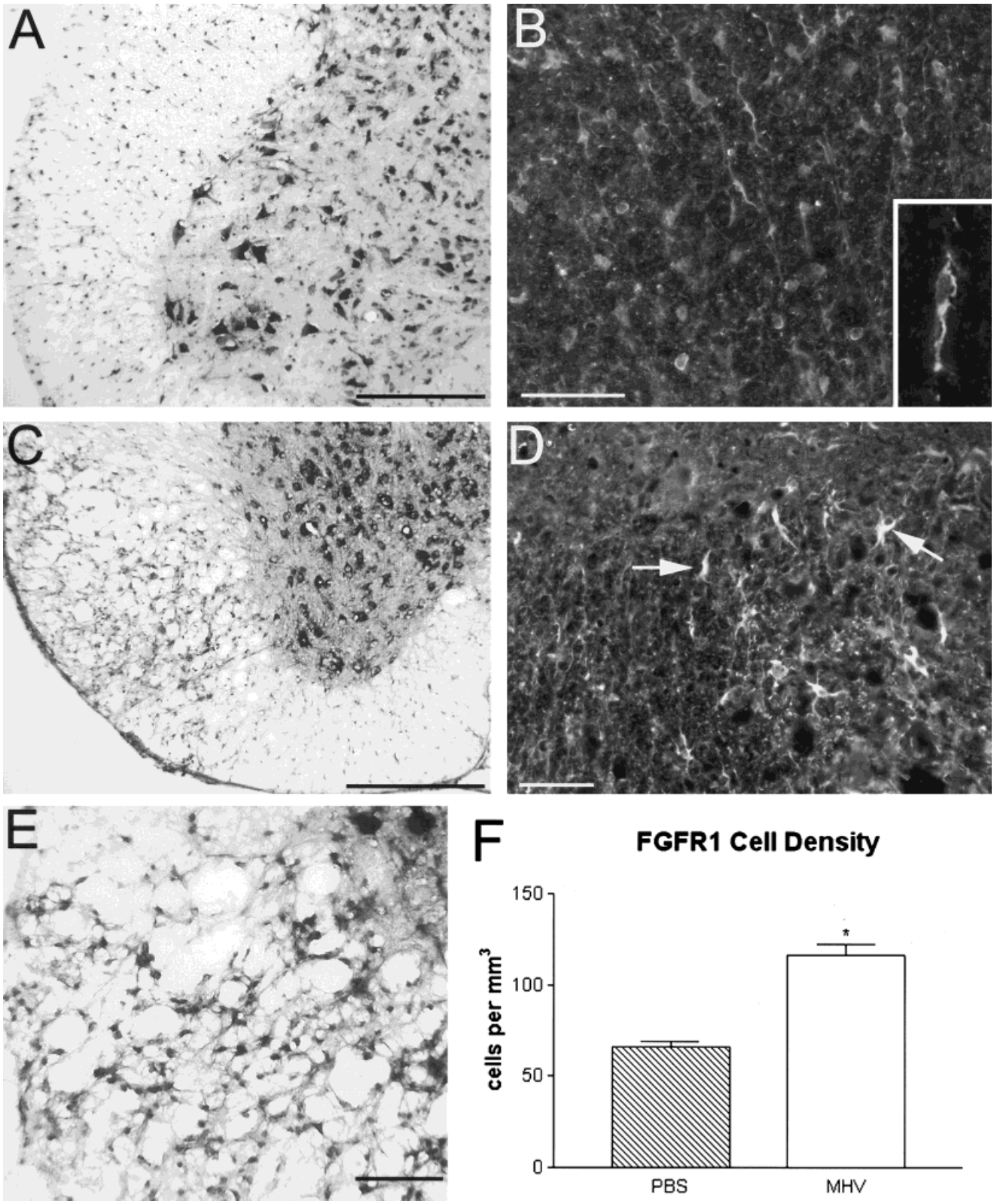

Figure 4.

severely affected mice, as determined from the hang time assay of motor function. Cells were grown in DMEM supplemented with 10% FBS (Life Technologies). After 2 days in vitro, cells were fixed with 4% paraformaldehyde and processed for in situ hybridization, as detailed above, with the omission of the proteinase K digestion. After incubation in NBT/BCIP, OPs were identified by immunostaining with anti-NG2 rabbit polyclonal antisera (kindly provided by Dr. Joel Levine, Stony Brook, NY and by Dr. William Stallcup, La Jolla, CA) detected with Cy3-conjugated anti-rabbit secondary antibody.

FGF2 Null Mice

FGF2 knockout mice (generously provided by Dr. Doetschman, University of Cincinnati; Zhou et al., 1998) were used as controls for testing the specificity of the in situ hybridization detection of FGF2 mRNA transcripts and of the immunofluorescence detection of FGF2 protein. These mice do not have detectable FGF2 wild-type messages or transcripts containing exon 2 and 3 sequences (Zhou et al., 1998).

Imaging and Documentation

Immunostaining and in situ hybridization results were captured with either 400 ASA Ektachrome color film or a Spot 2 digital camera (Diagnostic Instuments, Sterling Heights, MI) using single channels filter sets for Cy3, FITC, and AMCA or a triple-bandpass filter (Chroma Technologies, Brattleboro, VT) to view multiple channels simultaneously. At least three different MHV affected mice and three different PBS-injected mice were examined for each result presented and exhibited similar results to the representative areas shown.

Unbiased Stereology Analysis

Expression of FGFR1 or FGFR3 mRNA was quantitated using the optical "disector" method of morphometric analysis (Bjugn, 1993; Bjugn and Gundersen, 1993; Long et al., 1998). The cell density was estimated using the Stereologer System (Systems Planning and Analysis Inc., Alexandria, VA). Analysis was performed on lumbar spinal cord sections within ventrolateral white matter lesions from MHV-injected mice and within corresponding ventrolateral white matter areas of matched PBS-injected control spinal cords, prepared on the same slide. Sections from three MHV-injected and three PBS-injected mice were analyzed for each nucleotide riboprobe. Optical "disectors" composed of mainly vacuolar spaces within lesions were not included in the analysis.

