

Elevated Levels of the Chemokine GRO-1 Correlate with Elevated Oligodendrocyte Progenitor Proliferation in the *Jimpy* Mutant

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The dysmyelinating mutant *jimpy* (*jp*) arises from a point mutation in the mouse gene encoding proteolipid protein and is characterized by severe dysmyelination attributable to oligodendrocyte death. This mutant was used to investigate the regulation of oligodendrocyte progenitor proliferation in the postnatal spinal cord. At postnatal day 18, *jp* spinal cord contained a three- to eightfold greater number of proliferating oligodendrocyte progenitor cells than did *wild-type* (*wt*) spinal cord. Increased proliferation in *jp* spinal cord was accompanied by a twofold increase in the number of progenitor cells. Semi-quantitative reverse transcriptase-PCR revealed no change in the level of mRNA encoding the platelet-derived growth factor A, transforming growth factor- β , or insulin-like growth factor-I, all of which have been implicated as regulators of proliferation and differentiation of oligodendrocyte progenitor cells. There

was, however, a 17-fold increase in the level of mRNA encoding the chemokine GRO-1 and a 5- to 6-fold increase in GRO-1 protein in the *jp* spinal cord. Double immunofluorescence labeling revealed elevated levels of GRO-1 in reactive astrocytes in *jp* spinal cord white matter. *In vitro* studies indicated that extracts from *jp* spinal cord stimulated oligodendrocyte progenitor proliferation. Furthermore, removal of GRO-1 from *jp* extracts by immunoprecipitation reduced the proliferation of progenitor cells to a level similar to that achieved by *wt* extracts. These findings suggest a novel mechanism by which proliferation of oligodendrocyte progenitor cells is regulated in the postnatal spinal cord in response to insult.

Key words: oligodendrocyte progenitor; *jimpy*; glia; myelin; chemokine; GRO-1; NG2; PDGF; PDGF receptor

Oligodendrocytes are generated from progenitor cells that arise in the ventral ventricular zone of the embryonic rodent spinal cord (Warf et al., 1991; Pringle and Richardson, 1993). These progenitor cells proliferate and migrate to occupy the entire CNS by the end of the first postnatal week (Hirano and Goldman, 1988; Pringle et al., 1992; Pringle and Richardson, 1993; Timsit et al., 1995; Nishiyama et al., 1996a). Platelet-derived growth factor (PDGF) AA is secreted by astrocytes and neurons and stimulates oligodendrocyte progenitor cell (OPC) proliferation via its α receptor (PDGF α R) (Raff et al., 1988; Richardson et al., 1988; Sasahara et al., 1991; Yeh et al., 1991). PDGF AA may be primarily responsible for establishing the number of OPCs in prenatal and early postnatal mouse spinal cord (Calver et al., 1998).

PDGF α R is coexpressed with the NG2 proteoglycan on OPCs *in vitro* and *in vivo*, but both molecules are lost as the progenitors

differentiate into mature oligodendrocytes (Levine et al., 1993; Levine and Nishiyama, 1996; Nishiyama et al., 1996a, b). The NG2 proteoglycan forms a multimolecular complex that includes PDGF α R and enhances OPC proliferation (Nishiyama et al., 1996b).

Further modulation of the proliferative response of OPCs to PDGF was recently described by Robinson et al. (1998), who demonstrated that the chemokine GRO-1 has a synergistic effect on PDGF-driven OPC proliferation. Chemokines (chemoattractant cytokines) are a family of small, secreted proteins that function as chemoattractants for leukocytes via G-protein-coupled seven-transmembrane receptors (Rollins, 1997; Luster, 1998). GRO-1 belongs to the CXC family of chemokines and includes molecules identified previously as mouse KC (Cochran et al., 1983; Oquendo et al., 1989), hamster GRO- α (Anisowicz et al., 1987), and human melanoma growth-stimulating activity (MGSA) (Richmond et al., 1988). These chemokines are considered to be homologs (see web site <http://cytokine.medic.kumamoto-u.ac.jp/CFC/CK/CXCG/CXCG.html> for a review of the nomenclature).

The proliferation and survival of oligodendrocyte lineage cells are significantly altered in *jimpy* (*jp*) mutants. *Jp* is an X-linked recessive mutation in the proteolipid protein (PLP) gene (Nave et al., 1986, 1987; Ikenaka et al., 1988). White matter tracts of the affected males show dysmyelination accompanied by increased proliferation of morphologically identified immature oligodendrocytes (Skoff, 1982) and increased oligodendrocyte death (Knapp et al., 1986), possibly attributable to abnormal PLP transport and accumulation (Gow et al., 1998).

To define oligodendrocyte lineage cell proliferation and mo-

Received May 18, 1999; revised Jan. 21, 2000; accepted Jan. 27, 2000.

This work was supported by National Institutes of Health Grants NS 35136 to A.N. and NS 30800 to R.H.M. and by the National Multiple Sclerosis Society Research Grant RG 2826 to A.N. We thank Dr. Judy Drazba (Cleveland Clinic, Cleveland, OH) for her help with confocal microscopy and Drs. Bruce Trapp and Wendy Macklin (Cleveland Clinic) for helpful discussion. We thank Drs. William Stallcup (The Burnham Institute, La Jolla, CA), Joel Levine (State University of New York, Stony Brook, NY), Tom Hamilton (Cleveland Clinic), Carl-Henrik Heldin (Uppsala, Sweden), and Toshiro Kumanishi (Niigata University, Niigata, Japan) for antibodies.

Parts of this paper have been presented previously at the Keio University Symposium for Life Science and Medicine, Tokyo, December, 1997, and the 1091st Meeting of the Keio Medical Society, Tokyo, June, 1998.

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lecular changes in *jp* spinal cord, we compared oligodendrocyte progenitor NG2-immunoreactive (NG2+) cell number and proliferation in *jp* and *wild-type* (*wt*) mouse spinal cord. We show increased proliferation of NG2+ cells in *jp* spinal cord white matter compared with that in *wt* spinal cord. Although no change was detected in the level of mRNA encoding PDGF A, transforming growth factor (TGF)- β 2 or - β 3, or insulin-like growth factor-I (IGF-I) in *jp* spinal cord, there was a significant increase in the expression of the chemokine GRO-1, which was present in *jp* astrocytes. Furthermore, extracts from *jp* spinal cord enhanced OPC proliferation *in vitro*, whereas extracts from *wt* spinal cord had no effect. The growth-stimulatory activity in *jp* extracts could be eliminated by immunodepleting GRO-1 from the extracts. These data suggest that in addition to classical peptide growth factors, chemokines, which are synthesized in the CNS, contribute to the regulation of OPC proliferation in the postnatal spinal cord in response to insult.