RESULTS

Increased Expression of FGF2 Peaks During the Early Remyelination Stage of MHV-A59 Disease Progression

Kinetic RT-PCR analysis (Fig. 1) was used to measure changes in the abundance of FGF2 mRNA transcripts during the progression of demyelination and subsequent remyelination in mice intracranially infected with MHV-A59 virus or PBS. Motor function was monitored to demonstrate loss of function and subsequent recovery (Fig. 1A), which corresponds histopathologically to the progression of demyelination and remyelination (Redwine and Armstrong, 1998). During the stage of maximal demyelination, affected mice exhibited impaired limb movement (clinical score average of 2.0 at 10 days postinjection, dpi; N = 7). The ability of MHV-A59-infected mice to support their weight while hanging upside down from a wire cage top was significantly impaired at 10 dpi, in comparison to prior to the MHV injection, when they easily performed the task. By 4 wpi, in correlation with histological signs of early remyelination (Redwine and Armstrong, 1998), mice almost completely recovered motor function. FGF2 mRNA transcript levels (Fig. 1B) began to increase after the period of maximal motor impairment (10 dpi) and increased to 533% of control values during the early remyelination phase (28 dpi). Subsequent to the initial remyelination phase and associated functional recovery (42 dpi), FGF2 mRNA transcript abundance decreased to 213% of control levels.

Increased Expression of FGF2 Is Localized to Focal White Matter Lesions

This 4 wpi time point, with associated motor function improvement, defined a reproducible stage of early remyelination for examining the peak expression of FGF2 mRNA transcripts to determine the localization of FGF2 mRNA transcripts in lesioned vs. nonlesioned spinal cord tissue (Fig. 2A,B). In both PBS (control)- and MHV-A59-injected mice at 4 wpi, FGF2 mRNA transcripts were readily detected in the perinuclear cytoplasm of neurons in spinal cord gray matter. Focal areas of demyelinated white matter lesions in MHV-infected spinal cords were identified by loss of myelin, using darkfield microscopy (not shown; Redwine and Armstrong, 1998; Armstrong, 2000), as well as by signs of vacuolation, gliosis, and cellular infiltrates. Lesioned white matter consistently ex-

Fig. 4. Increased expression of FGFR1 is localized to white matter lesions. In situ hybridization for FGFR1 mRNA (A,C,E) and immunostaining for FGFR1 protein (B,D). **A:** In sections from normal spinal cord (PBS, 4 wpi), FGFR1 mRNA is most abundant in gray matter cells with a neuronal morphology and is also detected in distributed white matter cells. **B:** Immunofluorescence of FGFR1 protein in normal spinal cord (PBS, 4 wpi) showing expression in cells of multiple glial morphologies, including an apparently bipolar cell (enlarged in **inset**). **C:** In sections from lesioned spinal cord (MHV, 4 wpi), FGFR1 mRNA is more abundant in the vacuolated lesioned white matter (lateral funiculus) than in normal-appearing white matter (ventral funiculus). **D:** A similar increase of FGFR1 immunofluorescence is evident in lesioned (right side of field) relative to nonlesioned white matter (left side of field) in sections from mice 4 wpi with MHV. Signal is most intense in cells with a reactive astrocyte morphology (arrows). **E:** Higher magnification of FGFR1 mRNA in lesioned white matter shows a high density of labeled cells. **F:** Numerical density of cells expressing FGFR1 mRNA in ventrolateral spinal cord white matter in sections from mice injected with MHV-A59 (open bar) or PBS-injected control (hatched bar). Numerical density of cells in lesioned white matter that are expressing FGFR1 mRNA is significantly increased over that of a similar region of control white matter from matched PBS-injected mice (N = 3 MHV mice and 3 PBS mice; $\star P = 0.002$, ANOVA). All stereological analyses represented here had a coefficient of error (CE) of 0.12 or less. Error bars show SEM. Scale bars = 100 μm in A,C; 50 μm in B,D,E.

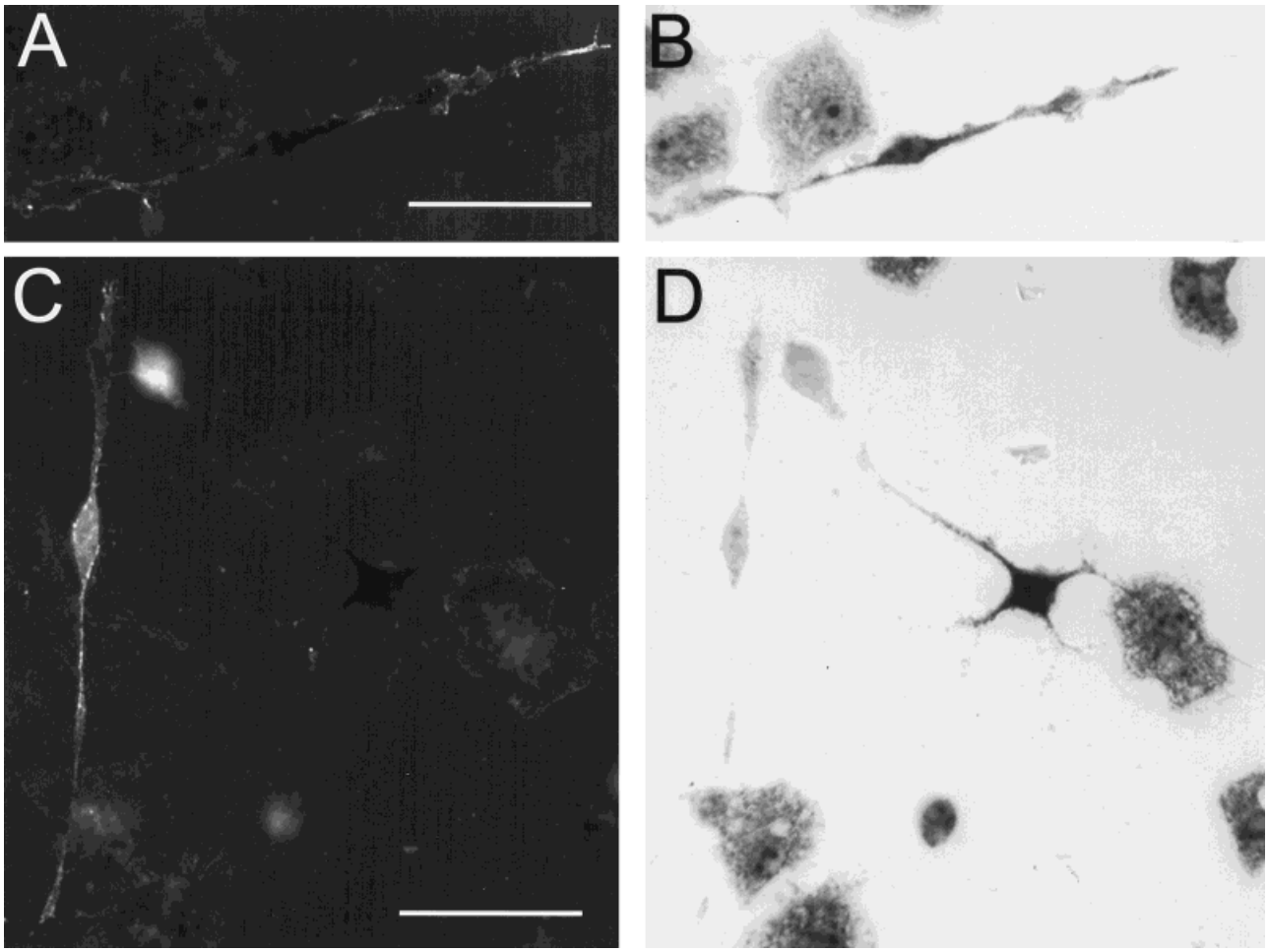

Fig. 5. FGFR1 mRNA expression in oligodendrocyte lineage cells cultured from lesioned spinal cords. Cells isolated from MHV lesioned mice at 4 wpi, grown in culture for 2 days to allow process extension, and then labeled with anti-NG2, detected with immunofluorescence (A,C) and in situ hybridization for FGFR1 mRNA detection with NBT/BCIP substrate (B,D). **A,B:** A bipolar oligodendrocyte progenitor cell with cell surface immunofluorescence for NG2 (A) evident in the processes and strong FGFR1 mRNA signal (B) in the cytoplasm, which blocks imaging of the majority of NG2 epifluorescence. **C,D:** A bipolar oligodendrocyte cell with cell surface immunofluorescence for NG2 (C, left side) does not have FGFR1 mRNA signal (D). An adjacent multipolar cell has strong FGFR1 mRNA signal (D). The ameboid cells are microglia, which are abundant in cultures of lesioned spinal cord (Armstrong et al., 1990). Microglia exhibited variable levels of FGFR1 mRNA transcripts but were never as intensely labeled as were the oligodendrocyte lineage cells. Scale bars = 50 μm.

hibited a high density of cells with intense signal from hybridization with the FGF2 antisense ribonucleotide probe. In comparison, nonlesioned white matter exhibited a relatively low FGF2 signal associated with glial-like cells distributed throughout spinal cord sections. Similar results were obtained with two different antisense FGF2 ribonucleotide probes (not shown). Ribonucleotide probes transcribed as the sense strand were not used as controls for nonspecific hybridization, because expression of antisense FGF2 mRNA transcripts has been reported (Grothe and Meisinger, 1995). The specificity of the FGF2 antisense hybridization was confirmed by the marked decrease of signal in tissue sections from knockout mice lacking FGF2 mRNA transcripts (Fig. 2C).

Immunostaining of similar sections (4 wpi with MHV-A59 or PBS) with an antibody against FGF2 ligand (Fig. 2D) demonstrated increased FGF2 immunoreactivity in lesion areas that corresponded to the increased expression of FGF2 mRNA transcripts. Intense FGF2 immunoreactivity was associated with cells that appeared to be large reactive astrocytes in the lesions and also in astrocyte-like cells in adjacent gray matter. The specificity of the FGF2 immunoreactivity was confirmed by lack of signal after peptide antigen absorption as well as by marked loss of immunostaining using tissue sections from FGF2 null mice (not shown).

Reactive Astrocytes Exhibit Intense FGF2 mRNA Signal in Lesions

Although a previous study in a rat model of experimental demyelination reported a lack of FGF2 mRNA expression by astrocytes (Liu et al., 1998), our results