MATERIALS AND METHODS

Animals. Heterozygous female mice (Ta Plp^{jp/+}) carrying the *jp* mutation and *wt* males (B6CBACa-A^{W⁻¹}/A F1) were obtained from The Jackson Laboratory (Bar Harbor, ME) and used as breeding pairs. At postnatal day 18 (P18), *jp* males were identified by their characteristic tremor. Unaffected male littermates were used as controls.

Sprague Dawley rats were obtained from Charles River Laboratories (Raleigh, NC). Newborn (P0–P2) rats were used to isolate oligodendrocyte progenitor cells.

Antibodies. Antibodies were obtained from the following sources. Rabbit anti-rat NG2 antibodies were gifts from Drs. William Stallcup (The Burnham Institute, La Jolla, CA) and Joel Levine (State University of New York, Stony Brook, NY). Rabbit anti-human PDGF α R (R7) antibody was a gift from Dr. Carl-Henrik Heldin (Uppsala, Sweden). Monoclonal antibodies to bovine glial fibrillary acidic protein (GFAP), S100 β , and GRO- α were obtained from Sigma (St. Louis, MO). Rabbit anti-bovine GFAP antibody was obtained from Dako (Carpinteria, CA). Mouse monoclonal antibody to 5-bromo-2'-deoxyuridine (BrdU) was obtained from Amersham (Arlington Heights, IL). Rabbit anti-bovine S100 β was a gift from Dr. Toshiro Kumanishi (Niigata University, Niigata, Japan). Rat monoclonal antibodies to mouse F4/80 and CD45 were obtained from Accurate Chemicals (Westbury, NY). Rat monoclonal anti-mouse KC antibody was obtained from R & D Systems (Minneapolis, MN). Rabbit anti-mouse KC antibody was a gift from Dr. Tom Hamilton (Cleveland Clinic, Cleveland, OH).

BrdU labeling. To identify proliferating cells, 0.05 mg of BrdU (Boehringer Mannheim, Indianapolis, IN)/gm of body weight was injected intraperitoneally into P18 mice three times at 2 hr intervals. Two hours after the final injection, mice were perfused with 2% paraformaldehyde solution containing 0.01 M sodium metaperiodate and 0.1 M lysine (paraformaldehyde-lysine-periodate fix). Brains and spinal cords were post-fixed overnight in the same fixative. After cryoprotection in 0.1 M phosphate buffer, pH 7.3, containing 15% sucrose, the tissues were embedded in OCT compound. Ten micrometer sections were cut using a cryostat (Jung Frigocut 2800N; Leica, Nussloch, Germany) and processed for immunohistochemistry. Some sections were stained with hematoxylin and eosin.

Immunohistochemistry. For immunoperoxidase labeling, sections were rinsed in PBS and incubated in 1% hydrogen peroxide (H₂O₂) to block endogenous peroxidase activity. Nonspecific protein-binding sites were blocked by incubation in PBS containing 5% normal goat serum (Life Technologies, Grand Island, NY) for 1 hr at room temperature. Sections were then incubated in primary antibodies diluted in 5% normal goat serum overnight at 4°C. Irrelevant primary antibodies were used as controls. After rinsing for 1 hr in PBS, sections were incubated in biotinylated secondary antibodies (Vector Laboratories, Burlingame, CA; diluted 1:500) for 1 hr at room temperature followed by incubation in avidin-biotin complex conjugated with horseradish peroxidase (Vector Laboratories; diluted 1:1000) for 1 hr at room temperature. Peroxidase activity was visualized by incubation in 0.1% 3,3'-diaminobenzidine and 0.03% H₂O₂.

For double-immunofluorescent labeling, the two primary antibodies and secondary antibodies (Jackson ImmunoResearch, West Grove, PA)

were applied to the sections simultaneously. Labeled sections were mounted in Vectashield (Vector Laboratories) and examined using a Zeiss Axiophot or Leica DMR epifluorescence microscope or a Leica TCS-NT confocal laser-scanning microscope.

For detection of BrdU, sections were pretreated with 2N HCl at room temperature for 10 min followed by neutralization in 0.1 M sodium borate buffer, pH 8.2, at room temperature for 10 min. After washes in PBS, the sections were processed for double immunohistochemistry as described above.

Cell counts. The number of cells double-labeled for NG2 and BrdU was estimated in the white matter of *wt* and *jp* spinal cord at P18 by counting double-labeled cells in the white matter of 10 μ m transverse sections at cervical, thoracic, and lumbar levels. Areas of the spinal cord white matter in which cells were counted were measured by use of the NIH Image program. Three to five sections from each tissue were counted, and mean values were obtained for each tissue. A total of four to six *wt* and *jp* mice were analyzed. The numbers are expressed as the numbers of NG2+/BrdU+ cells per unit area of spinal cord white matter, which includes dorsal, lateral, and anterior columns. Only cells with BrdU staining over the entire nucleus surrounded by a closed ring of NG2 immunoreactivity were scored as NG2+/BrdU+ (see Fig. 2A,B). Cells with one or two small puncta of BrdU immunoreactivity or BrdU+ cells that were only partially surrounded by NG2 immunoreactivity were not scored as positive.

The number of OPCs in the dorsal column and dorsal horn of *jp* and *wt* spinal cord was estimated by counting the number of PDGF α R-immunoreactive cells in immunoperoxidase-labeled sections. Cell bodies with a distinct ring of PDGF α R immunoreactivity were scored as positive. The area of the dorsal column and dorsal horn was estimated by use of the NIH Image program.

Reverse transcriptase-PCR. Total RNA was isolated from P18 *jp* and *wt* spinal cords using the guanidinium isothiocyanate method (Chirgwin et al., 1979). Five micrograms of total RNA were used in the reverse transcriptase (RT) reaction using SuperScript II (Life Technologies). One-tenth of the synthesized cDNA was subjected to PCR in 100 μ l containing 0.2 μ Ci of [α -³²P]dCTP (New England Biolabs, Beverly, MA). The primers used were as follows: mouse PDGF A, forward, 5'-ctgtg cccat tcga gg, and reverse, 5'-accgc acga cattg (Mercola et al., 1990); mouse TGF- β 2, forward, 5'-ttcac caca agaca gg, and reverse, 5'-tttc atca agate cc (Miller et al., 1989a); mouse TGF- β 3, forward, 5'-ttcga catga tccag gg, and reverse, 5'-gcgga agcag taatt gg (Miller et al., 1989b); mouse IGF-I, forward, 5'-acctg gcgct ctgct tgc, and reverse, 5'-tgggc atgtc agtgt ggc (Bell et al., 1986); mouse KC, forward, 5'-tgcac ccaa cga gtcag, and reverse, 5'-gtggt tgaca cttag tggtc tc (Oquendo et al., 1989); and mouse cyclophilin, forward, 5'-cgtgg gctcc gctc tt, and reverse, 5'-ccggc tgtct gctt ggtgc tctc (Hasel et al., 1991). Amplified products were separated on 6% polyacrylamide gels containing 8 M urea. After electrophoresis, the gels were dried and exposed to x-ray film for autoradiography or to a phosphorimager screen for quantitation of the density of the bands.