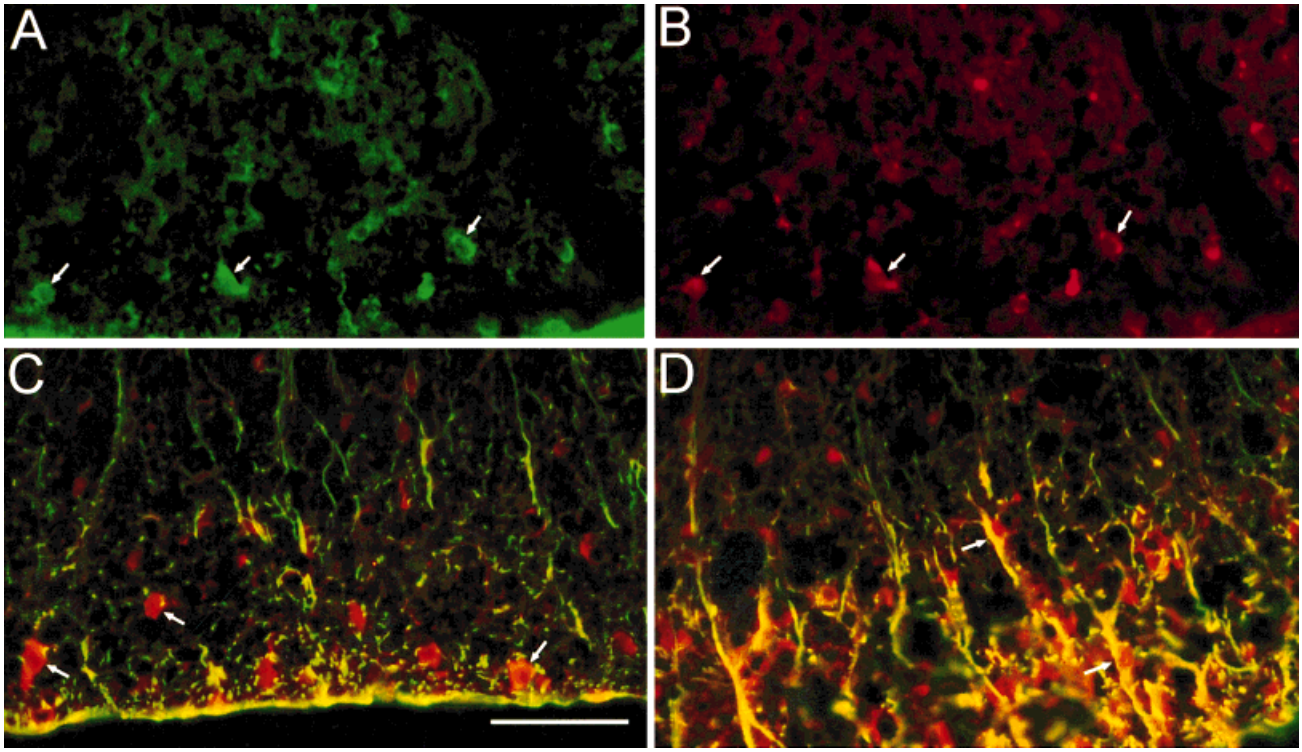

Fig. 6. Cell type-specific expression of FGFR2 is altered in lesioned white matter. Immunofluorescence detection of FGFR2 (A,C,D) with cell type-specific markers using PLP mRNA detection for oligodendrocytes (B) or double immunofluorescence with anti-GFAP for astrocytes (C,D). **A,B:** In normal spinal cord white matter (PBS, 4 wpi), FGFR2 immunofluorescence was present (green in A) in the same cells that exhibited PLP mRNA (red in B; arrows indicate examples of double labeled oligodendrocytes). **C:** In normal spinal cord white matter (PBS, 4 wpi), astrocytes labeled with anti-GFAP (green) are not double immunolabeled for FGFR2 (red). **D:** In lesioned white matter (MHV, 4 wpi), anti-GFAP immunoreactivity (green) increases consistent with gliosis and FGFR2 immunoreactivity (red) is now found in cells that include astrocytes (double immunolabeled cells appear yellow). Scale bar = 50 μm.

indicated FGF2 mRNA transcripts were associated with multiple cell types, including extremely high signal in cells with an astrocytic morphology (Fig. 2). Therefore, FGF2 mRNA expression by astrocytes was examined by in situ hybridization for FGF2 mRNA followed by immunostaining for GFAP, an intermediate filament protein used to identify astrocytes (Fig. 3). In normal spinal cord tissue, FGF2 mRNA signal was detected in GFAP-immmunolabeled cells mainly in the gray matter, less frequently in white matter, and also in cells along the central canal (Fig. 3A,B). Sections from MHV-A59-affected mice exhibited increased GFAP immunoreactivity, which was mildly increased throughout the gray matter and severely increased in focal white matter lesions (Fig. 3C). Intense FGF2 hybridization signal was clearly associated with GFAP-immunolabeled astrocytes within and near the lesions (Fig. 3C, solid arrows). Small microglia-like cells also exhibited FGF2 hybridization signal within and near the lesions at 4 wpi (Fig. 3C, open arrows), consistent with the study by Liu et al. (1998), which used several markers to identify microglia expressing FGF2.

Expression of FGFR Types in Normal and Lesioned Spinal Cord Tissues

To determine the range of potential cell types within the spinal cord environment that could potentially be regulated by local FGF2 levels during remyelination, we characterized the expression pattern of FGFR1, FGFR2, and FGFR3 in normal and lesioned spinal cord tissue sections using in situ hybridization and/or immunodetection.

FGFR1 was expressed in multiple cell types with particularly high levels in neurons, which were evident from the distinctive morphology and organization within the gray matter laminae (Fig. 4A). Within normal white matter (Fig. 4A,B), FGFR1 was present in distributed cells of various morphologies, which is consistent with our previous report using cell type-specific markers to demonstrate that oligodendrocytes, OPs, and astrocytes exhibited immunolabeling with a monoclonal antibody recognizing mainly FGFR1 (Redwine et al., 1997). In areas of demyelinated lesions at 4 wpi, there was a significant increase of cells detected with in situ hybridization for FGFR1 (Fig. 4C,E,F) and a corresponding increase in

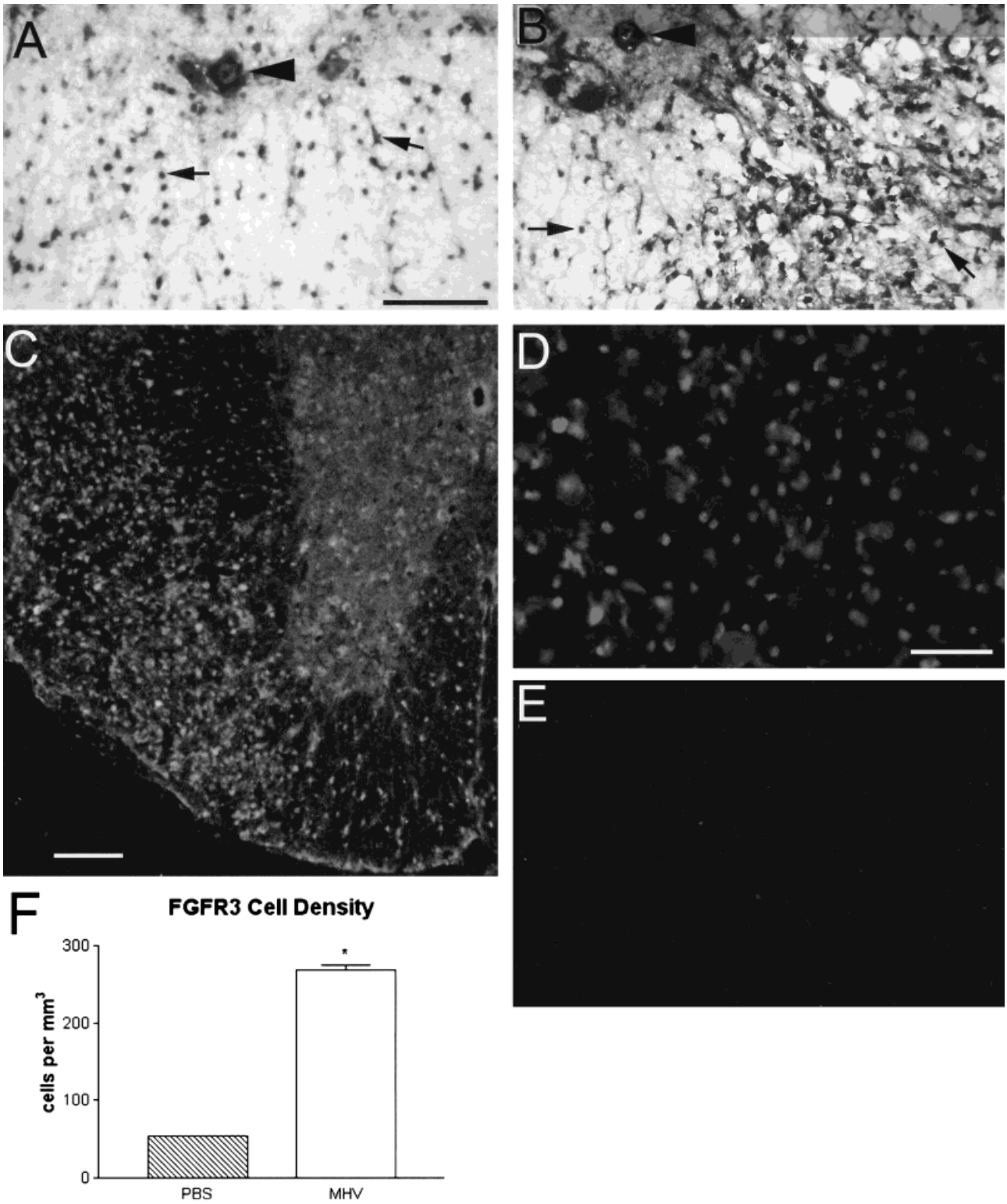

Figure 7.

FGFR1 immunostaining intensity (Fig. 4D). Similar results were obtained with two different FGFR1 ribonucleotide probes, but signal was not present when the sense strand was used as the probe in parallel in situ hybridization reactions (not shown). In lesions, as in normal tissue, FGFR1 appeared to be expressed by cells of multiple glial morphologies, the most intense immunoreactivity being apparent in cells with the distinctive morphology of reactive astrocytes (Fig. 4D). We previously reported that OPs exhibit increased density in lesion areas and are immunolabeled by a monoclonal antibody recognizing mainly FGFR1 (Redwine and Armstrong, 1998). We wanted to characterize more specifically FGFR1 expression by adult OPs because of the proposed requirement of FGFR1 for neonatal OP migration (Osterhout et al., 1997). We detected FGFR1 mRNA in many, but not all, bipolar NG2-immunolabeled OPs cultured from spinal cords at 4 wpi with MHV-A59 (Fig. 5A,B). FGFR1 mRNA was actually more consistently detected in cells with several processes than in bipolar cells (Fig. 5C,D).

In normal white matter, FGFR2 appeared to be expressed by cells with an oligodendrocytic morphology and distribution, as previously reported (Asai et al., 1993; Yazaki et al., 1994; Miyake et al., 1996; Belluardo et al., 1997). We confirmed the oligodendroglial phenotype of FGFR2-immunolabeled cells by simultaneous in situ hybridization detection of PLP mRNA (Fig. 6A,B). For early remyelinating lesions at 4 wpi, we expected decreased expression of FGFR2, insofar as the density of mature oligodendrocytes, identified by detectable by PLP mRNA, is still decreased within lesions (Redwine and Armstrong, 1998). Surprisingly, in contrast to the lack of FGFR2 immunolabeling by astrocytes in normal white matter, reactive astrocytes in these lesions exhibited strong FGFR2 immunolabeling (Fig. 6C,D).