The optimal amplification cycle number for PCR was determined to ensure that the amount of amplified product was proportional to the amount of input cDNA for both abundant (cyclophilin) and less abundant (TGF- β 2) mRNA species. To quantify the relative amount of mRNA encoding each growth factor, we normalized the density of the bands to that of cyclophilin mRNA. The mean values for each growth factor were obtained from 3 to 5 *wt* and *jp* RNA samples.

Tissue extracts. Spinal cords from *wt* and *jp* mice were homogenized in DMEM (300 μ l/spinal cord), extracted at 4°C for 15 min, and centrifuged at 13,000 rpm for 5 min to remove insoluble material. GRO-1 was immunodepleted from the extracts by immunoprecipitating sequentially with rabbit and rat anti-mouse KC antibodies (obtained from Dr. Tom Hamilton, Cleveland Clinic, and R & D Systems, respectively). Control immunodepletion was performed using rabbit anti- β -galactosidase (anti- β -gal) antibody or normal rabbit serum. Antigen-antibody complexes were precipitated using protein A-Sepharose (Pharmacia, Piscataway, NJ). Supernatants were concentrated using Microcon concentrators (3 kDa cutoff; Millipore, Bedford, MA). The protein concentration of each fraction was assayed using the Lowry method (Bio-Rad, Hercules, CA). The levels of GRO-1 in the extracts were assayed by ELISA using polyclonal antibodies to mouse KC, according to the instructions provided by the manufacturer (R & D Systems). Equal amounts of protein were added to cultures of oligodendrocyte progenitor cells.

Cultures of oligodendrocyte progenitor cells. OPCs were isolated and purified from neonatal rat spinal cords by immunopanning with A2B5

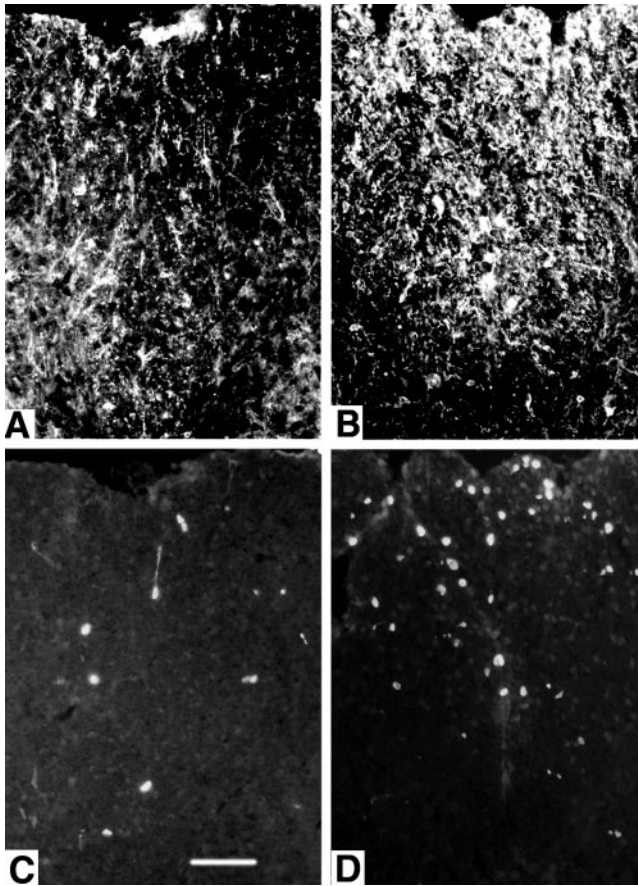


Figure 1. Double-immunofluorescence labeling for NG2 (*A, B*) and BrdU (*C, D*) in the dorsal column of *wt* (*A, C*) and *jp* (*B, D*) spinal cord at P18. A greater number of cells has incorporated BrdU in *jp* white matter than in *wt* white matter. There is increased immunoreactivity for NG2 in *jp* spinal cord compared with *wt* spinal cord. Scale bar, 50 μ m.

monoclonal antibody as described by Robinson and Miller (1996). Immunocytochemistry of purified cells revealed the population to be 95% homogeneous for A2B5+ and NG2+ cells. Contaminating GFAP+ astrocytes comprised 4–5% of the population, whereas microglia constituted <1% of the population. Cells were plated in 48-well plates at a density of 80,000 cells/well. After 18 hr, the medium was changed to serum-free DMEM containing N2 supplement (Life Technologies), PDGF AA, and *jp* or *wt* extracts. Forty-eight hours later, 0.5 μ Ci of [³H]thymidine (NEN Life Science Products, Boston, MA) was added to each well. After a labeling period of 4 hr, cells were washed twice with PBS, washed twice with cold 5% trichloroacetic acid, and lysed with 0.02 M KOH, and radioactivity was measured using a scintillation counter. In some experiments, cells were plated on glass coverslips in 24-well plates in the presence of control or GRO-1-immunodepleted *jp* extracts, labeled with BrdU, and assayed for the percentage of A2B5+ cells that had incorporated BrdU as described previously (Robinson et al., 1998).

RESULTS

Increased progenitor proliferation in *jp* spinal cord

OPC proliferation was examined in spinal cords of P18 *wt* and *jp* mice. P18 represents an age past the peak in oligodendrogenesis, when myelination has occurred at all levels of the spinal cord, and thus OPC proliferation is expected to have declined to a low level in *wt* mice. Because *jp* is an X-linked recessive mutation, *wt* and *jp* males were used for all the analyses.