The most striking overall difference in expression level of FGFR types between normal and demyelinated white matter was consistently observed for FGFR3 mRNA and protein detection (Fig. 7). In normal spinal cord, FGFR3 was detected in neurons of the gray matter and in distributed glial-like cells of the white matter (Fig. 7A). Within demyelinated white matter lesions (4 wpi), the density of cells labeled by FGFR3 mRNA was significantly increased (Fig. 7B,F), and the FGFR3 immunoreactivity was correspondingly increased (Fig. 7C,D). Immunolabeling with cell type-specific markers demonstrated that multiple cell types express FGFR3 mRNA and protein in normal and lesioned white matter (Fig. 8). In normal white matter, OPs identified by expression of PDGFαR were double immunolabeled for FGFR3 (Fig. 8A). Mature oligodendrocytes, identified by the CC1 monoclonal antibody, also were double immunolabeled for FGFR3 (Fig. 8B,C). A subset of white matter astrocytes and microglia, identified by GFAP and by Mac-1 immunoreactivity, respectively, also showed FGFR3 double immunolabeling in normal tissue (not shown). In each cell type, the subcellular distribution of FGFR3 immunoreactivity was stronger in the nucleus than the cytoplasm in the normal tissue. This subcellular distribution is consistent with previous reports of FGFR3 immunoreactivity (Johnston et al., 1995; Sogos et al., 1998). In the demyelinated lesions (4 wpi), reactive astrocytes exhibited strong nuclear and cytoplasmic FGFR3 immunoreactivity (Fig. 8D), as did many microglia (not shown), consistent with findings in kainic acid-lesioned rat brain (Ballabriga et al., 1997). We more specifically examined FGFR3 expression by OPs, because, as noted above, the density of OPs in early remyelinating lesions is increased, whereas that of identifiable mature oligodendrocytes is still decreased. In addition, FGFR3 is expressed by neural progenitors during development (Peters et al., 1993) and so might continue to be expressed by adult progenitors. To identify individual OPs clearly, we cultured cells from MHV-lesioned spinal cord at 4 wpi. OPs immunolabeled for NG2 consistently exhibited FGFR3 mRNA signal at the bipolar stage as well as after multiple processes were elaborated (Fig. 8E–H).

Fig. 7. Increased density of FGFR3-expressing cells in areas of white matter lesions. In situ hybridization for FGFR3 mRNA (A,B) and immunostaining for FGFR3 protein (C,D). **A:** In sections from normal spinal cord (PBS, 4 wpi) FGFR3 mRNA is detected in gray matter neurons (large cells, arrowhead) and in distributed small glial-like cells of the gray matter and white matter (arrows). **B:** In sections of lesioned spinal cord (MHV, 4 wpi), FGFR3 mRNA is expressed in both large neuronal cells (arrowhead) and small glial-like cells (arrows), with a marked increase in the density of labeled cells in the lesioned area of white matter (lesion associated with vacuolation; right side of field) relative to the nonlesioned white matter (left side; ventral to gray matter). **C:** A similar increase in the density of cells expressing FGFR3 within lesions is detected by immunofluorescence analysis of sections from lesioned spinal cord (MHV, 4 wpi). **D:** Higher magnification of FGFR3 immunofluorescence within a lesioned area of white matter shows variable intensities of nuclear and cytoplasmic signal. **E:** In sections prepared as in D, the immunoreactivity is abolished by incubation of the anti-FGFR3 antisera with the peptide antigen prior to immunostaining. **F:** Numerical density of cells expressing FGFR3 mRNA in spinal cord sections of mice injected with MHV-A59 (open bar) or PBS-injected control (hatched bar). Numerical density of cells in lesioned white matter that are expressing FGFR3 mRNA is significantly increased over that of a similar region of control white matter from matched PBS-injected mice (N = 3 MHV mice and 3 PBS mice; $\star P < 0.001$, ANOVA). All stereological analyses represented here had a coefficient of error (CE) of 0.12 or lower. Error bars show SEM. Scale bar = 100 μm in A,C (also applies to B); 50 μm in D,E.

DISCUSSION

Mice infected with the MHV-A59 coronavirus exhibit focal areas of transient demyelination throughout the spinal cord that are subsequently completely remyelinated, with corresponding recovery of motor function. Using this experimental model, we found that FGF2 ligand mRNA expression is significantly increased during an early stage of remyelination. This increased FGF2 expression is spatially localized to areas of demyelination, with especially intense FGF2 mRNA and protein signal associated with reactive astrocytes. We also found that FGFR expression is significantly increased within lesions during

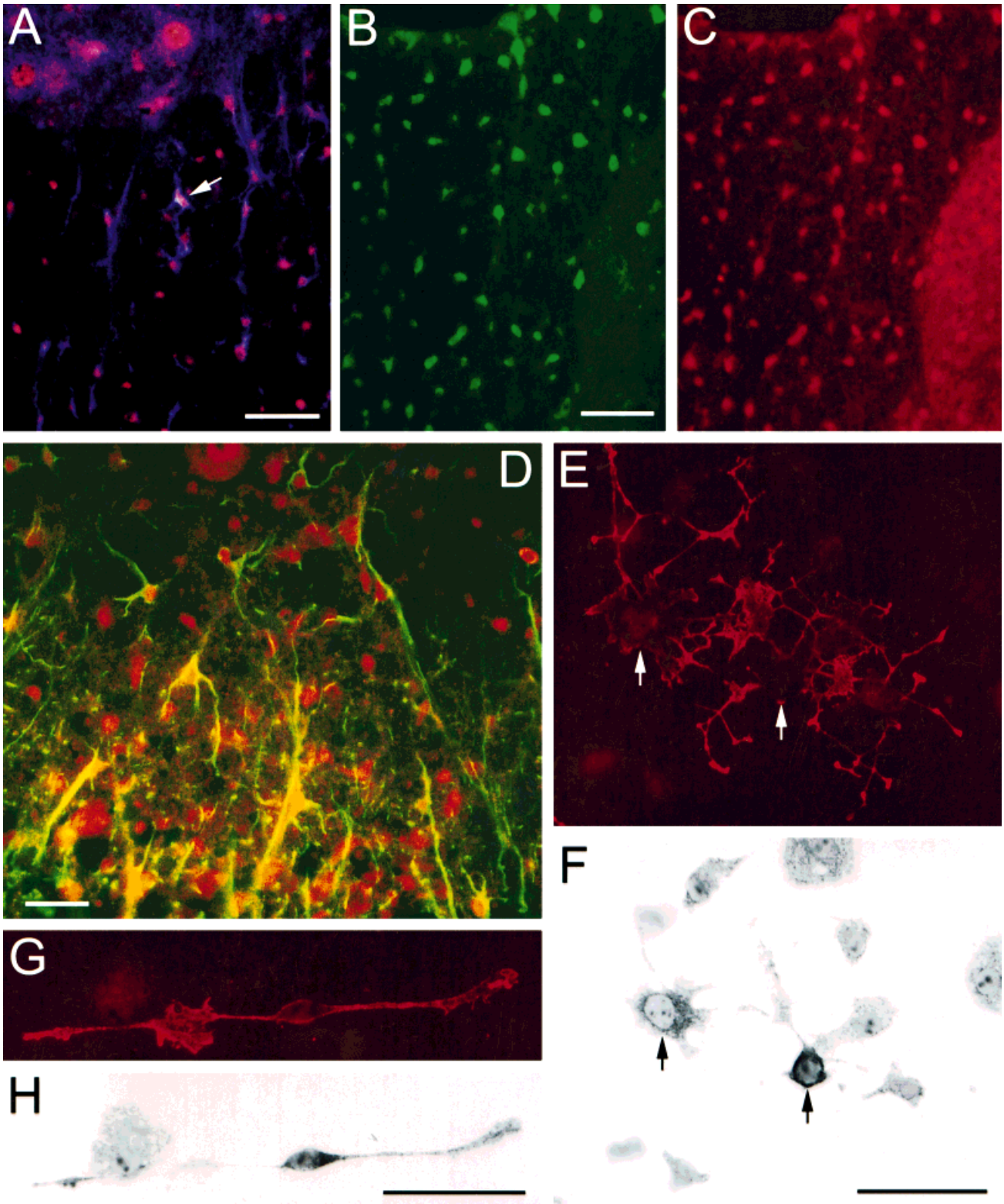

Figure 8.

this early stage of remyelination. With respect to remyelination, FGFR expression by OPs in the adult CNS is of particular interest in that these cells appear to be a source of remyelinating cells in experimental models (Franklin et al., 1997; Gensert and Goldman, 1997; Carroll et al., 1998; Keirstead et al., 1998; Redwine and Armstrong, 1998). We demonstrate FGFR1 and FGFR3 expression by OPs, which we have previously shown express PDGFαR and exhibit vigorous proliferation during remyelination in this model (Redwine and Armstrong, 1998). We also show that, in normal adult CNS, mature oligodendrocytes express FGFR3 in addition to the previously reported expression of FGFR1 and/or FGFR2 (Asai et al., 1993; Yazaki et al., 1994; Gonzalez et al., 1995; Miyake et al., 1996; Belluardo et al., 1997; Redwine et al., 1997; Jiang et al., 1999). The differential expression of FGFR types by oligodendrocyte lineage cells may provide a mechanism for FGF2 to influence differentially proliferation, migration, differentiation, and/or survival as required for oligodendrocyte lineage cells to repopulate lesions and accomplish stable remyelination.

FGF2 expression is subject to both transcriptional and translational control mechanisms (Touriol et al., 1999; Willis, 1999; Ueba et al., 1999), which may be influenced by environmental factors (Gomez-Pinilla and Dao, 1999; Jiang et al., 1999). Although we have not yet identified the molecular signals that trigger the observed up-regulation of FGF2, the increased expression of FGF2 in this MHV-A59 model does not appear to reflect a response that is specific to this method of inducing demyelination or to components specific to this lesion environment. Inflammatory demyelination of experimental autoimmune encephalomyelitis (Liu et al., 1998) and toxin-induced demyelination using lysolecithin injection (Hinks and Franklin, 1999) each exhibit increased FGF2 expression. Whether increased expression of FGFRs also occurs in these other demyelinating models has not been addressed.

FGF2 synthesized by cells within and near lesions may be released to act in a paracrine and/or autocrine manner in the remyelination process. Although lacking a signal sequence for secretion, FGF2 can be released from cells via mechanisms that are not yet clear but may involve an ATP-dependent non-ER/Golgi pathway and interactions with the 27 kDa heat shock protein (HSP27; Mignatti et al., 1992; Florkiewicz et al., 1995; Piotrowicz et al., 1997). Our preliminary studies of MHV-A59 lesions demonstrate that reactive astrocytes have increased immunoreactivity for HSP27 (unpublished observation), which may thus facilitate FGF2 secretion through a molecular chaperone mechanism.

A potentially important interaction relative to growth factor regulation of remyelination is that FGF2 increases expression of PDGFαR (McKinnon et al., 1990) and NG2 on neonatal OPs (Nishiyama et al., 1996b). In response to demyelination, increased local expression of FGF2 (present results) might augment OP expression of PDGFαR and NG2. Indeed, our previous studies of MHV-A59 lesions, similarly analyzed during early remyelination at 4 wpi, demonstrated increased local expression of PDGFαR and NG2 as well as a dramatic increase of proliferating OPs detected by in vivo incorporation of bromodeoxyuridine (Redwine and Armstrong, 1998). In these lesioned tissues, PDGF ligand was up-regulated (Redwine and Armstrong, 1998) but was not as markedly increased or as distinctly localized to the lesions as has been found in the present analysis of FGF2. PDGF and FGF2 acting in combination induce OPs from neonatal CNS to grow as a self-renewing line and induce OPs from adult CNS to display proliferative, migratory, and morphological characteristics similar to those of neonatal cells (Bögler et al., 1990; Wolswijk and Noble, 1992; Engel and Wolswijk, 1996). Thus, in response to demyelination, local increases of FGF2 might also play a role in remyelination by enhancing OP responses to PDGF.