BrdU labeling revealed a significant increase in the number of proliferating cells in white matter tracts of the *jp* spinal cord

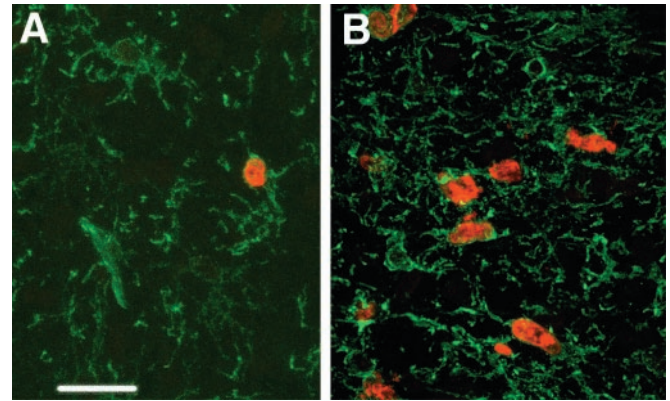


Figure 2. Confocal laser-scanning images of a section through P18 *wt* (*A*) and *jp* (*B*) spinal cord double-labeled for BrdU (red) and NG2 (green). The majority of BrdU+ nuclei are surrounded by NG2+ cell bodies and processes, indicating localization of BrdU and NG2 in the same cells. Scale bar, 30 μ m.

Number of NG2+/BrdU+ cells per area

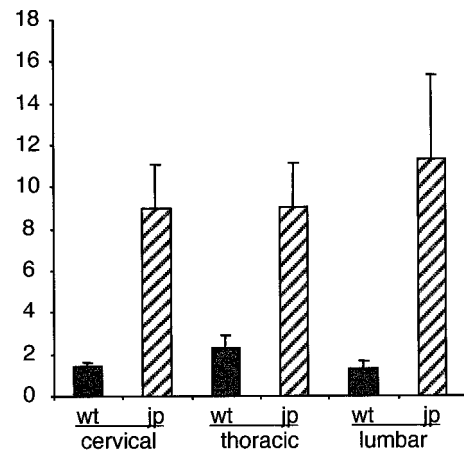


Figure 3. The number of BrdU+/NG2+ cells in *wt* (filled bars) and *jp* (hatched bars) spinal cord. The numbers represent the number of double-labeled cells per unit area of white matter in a 10- μ m-thick transverse section from cervical, thoracic, and lumbar spinal cord. Error bars represent SD ($n = 4$). There is a three- to eightfold increase in the number of BrdU+/NG2+ cells in *jp* spinal cord.

(Fig. 1). The vast majority of cells that incorporated BrdU were NG2+-presumptive OPCs (Fig. 2). In *wt* spinal cord 90–95% of the BrdU+ cells were NG2+, whereas only 70–80% of the BrdU+ cells in the *jp* spinal cord were NG2+. It is likely that 20–30% of BrdU+ cells that were NG2-negative in *jp* spinal cord represent locally proliferating microglia (Vela Hernandez et al., 1997). To determine the extent of the increase in NG2+ cell proliferation in *jp* spinal cord, we estimated the number of BrdU+/NG2+ cells per unit area of white matter in cervical, thoracic, and lumbar spinal cord. The number of NG2+ cells undergoing DNA synthesis was three- to eightfold higher in all three segments of *jp* spinal cord compared with that of *wt* spinal cord (Fig. 3). In *jp* spinal cord the increased BrdU incorporation in NG2+ cells was seen only in white matter, although NG2+ cells were present in both white and gray matter. Within white matter, NG2+ cells in *jp* spinal cord appeared to have increased numbers of processes and elevated levels of NG2 immunoreactivity (Figs. 1*A, B*, 2).

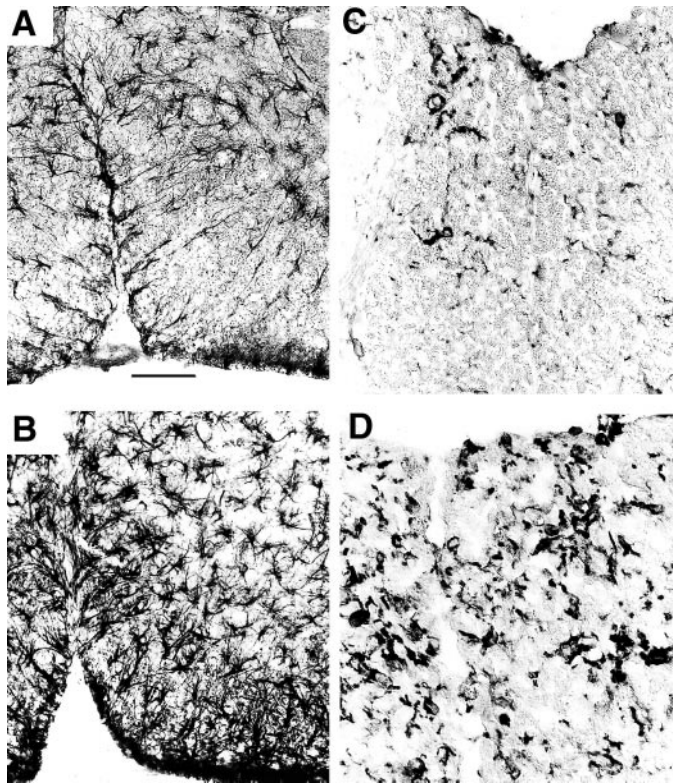


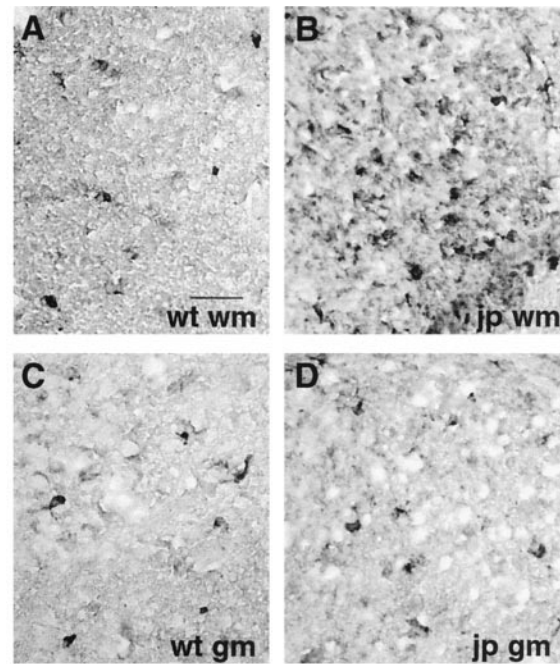
Figure 4. Immunoperoxidase labeling for GFAP (*A, B*; rabbit anti-bovine GFAP) and the microglial antigen F4/80 (*C, D*) in *wt* (*A, C*) and *jp* (*B, D*) spinal cord at P18. *A, B*, There is increased intensity of GFAP immunoreactivity in astrocytes in the anterior column of *jp* spinal cord. *C, D*, There is an increase in the number of F4/80+ microglial cells and increased intensity of immunoreactivity of cells in the *jp* dorsal column. Scale bar, 50 μ m.

Astrogliosis and microglial activation in *jp* spinal cord

Previous studies demonstrated astrogliosis and microglial activation in *jp* spinal cord, and these observations were confirmed in the present study. *jp* spinal cord exhibited elevated GFAP immunoreactivity compared with that of *wt* (Fig. 4*A, B*), which appeared to be primarily a result of hypertrophy rather than elevated astrocyte cell number. A significant increase in the number of microglial cells labeled with an antibody to the microglia- and macrophage-specific integral membrane protein F4/80 (Austyn and Gordon, 1981; McKnight et al., 1996) was seen in *jp* compared with *wt* spinal cords (Fig. 4*C, D*). Together these data suggest that there is a widespread activation and proliferation of macroglia and microglia in *jp* spinal cord.

Increased numbers of oligodendrocyte progenitor cells in *jp* spinal cord

To determine whether the increased proliferation of NG2+ cells in *jp* spinal cord was accompanied by increased numbers of OPCs, progenitor cell numbers were estimated by counting PDGF α R-immunoreactive cells. PDGF α R immunoreactivity was used because NG2+ cells in the postnatal CNS parenchyma express PDGF α R (Nishiyama et al., 1996a, 1997) and PDGF α R is detected more prominently on cell bodies than is NG2, whereas NG2 is localized on processes as well as cell bodies. There was a greater number of PDGF α R+ cells in the white matter of *jp* spinal cord compared with *wt*, and the individual cells were more intensely labeled (Fig. 5*A, B*). Quantitation of cell number dem-



E. Number of PDGF R+ Cells

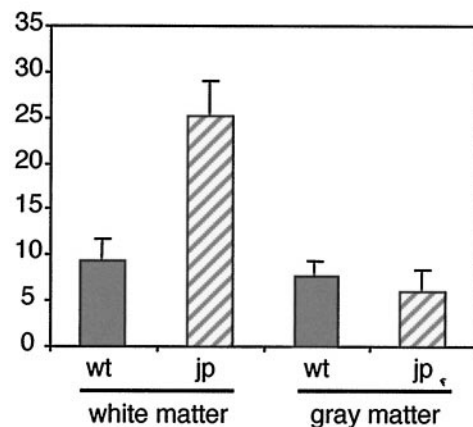


Figure 5. *A–D*, Immunoperoxidase labeling for PDGF α R in the dorsal column (*wm*; *A, B*) and dorsal horn (*gm*; *C, D*) of *wt* (*A, C*) and *jp* (*B, D*) cervical spinal cord at P18. There is an increased number of PDGF α R+ cells in *jp* dorsal column but not dorsal horn. Scale bar, 50 μ m. *E*, Number of PDGF α R-immunoreactive cells per unit area in the cervical dorsal column (white matter) and dorsal horn (gray matter) of *wt* and *jp* spinal cord at P18. Error bars represent SD ($n = 3$). *gm*, Gray matter; *wm*, white matter.

onstrated a 2.5-fold increase in PDGF α R+ cells in the *jp* dorsal column compared with those in the *wt* cervical dorsal column (Fig. 5*E*). In the gray matter, there was no significant difference in the number of PDGF α R+ cells between *wt* and *jp* (Fig. 5*C–E*). These data indicate that the elevated level of progenitor proliferation in the white matter of *jp* spinal cord is accompanied by elevated numbers of progenitor cells.

The levels of PDGF-A, IGF-I, and TGF- β mRNA are not altered in *jp* spinal cord

Several growth factors are known to modulate the proliferation of OPCs. To understand the mechanism for enhanced OPC proliferation in *jp*, we examined by semiquantitative RT-PCR the levels of mRNA encoding these growth factors.

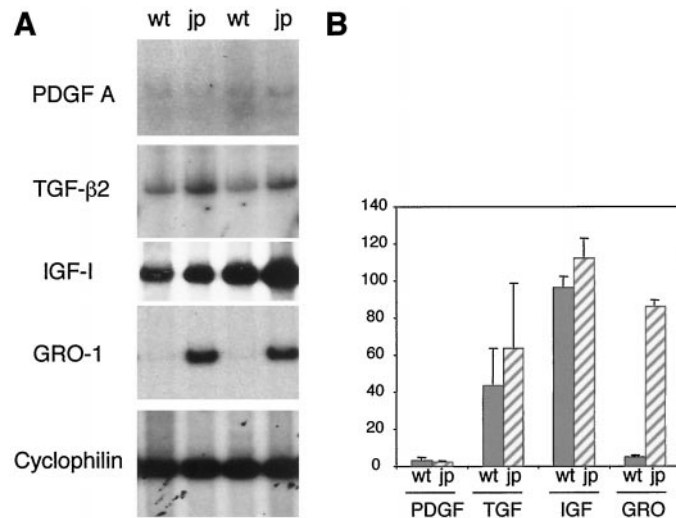


Figure 6. Semiquantitative RT-PCR to compare PDGF A, TGF- β 2, IGF-I, and GRO-1 mRNA levels between *wt* and *jp* spinal cord. *A*, Representative results of RT-PCR using 5 μ g of total RNA from P18 *wt* or *jp* spinal cord and oligonucleotide primers for PDGF A chain, TGF- β 2, IGF-I, GRO-1 (mouse KC), and cyclophilin. Each lane represents RNA extracted from one spinal cord. *B*, Quantitation of the intensity of the bands in *A* using a phosphorimager. The values represent the intensity of the bands for PDGF A, TGF- β 2, IGF-I, and GRO-1 mRNA normalized to the intensity of the bands for cyclophilin mRNA. Error bars represent SD ($n = 3$). There is no significant difference in the amount of PDGF A, TGF- β 2, or IGF-I mRNA between *wt* and *jp* spinal cord. There is a 17-fold increase in GRO-1 mRNA in *jp* spinal cord.

There was no significant increase in the level of PDGF A mRNA in *jp* spinal cord at P18 compared with that in *wt* spinal cord (Fig. 6). Relatively low levels of PDGF A mRNA were expressed in both *jp* and *wt* spinal cord. Similarly, there was no significant difference in the level of mRNA encoding IGF-I (Fig. 6), another putative oligodendrocyte progenitor cell mitogen (McMorris et al., 1986, 1988; Carson et al., 1993; Beck et al., 1995). These data suggest that increased OPC proliferation in *jp* spinal cord is not simply the result of elevated mitogen levels.