Our results indicate that oligodendrocyte lineage cells in normal and lesioned white matter express a repertoire of FGFRs. FGFR expression can be modulated by CNTF and FGF2 (Bansal et al., 1996; Bansal and Pfeiffer, 1997; Jiang et al., 1999) and, therefore, may be regulated by these and other cytokines and growth factors in the lesion environment. The functional results of FGF2 exposure can be diverse and may depend on the FGFR types expressed in conjunction with differential activation of intracellular signalling pathways within a given cell (Vaccarino et al., 1999). In studies of normal CNS tissues, FGFR1 is expressed at relatively high levels by neurons (Asai et al., 1993; Yazaki et al., 1994; Gonzalez et al.,

Fig. 8. Expression of FGFR3 by multiple glial cell types. FGFR3 expression detected by immunofluorescence (A,C,D) or in situ hybridization (F,H). Immunofluorescence identification of oligodendrocyte progenitors with PDGFαR (A) and anti-NG2 (E,G), mature oligodendrocytes with CC1 monoclonal antibody (B), and astrocytes with anti-GFAP (D). **A:** In sections of normal spinal cord, FGFR3 immunofluorescence (red) is present in the nucleus and cytoplasm of cells (example at arrow) with a characteristic small, elongated oligodendrocyte progenitor morphology that express PDGFαR (blue). **B,C:** In sections of normal spinal cord, mature oligodendrocytes in the white matter that are immunolabeled with CC1 (B, green) are also immunolabeled with anti-FGFR3 (C, red channel of same field as B). **D:** In lesioned white matter (MHV, 4 wpi), anti-GFAP immunoreactivity increases consistent with gliosis and FGFR3 immunoreactivity (red) is intense in the astrocyte cell body and processes (double-immunolabeled cells appear yellow). E–H show cells isolated from MHV lesioned mice at 4 wpi, grown in culture for 2 days to allow process extension, and then labeled with anti-NG2 and detected with immunofluorescence (E,G), and in situ hybridization for FGFR3 mRNA detection with NBT/BCIP substrate (F,H). **E,F:** Cells with multiple processes that have not yet lost NG2 expression (E, red) exhibit perinuclear NBT/BCIP reaction product for FGFR3 mRNA (F) that blocks the NG2 epifluorescence from the cell bodies (arrows indicate cell bodies). **G,H:** Bipolar cells immunolabeled for NG2 (G, red) consistently exhibited perinuclear FGFR3 mRNA signal (H). The ameboid cells (F,H) are microglia, which are abundant in cultures of lesioned spinal cord (Armstrong et al., 1990). Microglia exhibit variable FGFR3 mRNA signal intensity. The ameboid cell in H shows only gray associated with phase-contrast imaging and serves as an indicator of a cell that is not labeled for FGFR3 mRNA. Scale bars = 50 μm.

1995; Belluardo et al., 1997). In contrast, FGFR2 expression is not detected in neurons but has been well characterized as being expressed in mature oligodendrocytes (Asai et al., 1993; Belluardo et al., 1997; Bansal et al., 1996). However, oligodendrocytes and/or OPs have also been reported to express FGFR1 (Gonzalez et al., 1995; Bansal et al., 1996; Redwine et al., 1997; Jiang et al., 1999). FGFR1 has been proposed to play a role in migration of neonatal OPs (Osterhout et al., 1997) and may modulate myelination by mature oligodendrocytes (Harari et al., 1997). Our findings from remyelinating tissue support the possibility of a role for FGFR1 in some OPs, at a bipolar and thus potentially migratory stage, as well as in more differentiated cells.

FGFR3 is most abundantly expressed in germinal epithelium during CNS development and then is detected in distributed glial-like cells in the adult (Peters et al., 1993; Yazaki et al., 1994). Differences in the sensitivity of detection methods may explain why oligodendrocyte lineage cells and astrocytes were identified as expressing FGFR3 in our work whereas a previous study reported that only astrocytes express FGFR3 in adult rat brain (Miyake et al., 1993). In vitro analysis has demonstrated that the IIIc variant of FGFR3 is associated with a late oligodendrocyte precursor stage as neonatal OPs differentiate (Bansal et al., 1996). The FGFR3 splice variant IIIc can be activated by several FGF family members, including FGF2, whereas the IIIb isoform is preferentially activated by FGF1 (Ornitz et al., 1996). In addition, the IIIc form is present in brain, but FGFR3 IIIb is not detected (Chellaiah et al., 1994), supporting the potential for FGF2 to serve as a relevant ligand for the FGFR3 we observed in situ.

The effects of FGF2 in lesions may be extended by the potential to modulate astrocytic responses, in that astrocytes express multiple FGFR types (Gonzalez et al., 1995; Bansal et al., 1996; Ballabriga et al., 1997; Redwine et al., 1997; present results). In response to demyelination, astrocytes produce growth factors that may regulate oligodendrocyte lineage cells, such as FGF2, insulin-like growth factor-I, and PDGF-A (Komoly et al., 1992; Redwine and Armstrong, 1998; present results). Thus, FGF2 might directly and/or indirectly contribute to oligodendrocyte lineage cell responses during remyelination.

The present findings demonstrate that the appropriate combinations of ligand and receptor expression patterns are present in remyelinating lesions for hypothesizing that FGF2 could serve a potential regulatory role in the oligodendrocyte lineage response during remyelination. Attempts to use FGF2 null mice to determine the in vivo role of FGF2 in this remyelination model have not been possible because of morbidity of the MHV-A59 virus in this strain of mice (unpublished observation). Further analyses will be required to reveal positive, or possibly even negative, regulatory effects that may be mediated by FGF2, presumably interacting with specific FGFR splice variants and acting in combination with other factors. Detailed understanding of the cellular and molecular events that bring about successful remyelination in such model systems should facilitate the development of therapies for manipulating the cell populations and/or the lesion environment to promote repair in human demyelinating conditions, such as multiple sclerosis.

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