Members of the TGF- β family are known to be present in the CNS (Unsicker et al., 1991) and inhibit OPC proliferation (McKinnon et al., 1993). To determine whether the enhanced proliferation seen in *jp* spinal cord reflected a reduction in inhibition, the relative levels of mRNA for TGF- β 2 and - β 3 were assayed. No significant decrease in the level of TGF- β 2 (Fig. 6) or - β 3 (data not shown) mRNA was detected in the *jp* spinal cord, although the level of TGF- β 2 mRNA was slightly increased in *jp* compared with *wt* spinal cord. These findings suggest that altered levels of well known oligodendrocyte lineage growth factors are unlikely to be responsible for the increase in proliferation of NG2+ cells in *jp* spinal cord.

Expression of the chemokine GRO-1 is elevated in *jp* spinal cord

The elevated levels of OPC proliferation and the normal levels of OPC mitogens in *jp* spinal cord suggest that additional mechanisms regulate local proliferation of OPCs. The chemokine GRO-1 has been shown to be expressed by spinal cord astrocytes and to stimulate proliferation of OPCs (Robinson et al., 1998). To determine whether elevated levels of GRO-1 expression correlated with the increased proliferation of OPCs in *jp* spinal cord, the level of mRNA for GRO-1 was compared in *jp* and *wt* spinal

cord using oligonucleotide primers derived from the mouse GRO-1 (identified previously as KC) cDNA sequence (Oquendo et al., 1989). There was a 17-fold higher level of GRO-1 mRNA in P18 *jp* spinal cord compared with that in *wt* spinal cord (Fig. 6). Thus, the elevated OPC proliferation in *jp* spinal cord correlated with elevated levels of GRO-1 mRNA. In contrast to the elevated levels of GRO-1 mRNA in *jp* spinal cord, the levels of GRO-1 mRNA in *jp* cerebrum containing corpus callosum were not significantly higher than those in *wt* cerebrum (data not shown).

To determine whether increased GRO-1 mRNA in *jp* spinal cord was accompanied by increased levels of GRO-1 protein, the GRO-1 content in spinal cord extracts was measured by ELISA. Table 1 shows a representative result of an ELISA assay, which demonstrates a five- to sixfold increase in GRO-1 in *jp* spinal cord extracts compared with that in *wt* extracts (*wt*, 4.24 pg of GRO-1/mg of protein; *jp*, 25.1 pg of GRO-1/mg of protein). This finding is consistent with the results of Western blotting using rat anti-mouse GRO-1 antibody (data not shown). Because antibodies to GRO-1 recognize the antigen only under nonreducing conditions, we failed to detect GRO-1 monomer on Western blots. The high-molecular weight immunoreactive material on Western blots had an electrophoretic mobility similar to that of purified GRO-1 under nonreducing conditions, suggesting that the antibodies used in these studies reacted specifically with GRO-1.

GRO-1 is present in astrocytes

In normal developing rat spinal cord, GRO-1 is expressed in astrocytes in a spatially and temporally regulated manner (Robinson et al., 1998). To determine whether the elevated levels of GRO-1 seen in *jp* spinal cord were astrocyte-derived, sections of spinal cord were double-labeled with antibodies to GRO-1 and GFAP or S100 β . In *wt* spinal cord at P18, low levels of GRO-1 were detected in scattered cells in the dorsal columns (Fig. 7A). In parallel sections from *jp* spinal cord there were elevated levels of GRO-1 immunoreactivity in cell bodies and a more diffuse punctate staining throughout the neuropil, suggesting an extracellular localization of the chemokine (Fig. 7B). Double-labeling studies revealed that GRO-1 was present in GFAP+ astrocytes (Fig. 8A–C). Because GFAP immunoreactivity was primarily localized in astrocyte processes whereas the chemokine was localized in the cell body, colocalization studies were repeated using antibodies to S100 β , a calcium-binding protein expressed by astrocytes (Kligman and Hilt, 1988). Figure 8D–F demonstrates that GRO-1 is expressed in S100 β + astrocytes.

Table 1. ELISA for GRO-1 levels in *wt* and *jp* spinal cord extracts

	GRO-1/protein (pg/mg)
<i>wt</i> total	4.24
<i>jp</i> total	25.06
<i>wt</i> GRO-depleted	2.81
<i>wt</i> β -gal-depleted	6.36
<i>jp</i> GRO-depleted	10.93
<i>jp</i> β -gal-depleted	32.91

This is a representative set of results from three experiments, which showed an average of 6.9-fold higher GRO-1 concentration in *jp* extracts compared with that in *wt* extracts. Immunodepletion of GRO-1 from *jp* extracts (*jp* GRO-depleted) resulted in a 2.3- to 3.3-fold reduction in the concentration of GRO-1.

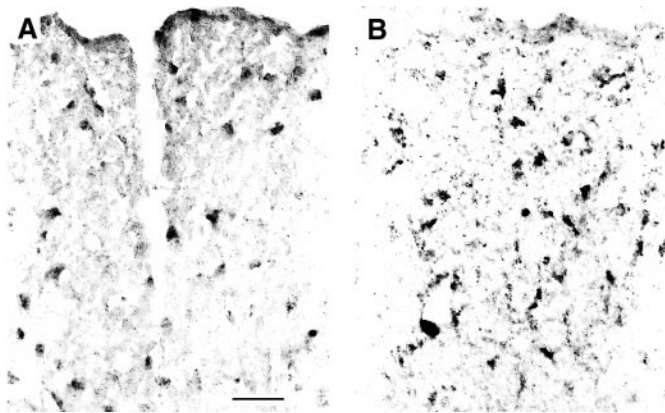


Figure 7. Immunoperoxidase labeling for GRO-1 in *wt* (A) and *jp* (B) spinal cord at P18, using mouse anti-human GRO-1 antibody (Sigma). There is increased immunoreactivity for GRO-1 in the *jp* dorsal column. Punctate GRO-1 immunoreactivity that is not associated with cells is more pronounced in *jp* cord. Scale bar, 50 μ m.

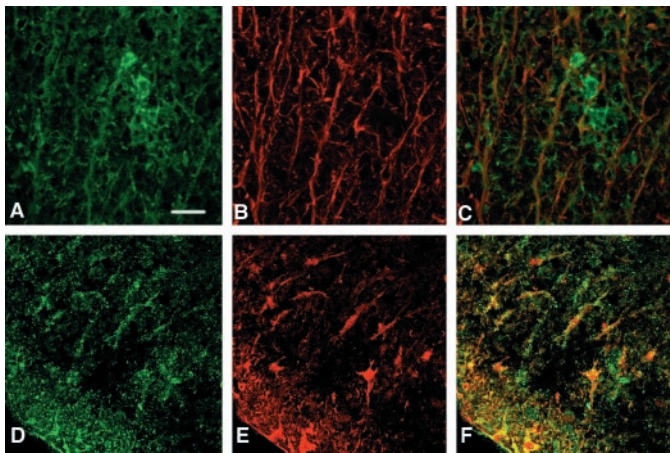


Figure 8. Confocal laser-scanning images of sections through P18 *jp* spinal cord double-labeled for GFAP (B, C; rabbit anti-bovine GFAP antibody) and GRO-1 (A, C; mouse anti-human GRO-1 antibody) or S100 β (E, F) and GRO-1 (D, F). GRO-1 is present in GFAP+ or S100 β + astrocytes. Scale bar, 35 μ m.

Increased expression of GRO-1 in *jp* spinal cord does not result in neutrophil infiltration

In other models of CNS pathogenesis, local increases in the expression of CXC chemokines such as GRO-1 result in local recruitment of neutrophils (Glabinski et al., 1997; Tani et al., 1996). Although *jp* spinal cord expressed elevated levels of GRO-1, there was no evidence of elevated recruitment of neutrophils into the spinal cord either by hematoxylin and eosin staining (data not shown) or by immunohistochemical analysis using an antibody to CD45, a leukocyte-specific receptor tyrosine phosphatase (data not shown). These data suggest that the influence of elevated levels of chemokines in the *jp* spinal cord is restricted to the CNS.

Jp spinal cord extracts stimulate progenitor proliferation

Purified OPC cultures (Robinson et al., 1998) were used to compare the effects of extracts from P18 *wt* and *jp* spinal cords on OPC proliferation. Preliminary observations revealed a greater proliferation of OPCs in the presence of *jp* extracts compared

Effects of *jp* extracts on OPC proliferation

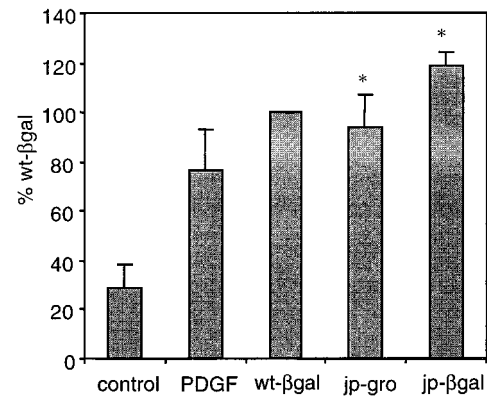


Figure 9. Effects of *wt* and *jp* spinal cord extracts on OPC proliferation. *Control*, Serum-free medium plus N2 supplement; *PDGF*, control plus 10 ng/ml PDGF AA; *jp-gro*, *jp* extracts immunodepleted of GRO-1 (300 μ g/ml protein); *jp- β gal*, *jp* extracts immunodepleted of β -galactosidase (300 μ g/ml protein); and *wt- β gal*, *wt* extracts immunodepleted of β -galactosidase (300 μ g/ml protein). All extracts were added in the presence of 10 ng/ml PDGF AA. [3 H]thymidine incorporation was assayed 2 d after the addition of extracts. Numbers indicate the ratio of [3 H]thymidine incorporation, measured in decays per minute, by OPCs under the indicated conditions to that in the presence of *jp- β gal* extracts (expressed as a percent). They represent average percent values from three separate culture experiments, and triplicate measurements for [3 H]thymidine incorporation were taken for each experiment. Error bars indicate SD. The difference in OPC proliferation by *jp-gro* and *jp- β gal* (*) is significant at $p < 0.03$ (t test).

with that in *wt* extracts. To determine whether the growth stimulatory effect of *jp* extracts was caused by GRO-1, we removed GRO-1 from *jp* extracts by sequential immunoprecipitation using two different anti-mouse GRO-1 (KC) antibodies (obtained from R & D Systems and Dr. Tom Hamilton). Cleared supernatants were assayed for GRO-1 by ELISA (Table 1) and added to OPC cultures. Table 1 shows a representative set of results of three immunoprecipitation experiments. Immunoprecipitation of *jp* extracts with anti-GRO-1 antibodies (GRO-1-depleted) resulted in a 2.3- to 3.3-fold reduction in the concentration of GRO-1. However, the concentration of GRO-1 in *jp* extracts after two rounds of GRO-1 immunodepletion was more than twofold higher than that in total *wt* extracts.

Addition of control β -gal-depleted extracts from *jp* spinal cords to OPC cultures resulted in increased cell proliferation (Fig. 9, *jp- β gal*). Addition of GRO-1-depleted *jp* extracts resulted in a level of OPC proliferation that was comparable with that observed with PDGF alone (Fig. 9, *PDGF*) or with β -gal-depleted extracts from *wt* spinal cords (Fig. 9, *wt- β gal*). Addition of GRO-1-depleted *jp* extracts resulted in an average 21.3% reduction in OPC proliferation compared with that in β -gal-depleted *jp* extracts in three independent experiments. Furthermore, in the presence of control *jp* extracts (*jp* extracts precipitated with normal rabbit serum), 58% of A2B5+ cells incorporated BrdU, whereas 11% of A2B5+ cells incorporated BrdU in the presence of GRO-1-depleted *jp* extracts. These findings suggest that the enhanced OPC proliferation in *jp* spinal cord is mediated in part by increased levels of GRO-1.

DISCUSSION

The proliferation of OPCs is tightly regulated during development. In normal mouse spinal cord the majority of oligodendro-

cytes are generated and myelin formation is well established by the third postnatal week (Matthews and Duncan, 1971), and glial cell proliferation declines beyond this stage. Previous studies demonstrated enhanced proliferation of immature oligodendrocytes in *jp* spinal cord identified by morphological criteria (Skoff, 1982). The current studies support and extend these observations. The low levels of BrdU incorporation seen in NG2+ OPCs in *wt* spinal cord, combined with the increased BrdU incorporation in *jp* spinal cord, strongly suggest that the NG2+ cells and previously morphologically defined immature oligodendrocytes represent overlapping cell populations. Quantitative analyses of NG2+ cell proliferation and the subsequent increase in the number of PDGF α R+ OPCs revealed an apparent discrepancy in the *jp* spinal cord. Although the increase in BrdU incorporation was greatly elevated (three- to eightfold), the increase in the number of OPCs was significantly lower (2.5-fold). Several factors may explain the differences. OPCs may rapidly differentiate into oligodendrocytes in *jp* spinal cord and therefore lose expression of the progenitor markers NG2 and PDGF α R. In agreement with this hypothesis, a significantly higher proportion of BrdU+ nuclei in *jp* spinal cord had only a partial, rather than a complete, ring of NG2 immunoreactivity around them, possibly because of accelerated progenitor cell differentiation. Alternatively, NG2+ cells may themselves die immediately after incorporation of BrdU. Such cell death of OPCs in *jp* spinal cord would be consistent with the observation that oligodendrocyte lineage cells die before they express detectable levels of PLP (Vermeesch et al., 1990). However, the clearance of dead cells would have to be very rapid, because it is not morphologically obvious.

Cell death in *jp* spinal cord occurs relatively late in the oligodendrocyte lineage. The spinal cord of *jp* animals is characterized by a reduced patchy presence of myelin. The reduction in myelin is directly correlated with a reduction in the number of differentiated oligodendrocytes in *jp* spinal cord compared with *wt* spinal cords (Kraus-Ruppert et al., 1973; Meier and Bischoff, 1975; Skoff, 1982). The reduction in the number of differentiated oligodendrocytes is even more dramatic in the context of the elevated levels of OPC proliferation that occur in *jp* spinal cord. Because the increase in proliferation of NG2+ cells is accompanied by an increase in the number of PDGF α R+ progenitor cells, the reduction in the number of differentiated oligodendrocytes must reflect cell death of late-stage progenitors or differentiated oligodendrocytes. This idea is consistent with morphological studies that have demonstrated that dying oligodendrocytes are more prevalent than dying progenitor cells in *jp* spinal cord (Skoff, 1982, 1995; Grinspan et al., 1998).

The elevation in OPC proliferation and death in *jp* animals is not uniform throughout the CNS. The increase in proliferation of NG2+ cells was more pronounced in the spinal cord than in the corpus callosum. This may reflect the chronology of OPC proliferation and myelination, which occur later in the corpus callosum than in the spinal cord (Matthews and Duncan, 1971; Sturrock, 1980; Skoff, 1982). Within the spinal cord, there was no significant increase in NG2+ cell proliferation in the gray matter, although NG2+ cells were abundant in both gray and white matter, suggesting that NG2+ cell proliferation in white and gray matter are regulated by different mechanisms. Consistent with this is our observation that OPC number is elevated in *jp* white matter but not gray matter. This raises the possibility that there are different lineages of oligodendrocytes in the CNS that are generated by different mechanisms, as suggested by recent studies of Spassky et al. (1998) and Marmur et al. (1998).

Studies *in vitro* and *in vivo* suggest that PDGF AA is a major mitogen for NG2+ OPCs (Raff et al., 1988; Richardson et al., 1988; Nishiyama et al., 1996b; Calver et al., 1998) and may regulate the final number of OPCs. The lack of elevation in PDGF A mRNA levels in *jp* spinal cord suggests that the enhanced proliferation in *jp* is not simply a reflection of elevated PDGF. Likewise, there is no obvious alteration in the expression patterns of two other peptide growth factors, TGF- β and IGF-I, both of which have been implicated in the regulation of proliferation and differentiation of OPCs (McMorris and Dubois-Dalcq, 1988; Carson et al., 1993; McKinnon et al., 1993; Beck et al., 1995). It seems likely that although these growth factors play a major role in regulating the initial production of oligodendrocytes and myelin, other mechanisms regulate the maintenance of myelin and oligodendrocyte numbers at later postnatal stages and in the mature CNS.

Several mechanisms have been demonstrated to regulate the proliferative response of OPCs to PDGF. For example, expression of NG2 enhances proliferation and migration of various cells in response to PDGF AA (Grako and Stallcup, 1995; Nishiyama et al., 1996b). Increased levels of NG2 and PDGF α R observed on *jp* progenitor cells may facilitate their response to limited amounts of PDGF AA. The chemokine GRO-1 has a synergistic effect on PDGF-driven OPC proliferation in the rat spinal cord (Robinson et al., 1998).

Chemokines are small, secreted proteins initially defined as chemoattractants for leukocytes that play an important role in inflammation (Rollins, 1997; Luster, 1998; Ransohoff and Tani, 1998). GRO-1 belongs to the α or CXC family of chemokines and functions as a chemoattractant for neutrophils. The mouse homolog KC was originally identified in 3T3 fibroblasts stimulated by PDGF (Cochran et al., 1983; Oquendo et al., 1989), and its human homolog MGSA stimulates proliferation of melanoma cells (Richmond et al., 1988). The hamster GRO- α was initially identified as a molecule present in tumorigenic cells but not in nontumorigenic cells (Anisowicz et al., 1987). These findings indicate that members of the GRO-1 family of chemokines are involved in the regulation of growth control. *jp* spinal cord contains elevated levels of GRO-1 mRNA and protein. GRO-1 in *jp* spinal cord is primarily localized in astrocytes, and the increased expression in *jp* spinal cord may reflect the activated state of these cells. Consistent with this hypothesis, the expression of other chemokines, such as monocyte chemoattractant protein-1, has been shown to be elevated in reactive astrocytes in experimental autoimmune encephalitis (Glabinski et al., 1997). In contrast to the greatly elevated levels of GRO-1 in *jp* spinal cord, the level of GRO-1 mRNA was not significantly elevated in *jp* cerebrum. This may be attributable to cortical gray matter present in the corpus callosum extracts. Alternatively, there may be inherent differences between corpus callosum and spinal cord white matter. This would be consistent with our observation that the number of proliferating OPCs is not as elevated in *jp* corpus callosum as in *jp* spinal cord.

We propose that the elevated proliferation of OPCs in the *jp* spinal cord is a reflection of increased expression of the chemokine GRO-1. In this model, the extensive oligodendrocyte death in *jp* CNS caused by the point mutation in the PLP gene triggers a chain of events including activation of microglia (Sidman et al., 1964; Vela Hernandez et al., 1997) and subsequent reactive astrogliosis (Skoff, 1976; Vela et al., 1996). One consequence of the local activation of astrocytes is the increased secretion of GRO-1 that allows local proliferation of NG2+ OPCs by synergistic

action with low levels of PDGF. This is supported by our *in vitro* studies in which *jp* spinal cord extracts showed a growth-stimulatory effect on OPCs and this effect could be inhibited by immunodepleting GRO-1 from the extracts. These studies demonstrate for the first time altered expression of chemokines in a naturally occurring noninflammatory lesion of the CNS and suggest that chemokines secreted by endogenous neural cells may exert their effects on endogenous CNS cells. This provides a novel mechanism by which the proliferation of OPCs is regulated in response to an insult in the postnatal CNS.

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